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The B7-CD28 Family Molecules

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Molecular Biology Intelligence Unit

The B7-CD28 Family Molecules

Lieping Chen, M.D., Ph.D.
Department of Immunology
Mayo Medical School and Graduate School
Mayo Clinic
Rochester, Minnesota, U.S.A.

Landes Bioscience / Eurekah.com
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U.S.A.

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www.Eurekah.com
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CONTENTS

Preface ix

1. **Structure Prediction and Binding Site Identification of the CD28 and B7 Family Molecules** 1
   Jürgen Bajorath
   Abstract 1
   The CD28 and B7 Families: Mediators of T Cell Costimulation 1
   The CD28 and B7 Families and the Immunoglobulin Superfamily 2
   Sequences, Topology and Binding Characteristics 2
   Structure-Function Analysis Based on Comparative Protein Models and Mutagenesis 4
   Comparative Modeling of IgSF Proteins Sharing Low Sequence Similarity 5
   Molecular Models of CTLA-4 and CD80/CD86 6
   Mapping of Binding Sites 7
   Experimentally Determined Structures 8
   Evaluation of Predictions 9
   Conclusions 9

2. **T Lymphocyte Signaling Pathways Regulated by CD28 and CTLA-4** 12
   David J. McKean and Matthew D. Griffin
   Introduction 12
   CD28 Costimulation 13
   CTLA-4 Signaling 23

3. **Costimulatory Molecules in T Cell Development, Activation and Effector Function: Similar Activity, Opposite Consequences** 38
   Yang Liu, Jian Xin Gao, Xue-Feng Bai, Xingluo Liu, Huiming Zhang and Pan Zheng
   Abstract 38
   Conceptual Evolution and Molecular Basis of the Second Signal for T Cell Activation 38
   Multiplicity of Costimulatory Molecules 39
   Expanding the Horizon of T Cell Costimulation: From Activation to Effector Function 39
   Costimulatory Molecules in T Cell Development 40
   Negative Costimulation? Paradigm Revisited 42
   B71/2 Mediated Costimulation in T Cell Activation and Development: Similar Activity, Opposite Consequences 42

4. **Negative Costimulatory Functions of B7-H1** 48
Haidong Dong, Hideto Tamura, Shengdian Wang and Lieping Chen
Introduction 48
Molecular Structure and Expression of B7-H1 48
Counter-Receptor of B7-H1 50
Immunological Functions of B7-H1 51
Effect of Tumor-Associated B7-H1 in Immune Evasion 52
Reverse Signaling of B7-H1 to Regulate T Cell Responses 54
Conclusion 55

5. **PD-1:PD-1 Ligand Pathway** 59
Gordon J. Freeman and Arlene H. Sharpe
Introduction 59
Structure and Expression of the PD-1 Receptor 59
Function of PD-1 60
Biochemistry Basis for PD-1 Function 61
Structure and Expression of PD-1 Ligands 62
Function of PD-1 Ligands 63
Summary and Concluding Remarks 65

6. **The ICOS/B7RP-1 Costimulation Pathway** 67
Steven K. Yoshinaga
Abstract 67
Introduction 67
Terminology 68
Structures of ICOS and B7RP-1 68
ICOS Expression 69
B7RP-1 Expression 69
Cytokine Regulation by ICOS/B7RP-1 70
Immunoglobulin Regulation by ICOS/B7RP-1 72
CD28 and CTLA-4 Regulate, but Are Not Required for, ICOS Function 72
The HpH4 HIV Antigen Is ICOS 72
ICOS and Apoptosis 73
B7RP-1-Fc and B7.2-Fc Transgenic Mice Exhibit Intestinal Inflammation ... 73
ICOS/B7RP-1 Therapeutic Modalities 74
Anti-Tumor Activities of B7RP-1 74
ICOS Blockade in Transplantation 75
ICOS Regulation of Secondary and Recall Responses 75
EAE Studies Reveal the Promise and the Complexity of ICOS/B7RP-1 Regulation ... 76
Differential ICOS/B7RP-1 Regulation 77
Summary 77

7. **Role of ICOS in T Cell Activation** **82**
Jeffrey A. Ledbetter, Erik Espling and Martha Hayden-Ledbetter
Introduction 82
Defective T Cell Help for Isotype Class Switching in ICOS Deficient Mice 82
Identification of a Novel ICOS Ligand Related to CD80 84
Role of ICOS in CD4 T Cell Activation 84
ICOS Costimulation of CD8 T Cells 87

8. **B7-H3** **91**
Andrei I. Chapoval and Lieping Chen
Introduction 91
Molecular and in Silico Cloning of B7-H3 91
Isoforms of Human B7-H3 92
B7-H3 Gene Analysis 93
B7-H3 Tissue Distribution and Expression of B7-H3R on Activated T Cells 94
B7-H3 Costimulates T Cell Growth in Vitro 96
B7-H3 Selectively Increases IFN-γ Production 97

9. **The Role of B7s in Transplantation** **100**
Didier A. Mandelbrot, Wayne R. Godfrey, Arlene H. Sharpe and Bruce R. Blazar
Introduction 100
The Absence of B7 Costimulation Renders T Cells Anergic in Vitro 100
Blockade of B7 Molecules Prolongs Organ Graft Survival 101
Allorecognition Pathways Affected by B7 Costimulation 101
The Role of B7 Molecules in Chronic Graft Rejection 102
Role of B7 in Xenotransplantation 102
The Role of B7-1 versus B7-2 Mediated Costimulation in Transplantation Responses 104
Combination Approaches to Improve Allograft Survival Based upon B7:CD28/CTLA-4 Blockade 104
CTLA4 Signaling Prolongs Graft Survival 105
B7 and CD28 Homologues 106
Overview of ex Vivo Blockade of the CD28/CTLA-4:B7 Pathway for Graft-versus-Host Disease (GVHD) Prevention 106
Ex Vivo Blockade of B7:CD28/CTLA-4 Interactions in Human Mixed Lymphocyte Reaction (MLR) Cultures 107
Human Clinical Trial of ex Vivo B7:CD28/CTLA4 Blockade for GVHD Prevention 107
Adjuvants to B7 Blockade 108
Modifications of ex Vivo B7:CD28/CTLA4 Costimulatory Pathway Blockade of Human MLR Cultures Designed to Improve the Efficacy of Tolerization 109

Ex Vivo Tolerization of Murine MLR Cultures by Costimulatory Pathway Blockade As a Means of Achieving GVHD Prevention 109
In Vivo Blockade of B7:CD28/CTLA4 for GVHD Prevention 110
Mechanism(s) of GVHD Inhibition by B7:CD28/CTLA-4 Blockade 111
Role of CD28/CTLA-4:B7 in Graft-versus-Leukemia (GVL) 112
Role of CD28/CTLA-4:B7 in Alloengraftment 112
Future Directions 113

10. B7 Family of Costimulatory Molecules in the Induction and Regulation of Autoimmunity 118
Claudia Jabs, Arlene H. Sharpe and Vijay K. Kuchroo
Introduction 118
EAE and IDDM 119
Manipulation of B7 Costimulatory Pathway 120
Blocking Negative Costimulatory Signals in Autoimmune Diseases 122
ICOS-B7h Costimulation in Autoimmune Diseases 123
PD-1:PD-L1/PD-L2 Pathway and Autoimmunity 124

11. Operation Enduring Costimulation: Modulation of B7 Receptors to Elicit Anti-Tumor Immunity 128
Eugene D. Kwon, Qingyong Ji and Arthur A. Hurwitz
Turning Up the Offensive 128
An Alliance of Mediators 130
Shutting Down the Defense 131
The New Breed… 133
Future Battles 134

Index 139

EDITOR

Lieping Chen, M.D., Ph.D.
Department of Immunology
Mayo Medical School and Graduate School
Mayo Clinic
Rochester, Minnesota, U.S.A.
Chapters 4, 8

CONTRIBUTORS

Xue-Feng Bai
Department of Pathology
Ohio State University Medical Center
Columbus, Ohio, U.S.A.
Chapter 3

Jürgen Bajorath
Albany Molecular Research, Inc.
Bothell Research Center
Bothell, Washington, U.S.A.
and
Department of Biological Structure
University of Washington
Seattle, Washington, U.S.A.
Chapter 1

Bruce R. Blazar
Department of Pediatrics
Division of Bone Marrow Transplantation
University of Minnesota Cancer Center
Minneapolis, Minnesota, U.S.A.
Chapter 9

Andrei I. Chapoval
Department of Immunology
Mayo Clinic
Rochester, Minnesota, U.S.A.
Chapter 8

Haidong Dong
Department of Immunology
Mayo Clinic
Rochester, Minnesota, U.S.A.
Chapter 4

Erik Espling
Pacific Northwest Research Institute
Seattle, Washington, U.S.A.
Chapter 7

Gordon J. Freeman
Department of Adult Oncology
Dana-Farber Cancer Institute
Harvard Medical School
Boston, Massachusetts, U.S.A.
Chapter 5

Jian Xin Gao
Department of Pathology
Ohio State University Medical Center
Columbus, Ohio, U.S.A.
Chapter 3

Wayne R. Godfrey
University of Minnesota
Cancer Center and Department of Pediatrics
Division of Blood and Marrow Transplantation
Minneapolis, Minnesota, U.S.A.
Chapter 9

Matthew D. Griffin
Department of Nephrology
Mayo Clinic
Rochester, Minnesota, U.S.A.
Chapter 2

Martha Hayden-Ledbetter
Pacific Northwest Research Institute
Seattle, Washington, U.S.A.
Chapter 7

Arthur A. Hurwitz
Department of Microbiology
and Immunology and Urology
State University of New York Health
Science Center
Syracuse, New York, U.S.A.
Chapter 11

Claudia Jabs
Departments of Neurology
Center for Neurologic Diseases
Harvard Medical School
Boston, Massachusetts, U.S.A.
Chapter 10

Qingyong Ji
Depts. of Microbiology and Immunology
SUNY Upstate Medical University
Syracuse, New York, U.S.A.
Chapter 11

Vijay K. Kuchroo
Department of Neurology
Center for Neurologic Diseases
Harvard Medical School
Boston, Massachusetts, U.S.A.
Chapter 10

Eugene D. Kwon
Department of Urology
Cardinal Bernardin Cancer Center
Loyola University Medical Center
Maywood, Illinois, U.S.A.
Chapter 11

Jeffery A. Ledbetter
Pacific Northwest Research Institute
Seattle, Washington, U.S.A.
Chapter 7

Xingluo Liu
Department of Pathology
Ohio State University Medical Center
Columbus, Ohio, U.S.A.
Chapter 3

Yang Liu
Department of Pathology
Ohio State University Medical Center
Columbus, Ohio, U.S.A.
Chapter 3

Didier A. Mandelbrot
Department of Medicine
University of Massachusetts
Worcester Massachusetts, U.S.A.
Chapter 9

David J. McKean
Department of Immunology
Mayo Clinic
Rochester, Minnesota, U.S.A.
Chapter 2

Arlene H. Sharpe
Department of Pathology
Brigham and Women's Hospital
Harvard Medical School
Boston, Massachusetts, U.S.A.
Chapters 5, 9, 10

Hideto Tamura
Department of Immunology
Mayo Clinic
Rochester, Minnesota, U.S.A.
Chapter 4

Shengdian Wang
Department of Immunology
Mayo Clinic
Rochester, Minnesota, U.S.A.
Chapter 4

Steven K. Yoshinaga
Amgen Inc.
One Amgen Center Drive
Thousand Oaks, California, U.S.A.
Chapter 6

Huiming Zhang
Department of Pathology
Ohio State University Medical Center
Columbus, Ohio, U.S.A.
Chapter 3

Pan Zheng
Department of Pathology
Ohio State University Medical Center
Columbus, Ohio, U.S.A.
Chapter 3

PREFACE

The B7-CD28 family molecules are probably the most intensively studied receptor-ligand systems in the field of immune regulation. This is evident by the explosive accumulation of literature, particularly in the last ten years. While extensive studies on the classic components of the B7-CD28 pathway, including CD80, CD86, CD28 and CTLA-4, will continue to take place, recent years witness rapid discoveries and characterization of new receptors and ligands in the family. These new pathways, although still in their infancy, have already brought much excitement to the field. The idea for publishing this book is to bring state-of-the-art information and critical thinking from the leading investigators in one volume. This book covers significant territory of this rapidly moving field from structural biology and biochemical signaling to immunological functions and their potential applications in the treatment of human diseases.

This book could not have been written without my coauthors preparing excellent contributions in a timely fashion and my secretary, Kathy Jensen, for patient assistance with editing. I thank them all wholeheartedly. I also like to thank the Mayo Foundation, American Cancer Society and National Institutes of Health for financial support.

Lieping Chen, M.D., Ph.D.
Rochester, Minnesota, U.S.A.
August 2003

Chapter 1

Structure Prediction and Binding Site Identification of the CD28 and B7 Family Molecules

Jürgen Bajorath

Abstract

The focal point of this contribution is a critical evaluation of studies designed to explore structure-function relationships of CD28- and B7-like proteins prior to experimental determination of three-dimensional structures. Based on a number of representative investigations, what has been learned about the structures and binding characteristics of members of these protein families by combining computer modeling and site-specific mutagenesis will be reviewed. The opportunities and limitations of these approaches will be discussed in light of recently determined three-dimensional structures of the extracellular domains of human CTLA-4 (CD152) and CD80/CD86. In an accompanying chapter, the X-ray structures of these proteins and their complexes will be discussed in greater detail. In retrospect, we find that binding sites in CTLA-4 and CD80/CD86 could be identified and mapped several years before experimental structures of receptor-ligand complexes were obtained. However, detailed prediction of dimerization modes and receptor-ligand complexes was beyond the limitations of model building. However, modeling of binding domains of CTLA-4 and CD80/CD86 provides an instructive example of protein structure prediction in the presence of very low sequence identity.

The CD28 and B7 Families: Mediators of T Cell Costimulation

Immune cell surface proteins belonging to the CD28 and B7 families have long been the target of intense studies in the context of T cell costimulation and regulation of immune responses (for representative reviews, see refs. 1-3). The interest in these proteins, their interactions, and specific functions remains strong, as exemplified by recent identification of new members of the expanding B7 family.[4,5] At least in part, the high level of interest in the CD28 and B7 families is a consequence of the significant therapeutic potential of modulating the interactions between these proteins in vivo.[6,7]

What is T cell costimulation? Immune responses to foreign antigens depend on specific interactions between T cells receptors (TCRs) and major histocompatibility complex class I and II molecules (MHCs) that present antigenic peptides. Signal transduction through TCRs is critical for the induction of T cell-dependent immune responses. TCR-MHC interactions

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are complemented by T cell coreceptors CD4 or CD8 that bind to MHC class II or class I, respectively. Nevertheless, these augmented TCR-MHC interactions alone are not sufficient to trigger effective immune responses. In addition, interactions between CD28-like proteins on T cells and B7-like proteins on antigen presenting cells are required to deliver important costimulatory signals. In contrast to antigen-specific TCR signals, costimulatory signals are not specific to antigen.

Both CD28 and cytotoxic T lymphocyte associated antigen 4 (CTLA-4, CD152) bind CD80 (B7-1) and CD86 (B7-2), albeit with different avidities and kinetic constants (on- and off-rates; see below). By contrast, inducible T cell costimulator or ICOS, a recently identified member of the CD28 family,[8] recognizes another member of the B7 family, B7-related protein-1 (B7RP-1)[9] or B7-H2.[10] Signaling through both CD28 and ICOS delivers strong costimulatory signals to T cells that are critical for effective activation and proliferation, whereas CTLA-4 signaling, upon binding to CD80 or CD86, negatively regulates (or balances) these effects.[11] Another more distant member of the CD28 family is the so-called programmed death 1 (PD-1) protein,[12] which is also a negative regulator of immune responses, similar to CTLA-4. For PD-1, different B7-like ligands have been identified, B7-H1 (PD-L1)[13,14] and, more recently, PD-L2.[15] Expression characteristics of CD28 and B7 proteins vary in part significantly but these properties are not very relevant for the discussion of direct binding interactions presented herein. By contrast, an important aspect for the analysis of these interactions is that the CD28 and B7 families utilize similar structural motifs in their extracellular regions to mediate networks of receptor-ligand interactions with in part overlapping and in part distinct specificity, as discussed below in more detail.

The CD28 and B7 Families and the Immunoglobulin Superfamily

Like many other lymphocyte antigens, both the CD28 and B7 families belong to the immunoglobulin superfamily (IgSF).[16] Assignments to the IgSF are generally made based on characteristic sequence motifs or key residues in extracellular domains of these proteins. The overall structure of Ig domains consists of a tightly packed sandwich of two curved beta-sheets with extensive hydrophobic core that often includes a canonical disulfide bond. Based on the beta-strand content of these sheets and the way the strands are connected by loops (i.e., their topology), different types of Ig domains are distinguished, most importantly, variable or V-type and constant or C-type domains, as first observed in the variable and constant regions of antibodies, and hybrid domains that combine V- and C-like features.[17,18] In V-domains, the two beta-sheets consist of five and four strands, respectively, that form the so-called GFCC'C" and ABED faces of the domain. However, in these domains, the N-terminal A-strand is often split into two halves, the A- and A'-segments, which switch between the sheets and thereby produce a six-strand A'GFCC'C" and a four strand ABED sheet. Compared to V-domains, C-type domains lack the C'-C" region and their beta-sheets thus consist of three (GFC) and four (ABED) strands, respectively. In structural terms, these Ig domain types and their hybrid domains like the I-domain[17] represent defined variations of a common theme, the canonical Ig fold, one of the most stable protein folds known to date. Figure 1 shows a schematic representation of the Ig V-like domain of CTLA-4 as an example.

Sequences, Topology and Binding Characteristics

Sequence similarity within protein superfamilies is low, by definition less than 50%. However, within the IgSF and their protein families, sequence identity is usually considerably lower, often 20% or less. This is in part a consequence of the fact that the Ig fold is capable of tolerating a high level of sequence variability or, in other words, many residue substitutions. In both the CD28 and B7 families, pair-wise sequence identity in extracellular regions is, on

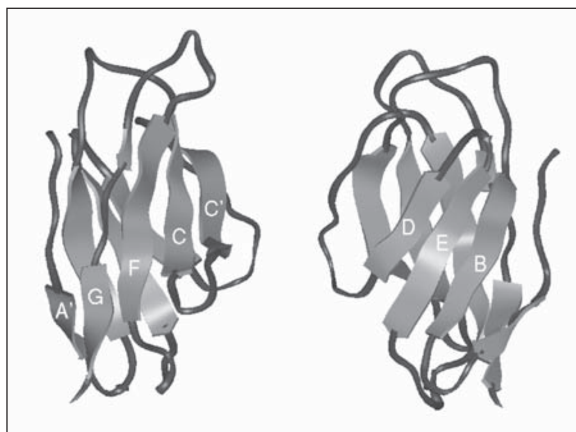

Figure 1. Schematic representation of an Ig V-like domain. The solution structure of CTLA-4 (ref. 36) is shown as an example. Beta-strands are represented as flat ribbons and labeled according to IgSF conventions. The two views are related by ~180° rotation around the vertical axis and thus focus on the opposite beta-sheet surfaces of the domain. The tube in the FG-loop at the top indicates the position of the characteristic -MYPPPY- sequence motif that is conserved in CTLA-4 and CD28.

average, approximately 20%-25%. For example, Figure 2 shows an alignment of sequences of the extracellular V-type domains of CD28, CTLA-4, and ICOS from different species. A number of conserved, or largely conserved, residue positions correspond to signature or consensus residues of the Ig fold that are often buried in the protein core and determine its three-dimensional structure.

CD28-like and B7-like proteins have similar molecular topology but differ in their extracellular domain structure. Like many other immune cell surface proteins,[19] these proteins consist of extracellular domains that are linked by a short stalk region to a single transmembrane segment that connects the extracellular binding domains to relatively short (but highly diverse) cytoplasmic regions. However, members of the CD28 family only have a single Ig V-like extracellular domain, whereas B7 proteins have two extracellular domains, an N-terminal V-like domain, followed by a C-like domain. In contrast to members of the B7 family, CD28-like domains form covalent homodimers on the cell surface, due to a disulfide bond in the stalk region outside the Ig V-domain.

In principle, interactions between CD28 and B7 proteins are also representative of other immune cell surface receptor-ligand interactions in that they are fairly transient and characterized by fast kinetic on- and off-rates of binding.[20,21] The stoichiometry of the CD28/CTLA-4—CD80/CD86 interactions is 1:1,[22] which means that a disulfide-linked CD28 or CTLA-4 homodimer binds two CD80 or CD86 monomers, thus forming a 2:2 molecular complex on the cell surface. The intrinsic affinity of these interactions, as studied using soluble recombinant forms of the proteins, is in general low, in the micromolar range, although CTLA-4 binds ligand 10 to 100-fold more strongly than CD28 (which is largely due to CTLA-4's faster kinetic on-rate of binding)[20]. In fact, the CTLA-4—CD80 interaction has an affinity in the high nanomolar range (~300 nM)[21] and is thus among the strongest interactions for immune cell surface proteins determined thus far. However, for effective signaling and costimulation to occur, effects such as receptor or ligand oligomerization or clustering on the cell surface are certainly important, as they transform molecular interactions of low intrinsic affinity into coordinated interactions of higher avidity.[23]

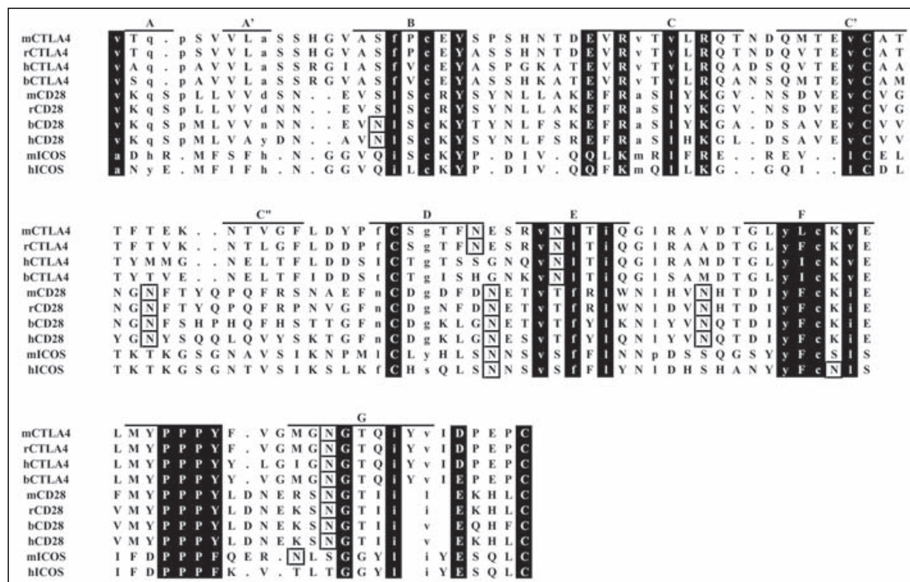

Figure 2. Structure-oriented alignment of extracellular Ig domain sequences of the CD28 family. V-domain sequences of CTLA-4, CD28, and ICOS from different species (m: murine, r: rat, h: human, b: bovine) are shown. The alignment takes structural information of the IgSF V-fold into account. Beta-strand segments are labeled and residues that map to positions in the core of the domain are shown in lower case. These positions are not available for binding interactions but often important for structural integrity. Potential N-linked glycosylation sites are boxed and residues conserved in all sequences (taking most conservative substitutions into account) are shown on a black background, illustrating the limited sequence similarity shared by these proteins. In fact, the majority of conserved positions maps to core regions and is therefore IgSF-characteristic but not CD28 family-specific. The absence of any sequence conservation in the C"-region of these proteins is noteworthy. The terminal cysteine residue following the G-strand is responsible for covalent dimerization of these proteins on the cell surface.

Structure-Function Analysis Based on Comparative Protein Models and Mutagenesis

In general, the goal of structure-function studies of the kind described herein is to identify residues that are important or essential for receptor-ligand interactions and provide a spatial outline of the binding sites. In the absence of experimentally determined three-dimensional structures, such investigations rely, by necessity, on molecular models that are usually produced by comparative structure prediction. The basic idea behind comparative protein modeling is to predict an unknown protein structure based on known structures of homologous or similar proteins (for reviews, see refs. 24 and 25). Thus, in essence, comparative modeling is an extrapolation from known structural data. First, topologically correct sequence alignments of target proteins with unknown and template proteins with known three-dimensional structure are generated. These alignments are then applied to predict structurally conserved regions. These regions provide the core of comparative models that are then complemented with predicted loop conformations and side chain conformations of non-conserved residues and subjected to various model refinement (e.g., energy minimization, molecular dynamics) and assessment (e.g., stereochemistry, sequence-structure-compatibility) calculations.

Once molecular models of target proteins have been constructed, they can be used to select residues for site-specific mutagenesis. Mutated residues can then be classified according

to their importance for the receptor-ligand interaction and, based on these findings, binding sites are mapped in three dimensions. What is the major advantage of using structures or models for residue selection and mapping? Simply put, structures make the selection of residues possible that are proximal in three dimensions but distant in sequence and thus provide a significant advantage for the identification of binding sites, compared to sequence-based or random mutagenesis approaches.

In any mutagenesis analysis of receptor-ligand interactions, a major problem is to determine whether mutations directly affect binding or indirectly by perturbing the structure of the mutant protein. For example, this would often be the case if core residues were mutated. Although the possibility of more or less significant structural perturbations can never be ruled out in mutagenesis studies, gross structural defects of mutant proteins can often be detected by use of panels of conformationally sensitive monoclonal antibodies and comparison of binding profiles of mutant and wild-type proteins. This has generally been the method of choice for the assessment of mutants in our structure-function studies on various immune cell surface receptors (for reviews, see refs. 26 and 27).

Comparative Modeling of IgSF Proteins Sharing Low Sequence Similarity

Generation of topologically correct sequence alignments of template and target proteins, the initial step in comparative modeling, is of critical importance for the accuracy of molecular models. Errors that occur at this stage (i.e., misalignments) result in the incorrect assignment of sequence segments in target proteins to secondary structure elements in templates and can not be corrected later on during the modeling process. Such inaccuracies typically represent the largest errors in comparative models. As long as sequence similarity between templates and targets is high (e.g., 50% or more), such misalignments are relatively easy to avoid. However, if sequence similarity is low, which is the case for the CD28 and B7 families and many other proteins belonging to the IgSF, the generation of correct alignments becomes quite difficult. For model building studies on CD28 and B7 proteins, Ig V-like or C-like structural templates with more than 20% sequence similarity could not be identified. This again reflects the fact that the Ig fold is very stable and capable of tolerating many different sequences, thus making low sequence similarity a characteristic feature shared by many IgSF proteins.

However, if molecular models are used for structure-function analysis and mapping of binding sites, they must be as accurate as possible. In order to identify residues critical for binding, selection of spatially adjacent residues on the protein surface (but not in the core regions) must be possible with confidence. Otherwise, incorrect "pictures" of binding sites are obtained and misleading conclusions are drawn. For IgSF proteins with low sequence identity, this represents a significant problem because the Ig fold exclusively consists of beta-strands and loops connecting these strands with specific topology. Beta-strands have stringent "up-down" side chain periodicity. This means that residues forming the strands of the two beta-sheets or faces of the Ig-domain occupy alternating positions in the core region and on the protein surface. It follows that even small alignment errors usually lead to modeled beta-strands that are "out of register". In this case, residues that are in reality located on the surface of the target protein are modeled in the core and vice versa surface, which renders such models useless for any kind of structure-function studies.

How can such errors be avoided? For IgSF proteins, the key is to focus on a limited number of signature or consensus residues that form patterns characteristic of different Ig-domain types. This is illustrated in Figure 3, which shows a sequence alignment of various V-type domains obtained by exact superposition of their three-dimensional structures. The alignment was subsequently annotated with IgSF V-set consensus positions. These largely conserved residues were then used as "anchor points" for the alignment of new protein sequences. Focusing

```
           ___A___ __A'___     ____B____             ___C___
                        *       * * *                 * *
REI    VL: DIQMTQS PSSLSAS VGD RVTITCQAS QDIIK----- YLNWYQQ TPGKA--
KOL    VH: EVQLVQS GG-GVVQ PGR SLRLSCSSS GFIFSSY--- AMYWVRQ APGKG--
CD8    V : -SQFRVS PLDRTWN LGE TVELKCQVL LSNPTS---- GCSWLFQ PRGAAAS
CD4    D1: ------- KKVVLGK KGD TVELTCTAS QKKSI----- QFHWKN- SN-----
CD2    D1: ------- SGTVWGA LGH GINLNIPNF QMTDDID--- EVRWER- GS-----

hCD86  D1: ------- -IQAYF- NE- TADLPCQFA NSQNQSLSEL VVFWQD- QE-----
mCD86  D1: ------- -TQAYF- NG- TAYLPCPFT KAQNISLSEL VVFWQD- QQ-----
hCD80  D1: ------- -VTKEV- KE- VATLSCGH- NVSVEELAQT RIYWQK- EK-----
mCD80  D1: ------- -LSKSV- KD- KVLVPCRY- NSPHEDESED RIYWQK- HD-----


           ___C'___      _C"_         __D___      ___E____
              **                      **            * * *    *   *
REI    VL: PKLLIYE- AS--- NLQA GVPS--- RFSGSG SG--- TDYTFTIS SLQPEDI
KOL    VH: PEWVAIIW DDGSD QHYA DSVKG-- RFTISR NDSK- NTLFLQMD SLRPEDT
CD8    V : PTFLLYLS QNKP- KAAE GLDTQ-- RFSGKR LG--- DTFVLTLS DFRRENE
CD4    D1: QIKILGNQ GS--- FLTK GPSKLND RADSRR SLWDQ GNFPLIIK NLKIEDS
CD2    D1: -TLVAEFK RKMK- PFLK SG----- AFEIL- A---- -NGDLKIK NLTRDDS

hCD86  D1: -NLVLNEV YLGKE KFDS VHSKYMG RTSFD- SD--- -SWTLRLH NLQIKDK
mCD86  D1: -KLVLYEH YLGTE KLDS VNAKYLG RTSFD- RN--- -NWTLRLH NVQIKDM
hCD80  D1: -KMVLTMM S---- GDMN IWPEYKN RTIFD- ITN-- -NLSIVIL ALRPSDE
mCD80  D1: -KVVLSVI A---- GKLK VWPEYKN RTLYD- NT--- -TYSLIIL GLVLSDR
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Figure 3. Consensus residue method. The alignment illustrates how IgSF sequences can be topologically aligned, despite the presence of only very limited sequence similarity. The alignment of the top four sequences, representing diverse IgSF V-like domains, was obtained by accurate superposition of their X-ray structures. Thus, corresponding residues in secondary structure elements have equivalent spatial positions. The alignment is annotated with IgSF signature or consensus positions (labeled with asterisks and shown on a black background) that are characteristic of different domain types. Conserved IgSF V-set consensus residues are used here as "anchor points" for the alignment of new sequences (in this case, of B7 molecules) to compensate for the absence of significant overall homology. For clarity, only a subset of the V-domain alignment is shown to illustrate the idea behind the consensus residue approach. "VL" and "VH" indicate antibody variable light and heavy chains, respectively, and "D1" means the first or N-terminal V-like domain; "m" stands for murine and "h" for human.

on consensus residues as alignment anchors therefore compensates for the absence of significant overall sequence similarity, which would prohibit a meaningful statistical comparison of template and target sequences. Nevertheless, topologically correct alignment of IgSF sequence is often difficult and presented a significant challenge for the studies described in the following.

Molecular Models of CTLA-4 and CD80/CD86

In our laboratory, sequence analysis and model building studies have been carried out for all members of the CD28 and B7 families known to date. However, the discussion will be limited here to those proteins for which three-dimensional structures have also been determined, i.e., CTLA-4, CD80, and CD86. As indicated above, significant effort was required to align sequences of the extracellular regions of CTLA-4 and CD80/CD86 with known Ig structures. At the time CTLA-4 modeling was initiated, in the mid '90s, the knowledge base of Ig domain structures was much smaller than today and consisted to a large extent of antibody variable heavy (VH) and light (VL) chain domains. Careful IgSF consensus residue analysis suggested that the extracellular domain of CTLA-4 was indeed most similar to V-domains and independent models were built based on the VH domain of antibody HyHel-5[28] and the VL domain of REI,[29] respectively. Although the sequence similarity with REI, for example, was

less than 20% in the modeled region, the majority of beta-strands could be aligned with some degree of confidence based on consensus residue analysis. However, it was necessary to generate and analyze many alternative alignments before a final version of the CTLA-4 model was built, and sequence analysis and alignment studies required much more time than the structural modeling itself. The REI-based core structure of the model was complemented with loop and side chain conformations constructed by conformational search, and the model was energy minimized and assessed using sequence-structure-compatibility calculations.[29] Early predictions from CTLA-4 modeling included the formation of a non-classical disulfide bond linking the C'- and D-strands (Fig. 2), which is conserved across the CD28 family, and the mapping of the characteristic -MYPPPY- sequence to the F-G loop or, using antibody terminology, CDR3-analogous region (Fig. 1).[28] These predictions were later confirmed by experimental structures. The -MYPPPY- motif is conserved in CTLA-4 and CD28 and critical for binding and costimulatory functions.

Sequences of CD80 and CD86 were also analyzed in detail to provide a basis for model building. In the course of these studies, sequence similarity between the V-like domains of B7 molecules and several proteins encoded by the MHC gene cluster, for example, butyrophilin, was detected.[30] These observations suggested an evolutionary link between costimulatory and antigen presenting molecules and have led to an extension of the B7 gene family.[30,31] Furthermore, we predicted that the second domain of CD80 was most similar to C1-set domains, one of two subsets of IgSF constant domains, rather than to the C2-set.[32] Among available C1-set structures, greatest similarity was predicted to the structure of the β2-microglobulin domain[32], a part of MHC class I molecules, which further supported an evolutionary MHC-B7 link. However, this structural prediction was controversial because C1-set domains, which occur, for example, in the constant regions of antibodies, had not yet been found in IgSF cell surface proteins, the known C-type domains of which were all C2-like.[33]

To aid in the design and interpretation of mutagenesis experiments, we initially built a schematic model of the V-domain of CD80 and a detailed model of its C-like domain, based on the structure of β2-microglobulin.[34] Subsequently, a more detailed model of the V-like domain of CD86 was also generated.[35] The V- and C-domains of CD80 were modeled separately, not as a domain tandem, because of the uncertainties involved in modeling the domain interface and relative domain orientation.[34] It was also predicted,[35] and later confirmed by X-ray structures, that the V-like domains of CD80 and CD86 lack the A-strand segment (of the ABED sheet) and only contain an A'-strand segment (belonging to the A'GFCC'C" sheet).

Mapping of Binding Sites

Over the past several years, a variety of mutagenesis studies on CTLA-4 and CD28 have been reported (for review, see ref. 36 and references therein) and, in retrospect, the majority of these studies provided largely consistent findings regarding residues important for binding. The B7 binding site in CTLA-4 was first identified in our laboratory. Using our CTLA-4 models for residue selection and mapping, we could show that residues in the CDR3-analogous loop conserved in CTLA-4 and CD28, including the -MYPPPY- motif, were critical for binding and that binding was modulated by non-conserved residues in the spatially adjacent CDR1-analogous loop.[28] Other conserved residues on the A'GFCC'C" face of the domain important for binding were also selected and localized with the aid of the models. Mapping of the binding site was then completed using the NMR structure of CTLA-4,[36] and a comparison of the modeled and experimentally determined binding site region confirmed the predictions.[37] Figure 4 shows the modeled binding site. Essentially, the A'GFCC'C" domain surface of CTLA-4 is extended by the CDR3-analogous loop and residues critical for B7 binding cluster in this region. This surface patch is strictly conserved in CD28, suggesting that it also represents its B7 binding site (although the CD28 structure remains to be determined). For CD80 and CD86,

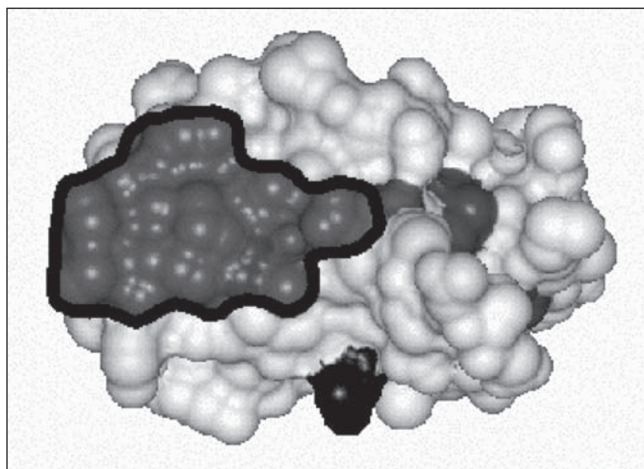

Figure 4. Prediction of the B7 binding site. The CTLA-4 model is shown with calculated molecular surface, and the view focuses on its A'GFCC'C" face. Residues conserved in CTLA-4 and CD28 are shown in dark gray. Most of these residues were subjected to site-specific mutagenesis, and the region outlined in black contains the residues that are critical for binding and form the B7 binding site. The black residue on the left is a potential N-linked glycosylation site in this region that would not interfere with binding. The B7 binding site in CTLA-4 was well predicted with the aid of the model prior to the availability of experimentally determined structures.

several independent studies implicated both V-like and C-like domains in CTLA-4/CD28 binding.[34,38-40] As it turned out, these predictions were only in part valid and less reliable than those for CTLA-4.

Experimentally Determined Structures

NMR[36] and X-ray structures[41] of CTLA-4 and X-ray structures of CD80[42] and the CTLA-4/CD86[43] and CTLA-4/CD80[44] complexes have by now been reported. The discussion of these structures will be limited here to the extent required to appreciate the results of model-based structure-function studies. The independently determined CD80 and CD86 structures are overall quite similar, despite limited sequence conservation. Their V-and C-like domains form an extended structure and a well defined non-covalent dimer. By contrast, experimental structures of CTLA-4 differ in some important details. In solution, CTLA-4 is monomeric[36] but the X-ray structures of murine[41] and human CTLA-4[42,43] display completely distinct dimerization modes. Formation of the murine dimer would interfere with N-linked glycosylation[45] and is therefore probably not physiologically relevant. Moreover, in the solution structure of CTLA-4, the C"-strand is not formed and the C"-region is largely disordered. By contrast, the C"-strand strand is formed in the X-ray structures but, different from standard V-domain topology, this strand switches the sheet and is located adjacent to the D-strand (and not the C'-strand). This topology is characteristic of TCR V-alpha domains and was first seen in the structure of an isolated TCR domain.[46] Finally, the conformation of the rigid triple proline sequence in the -MYPPY- motif was determined as "trans-trans-cis" by NMR but "cis-trans-cis" by crystallography, giving rise to some significant conformational differences in the CDR3-analogous region.

Evaluation of Predictions

What have these structures taught us about the predictive value of our structure-function studies? In the case of CD80, it was correctly predicted that the C-like domain is a C2-set domain, similar to β2-microglobulin. Furthermore, some of the residues important for binding have been accurately mapped to the C- and C'-strands[34] of the V-domain A'GFCC'C" face, similar to CTLA-4. This face has been found to represent the preferred binding site region in cell surface receptors and adhesion molecules belonging to the IgSF.[19,33] In retrospect, based on the V-domain model of CD86, the location and effects of a number of mutants could be rationalized (ref. 35 and unpublished findings). However, the complex structures clearly show that only the V- but not the C-domain of CD80/CD86 is involved in CTLA-4 binding, in contrast to various mutagenesis proposals. Mapping of mutated residues suggests that many C-domain mutants affect binding indirectly, either by perturbing the domain interface or by affecting the presentation of the V-domain for binding. Since accurate modeling of domain interfaces or relative domain orientations is very difficult, if not impossible, such effects could not be predicted with confidence.

For CTLA-4, binding site predictions were overall sound, including the proposed affinity-modulating role of CDR1 loop residues. A detailed comparison of a CTLA-4 model and NMR structure has been reported[37], providing a standard for model building in the presence of very low sequence similarity. Importantly, in the model, the beta-strands were well aligned and their periodicity was correctly predicted. Thus, residues on the protein surface could be confidently selected for mutagenesis. Overall, backbone root mean square (rms) deviations of model and NMR structure were between 2.1 and 2.9 Å, dependent on the superposition set. The largest error in the model was the incorrectly predicted conformation of the CDR1-analogous loop. Other loop conformations were reasonably well predicted (with a maximum backbone rms deviation of 1.5 Å). The conformation of the triple proline sequence in the -MYPPPY- motif was modeled as "cis-trans-cis", like in the crystal structures. Relative to the X-ray structures, the only misalignment in beta-strand regions was detected in the C"-region, where TCR V-alpha-like topology could not be predicted from sequence. For CTLA-4, the overall accuracy level, achieved in a difficult prediction, was clearly sufficient for meaningful structure-function studies.

Conclusions

Model-based structure-function studies on members of the CD28 and B7 families illustrate the opportunities and limitations of this approach. For single protein domains, such as the CTLA-4/CD28 binding domains, much can be learned from modeled structures and binding sites can be reliably predicted as long as models are carefully built and experiments are well designed. Model-based analyses are, by definition, less accurate than experiments but insights into structure and binding characteristics that are valuable for biological research can be obtained much faster than experimental structures are determined. A major limitation of the modeling approach is that domain interfaces, relative domain orientations, and molecular complexes are very difficult to predict with accuracy sufficient for practical applications. For example, details of the structures of CTLA-4 complexes with CD80 and CD86 could not have been modeled with certainty. It was possible to conclude, however, based on mapping of mutants, that interactions between these molecules must involve the A'GFCC'C" faces of their V-like domains. With the body of structural and mutagenesis data available by now, we are also beginning to better understand the binding characteristics of these cell surface proteins. Why do CTLA-4 and CD28 bind B7 molecules with different avidities and binding kinetics, although their B7 binding sites are conserved? First, we have obtained evidence that non-conserved residues in CTLA-4 and CD28 proximal to their conserved binding sites are likely to modulate

binding. In addition, it has now become clear that CD80 and CD86 utilize an only partly conserved set of residues for binding, thus providing a rationale for observed differences in their binding characteristics.

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Chapter 2

T Lymphocyte Signaling Pathways Regulated by CD28 and CTLA-4

David J. McKean and Matthew D. Griffin

Introduction

T lymphocytes in peripheral lymphoid tissues are inactive cells that must receive receptor-initiated signals to become activated and participate in an antigen specific immune response. The integration of intracellular signals from the antigen receptor and from one or more costimulatory receptors can stimulate cell division, cytokine production, differentiation and activation of effector cell functions in T lymphocytes. Positive and negative signals from various costimulatory receptors contribute to a dynamic T lymphocyte-mediated response when the immune system is challenged by a pathogen. CD28 is the primary costimulatory receptor in naïve T cells and it is activated by engaging B7-1 (CD80) or B7-2 (CD86) ligands on antigen presenting cells. The integration of signals from coengagement of TCR (signal #1) and CD28 (signal #2) receptors produces high levels of IL-2 production, augmented proliferation and provides essential survival signals for T lymphocytes. In the absence of signal #2, TCR signals are insufficient to fully stimulate previously unstimulated (naïve) T lymphocytes to undergo clonal expansion and differentiation and these T cells can become anergic.[1] Although previously stimulated (secondary) T lymphocytes also can respond to CD28 costimulatory signals, this costimulatory response is lost after successive rounds of antigen stimulation. While CD28 is the best-characterized costimulatory receptor, other CD28 family members (inducible costimulator (ICOS), cytotoxic T lymphocyte antigen-4 (CTLA-4; CD152), programmed death 1 (PD-1), the uncharacterized receptor for B7-H3) and TNF receptor family members (4-1BB, OX-40, HVEM) can differentially augment or inhibit select components of the activation response in secondary T lymphocytes. This review will focus on identifying the intracellular signaling events initiated by ligation of CD28 receptor and CTLA-4 receptors.

CD28 and related family members are disulfide-linked homodimeric glycoproteins that contain a single immunoglobulin variable region-like domain in their extracellular regions.[2] CD28 is constitutively expressed on $CD4^+$ $CD8^+$ thymocytes and naive peripheral T lymphocytes. CD28 expression levels are increased as a result of TCR engagement. B7 family members (B7-1, B7-2) are homodimeric molecules that are inducibly expressed on antigen presenting cells. Other CD28 receptor family members, including CTLA-4, ICOS, PD-1 and the B7H3 receptor, are selectively expressed on previously activated (secondary) T lymphocytes. Although CTLA-4 binds the same ligands as CD28, its expression is not constitutive but is induced following TCR engagement and functions to negatively regulate T cell activation.[2] The physiological importance of CTLA-4-mediated negative regulation is best illustrated by

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the phenotype of CTLA-4$^{-/-}$mice. These animals exhibit massive enlargement of lymphoid organs and die within weeks of birth due to lymphocytic infiltration of multiple organs.[3]

ICOS shares with CD28 the ability to enhance proliferation and lymphokine production in T lymphocytes stimulated with suboptimal signals from the antigen receptor. ICOS exhibits 39% sequence homology with CD28 but lacks the MYPPY amino acid sequence motif in the extracellular domain that is required for CD28 and CTLA-4 binding to B7-1 and B7-2.[4] The receptor binds B7-H2 (B7RP-1), which is expressed on B cells and human (but not murine) dendritic cells, and ligand expression is increased by INFγ.[5] Although ICOS is not constitutively expressed on naïve T cells, ICOS expression is induced within 24 hours by TCR engagement.[4] Anti-ICOS agonistic antibody enhances proliferation in TCR-activated primary T cells as well as augments the production of IL-4, IL-5, IL-10, INFγ but not IL-2.[5] Recent analysis of ICOS knock out mice indicates ICOS is important for humoral responses to T dependent antigens and CD40-dependent Ig class switch.[5] The ICOS null mice are hyper-responsive to autoimmune encephalomyelitis, suggesting that ICOS may regulate inflammatory cytokines. ICOS has a 37 residue cytoplasmic domain that has limited sequence homology to CD28 but has high homology (78% identity; 87% conserved) in comparisons of human and mouse ICOS. Although little has been reported about ICOS signaling mechanisms, the cytoplasmic domain does not contain the PXXP SH3 binding domain, which is an important component in CD28 signaling. ICOS does share the YMNM motif that regulates CD28 signaling and this SH2 binding motif may play an important role in regulating ICOS-initiated signaling events (Fig. 1).

PD-1 is expressed on thymocytes at the transition between $CD4^-$ $CD8^-$ and $CD4^+$ $CD8^+$ development and is inducibly expressed on T, B and myeloid cells.[6] It functions as a negative regulator of the immune response in that PD-1-deficient mice develop a variety of autoimmune-like diseases. PD-1 is a distant relative of the other CD28 family members, sharing little sequence homology in the cytoplasmic domain (see Fig. 1) and maps to a different chromosomal locus than CD28, CTLA-4 and ICOS.[7] The cytoplasmic domain contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) but does not have the YMNM motif shown to be important for CD28 signaling. The limited data available on PD-1 signaling was recently reviewed.[8]

CD28 Costimulation

Models of CD28 Costimulatory Effects

Coengagement of the TCR and CD28 receptor has been reported to initiate multiple signaling pathways that can potentially alter gene transcription and affect functional responses in T lymphocytes. However, no consensus has developed that explains how receptor-proximal signaling events from TCR and CD28 synergistically regulate T cell activation. Models that explain the biologically relevant signaling events initiated by CD28 ligation must be evaluated in the context of how this costimulatory receptor augments TCR-initiated activation responses at different stages of T lymphocyte differentiation. Although mice that do not express CD28 exhibit defects in T cell-mediated responses, these animals can mount T cell-dependent immune responses if challenged repeatedly with high doses of antigen.[9,10] Similarly, in vivo analysis of T cells stimulated with strong peptide agonists or high antigen doses are minimally enhanced by CD28 ligation.[11-14] CD28-independent T cell responses may be regulated by other costimulatory molecules (costimulation redundancy) or may be regulated by strong TCR signals in the absence of costimulation.

CD28 receptors have been proposed to provide costimulatory effects by amplifying TCR-initiated signals or alternatively by producing unique signals that synergize with TCR-initiated signaling pathways.[15] Consistent with the role of CD28 as a potentiator of TCR-induced signals, only a limited number of unique signaling events have been attributed

hCD28 PFWVLVVVGGVL ACYSLLVTVAF I I FWVRSKRSRLLH SD**YMNM**T**PRRP**GPT RKHYQ**PYAP** PRDFAAYRS
mCD28 LFWALVVVAGVL FCYGLLVTVALCV I WTNSRRNRLLQSD**YMNM**T**PRRP**GLT RKPYQ **PYAP**ARDFAAYRP

hCTLA-4 FLLWILAAVSSGLFFYSFL LTAVSLSKMLKKRSPLTTGV**YVKMPPTEP**ECEKQFQPYFIPIN
mCTLA-4 FLLWILVAVSLGLFFYSFLVTAVSLSKMLKKRSPLTTGV**YVKMPPTEP**ECEKQFQPYFIPIN

hICOS KFWLP I G CAAFVVVC I LGC I LICWLTKKKYSSSVHDPNGE**YMFM**RAVNT AKKSRLT DVTL
mICOS KLWLPV GCAAFVVVLL FGC I LI I WFSKKKYGSSVHDPNSE**YMFM**AAVNT NKKSRLAGV TS

hPD-1 T L VVGV VGGLLG S - LVLLV - WVLAVI CSRAA RGT IGA RRT GQPLKEDPSAVPVFSVDYGELDFQWREK
mPD-1 GMV I G I MSALVG I PVLLL L AWALAVFCST SMSE ARGAGS KDDTLKEEPSAAPVPSVAY EELDFQG REK

hPD-1 TPEPPVPCV PEQTEYAT I VFPSGMGTS S P ARRGSADG PRSAQPLRP ----EDGHCSWPL
mPD-1 TPELPTACVH ---TEYAT I VFTEGLGAS AMGRRGSADGL---- QGPRPP RHEDGHCSWPL

Figure 1. Sequences of the cytoplasmic domains of human and mouse CD28 (169), CTLA-4 (116), ICOS (170), and PD-1 (171). Underlined residues are the putative transmembrane regions identified in the original published sequence. There are a series of basic residues that follow the transmembrane regions (in bold) in both ICOS and CD28. The SH2 binding domain (YMNM) in ICOS and CD28 and the two SH3 binding motifs (PXXP) in all sequences are also in bold.

to CD28. However, CD28 signals do not amplify all TCR-initiated signals.[16,17] Thus, models of CD28 costimulation must explain how coengagement of TCR and CD28 can selectively alter TCR-proximal signaling events, resulting in the synergistic activation of select downstream signaling pathways. In contrast to the activation responses initiated by costimulation of peripheral T lymphocytes, CD28 signals initiate apoptosis in TCR-stimulated $CD4^+$ $CD8^+$ (double positive, DP) thymocytes.[18,19] In addition, although memory T cells express CD28 receptors, CD28 signals are less effective at augmenting TCR-initiated activation responses in memory T lymphocytes.[20,21] Thus, differences in the quantitative or qualitative organization of TCR/costimulatory receptor signaling complexes may exist at different stages of T lymphocyte development.

Effects of CD28 Signals on Lipid Raft Distribution

The lymphocyte plasma membrane contains sphingolipid-enriched subdomains (GEMs) or detergent-insoluble glycolipid (DIG) fractions that contain glycosphingolipids, cholesterol, sphingomyelin and glycosylphosphatidylinositol (GPI)-linked, palmitoylated, or myristoylated proteins.[22] These GEMs appear to correspond to the lipid rafts that are visualized in living cells by binding of cholera toxin B to GM1 ganglioside in the rafts. These lipid rafts selectively compartmentalize kinases, such as Lck and Fyn,[23] and adaptor proteins, such LAT,[24] which are required for TCR signaling. TCR engagement induces actin reorganization and the colocalization of TCRs with lipid rafts.[25] The polarization of rafts occurs concomitantly with the reorganization of the actin/cytoskeleton, resulting in the formation of an immunological synapse at the interface between the T cell and APC.[26] The redistribution of membrane receptors and signaling proteins within the synaptic region results in a small zone within the T cell-APC contact region, termed the supramolecular activation complex (SMAC).[27] It has been proposed that T cell signaling is enhanced and sustained by the colocalization of cell surface receptors and requisite signaling molecules needed to initiate the signaling cascades that control the transcriptional regulation of specific T cell activation responses.[28] One model suggests that the redistribution of cell surface receptors in the SMAC is based on the relative size of the receptors with larger proteins, such as CD45, being squeezed out of the SMAC .[29] Other data indicates that protein redistribution is temporally regulated, with CD45 being first depleted from the central zone but later reentering this region.[30] In contrast, CD4 initially co-localizes with the TCR in the central region but is subsequently excluded from the SMAC.[31]

Viola et al[15] reported that coengagement of TCR plus CD28 receptors stimulates an accumulation of rafts at the synapse whereas engagement of either TCR or CD28 alone does not alter raft distribution. Since raft redistribution requires an actin-myosin-driven transport process, these results suggest that the integration of CD28 and TCR signals can alter cytoskeletal interactions. These observations have led to the proposal that the CD28-dependent redistribution of rafts to the synapse is a general mechanism by which costimulation can amplify TCR signaling.[32] These studies were performed with resting human peripheral blood T lymphocytes and, if the results can be generalized, CD28 should have similar effects on other T cell subsets. Coengagement of TCR and CD28 on DP thymocytes results in synergistic activation of the cells (expression of CD69). However, Ebert et al[33] reported that coengagement of TCR and CD28 in DP thymocytes stimulates a polarization of their actin cytoskeleton but fails to recruit rafts to the synapse. Previously activated T lymphocytes are relatively unresponsive to CD28 costimulatory signals but at the same time these cells can respond to lower levels of TCR engagement. Thus, naïve and secondary T cells may exhibit distinct organization of their TCR signaling complexes on the plasma membrane. Previously activated effector and memory CD8 T cells have been reported to express Lck associated with CD8 at the cell membrane while Lck is homogenously distributed in the cytosol of naïve T cells.[34] By quantitatively and qualitatively augmenting Lck, signals from TCR engagement could be enhanced without initiating CD28-regulated differentiation events that may no longer be needed. Another report showed naïve T cells retain rafts in the cytoplasm rather than localized on the plasma membrane.[35] Three days after TCR stimulation, the rafts were targeted to the cell membrane and Lck was expressed in higher amounts than on naïve T cells. However, neither report showed if these differences between naïve and activated T cells correlated with altered CD28 responsiveness. Balamuth at al[36] showed that activated Th1 and Th2 cells have distinct pattern of membrane compartmentalization of rafts. TCR polypeptides were recruited into rafts and localized to the synapse at the T cell-APC contact site in Th1 cells. However, Th2 cells did not co-localize the TCR at the synapse. These results support the conclusion that Th1 and Th2 cells signal from distinct membrane compartments and that redistribution of TCR into rafts at the SMAC is not required to initiate T cell activation responses. The potential effects of costimulation on these Th1 and Th2 cells were not evaluated in this study. A model in which CD28 effects are mediated primarily from regulating lipid raft distribution explains how CD28 can augment TCR-proximal signaling responses in naïve T lymphocytes. Additional studies are needed to determine if there is a direct correlation between CD28 responsiveness and CD28-dependent reorganization of TCR polypeptides and signaling molecules on the membranes of different T lymphocyte subsets. CD28 can increase the T cell-APC contact but this adhesion property does not require signals generated by the CD28 cytoplasmic domain.[37] As will be discussed below, CD28 signals contribute to engaging the T cell cytoskeleton in reorganizing components of the membrane, but this enhanced synapse formation does not increase all TCR-dependent signals.[16] In contrast, the costimulatory effects of CD2 have been reported to promote raft aggregation and augment equally all of the TCR-initiated transcription factors known to be essential for IL-2 synthesis, presumably by potentiating TCR-initiated signaling pathways.[38] Thus, it seems unlikely that the quantitative increase in TCR signaling that results from CD28 costimulatory-mediated augmentation of synapse formation explains the selective CD28 effects on TCR responses.

CD28-Dependent Signaling Responses

Since neither the TCR nor CD28 receptors exhibit intrinsic kinase activity they must recruit protein-tyrosine kinases (PTKs) to phosphorylate substrates that function as signaling intermediates. The TCR signaling cascade is initiated by activation of Src kinases Lck and Fyn,

which phosphorylate ITAMS on CD3 polypeptides and activate PTKs such as Zap-70 (reviewed in ref. 39). CD28 receptors have been reported to either constitutively or inducibly associate with a variety of signaling molecules (Fig. 2). The cytoplasmic domain of the CD28 receptor consists of approximately 39 amino acids that are highly conserved between humans and mice. Two motifs within the cytoplasmic domain have been shown to regulate distinct T cell activation responses. There are two PXXP motifs in the CD28 cytoplasmic domain and one of these (PYAP, located in the C-terminal region of the cytoplasmic domain) has been reported to bind the SH3 domain of Lck, resulting in an increase in the kinase activity of Lck[40], and bind Itk[44] and Tec[45]kinases. The stimulus-dependent phosphorylation of tyrosine 173 by Lck or Fyn regulates binding of phosphatidylinositol-3-kinase (PI3K),[41] growth factor receptor-bound protein (Grb2),[42] and Grb2-related protein with insert domain (GRID)[43] via the SH2 binding motif YMNM. Together the inducible association of these molecules and their downstream signaling intermediates represent a potentially wide range of signaling options for CD28.

Signaling Events Regulated by Binding to the CD28 PXXP Motif

The two PXXP motifs in the CD28 cytoplasmic domain enable CD28 to function as an adaptor molecule by binding the SH3 motif on Lck. Since the specificity of this binding was determined in vitro using peptides from the CD28 cytoplasmic domain, these interactions presumably occur in a ligand-independent manner.[40] In addition, peptide binding also resulted in an increase in Lck kinase activity. Interestingly, a similar PXXP motif in the CD2 cytoplasmic domain was reported to bind to and activate Fyn. These results are consistent with the observation that crosslinking CD28 induces tyrosine phosphorylation in T cells, albeit a select subset of the substrates phosphorylated by TCR engagement.[37] Subsequent studies expressed different CD28 receptors in CD28-deficient T lymphocytes and showed that mutation of the C-terminal proline residues abrogated the proliferation and cytokine regulatory functions of CD28.[46] However, the CD28-dependent regulation of Bcl-X_L was not affected. These results document distinct roles for the PXXP and YMNM motifs in the CD28 cytoplasmic domain and emphasize the potential importance of the SH2 binding motif in regulating CD28-mediated functions.

The presence of the two PXXP motifs in the cytoplasmic domain also provides potential docking sites for other proteins that contain SH3 binding domains. The adaptor Grb2 has been reported to associate with the C-terminal diproline motif, thereby providing linkage to other signaling molecules regulated by the TCR complex.[47] The potential functions related to CD28-Grb2 interactions will be discussed below. Itk and Tec are evolutionarily related (Tec, Itk, Rlk, Btk) tyrosine kinases that are inducibly phosphorylated by CD28 crosslinking.[44] Itk associates with the N-terminal PXXP motif and Tec associates with both diproline motifs.[48-51] Although protein interactions via PXXP-SH3 domains are usually constitutive, ligation of CD28 increases CD28 association with Grb-2,[50] Itk[44] and Tec,[50] presumably as a result of a conformational change in the CD28 cytoplasmic domain. Analysis of Itk deficient mice (Itk $^{-}$) show that, although the TCR-initiated proliferative response was severely compromised in Itk^{-} mice, proliferation stimulated by coengagement of TCR + CD28 was stronger than observed in Itk wild-type littermates.[51] Itk^{-} mice also appear to have increased sensitivity to IL-2-mediated signaling. Thus, association of Itk with CD28 is not required for CD28 costimulation and Itk may down-regulate CD28-mediated signals. CD28 crosslinking is reported to initiate a Tec-dependent phosphorylation of p62^{dok}, an activation response that is not initiated by TCR engagement.[52] However, either TCR or CD28 engagement can initiate Tec activation, presumably by Src family kinases. Although Tec is reported to mediate CD28 costimulatory-dependent IL-2 gene transcription,[52] the mechanism by which this occurs is incompletely characterized.

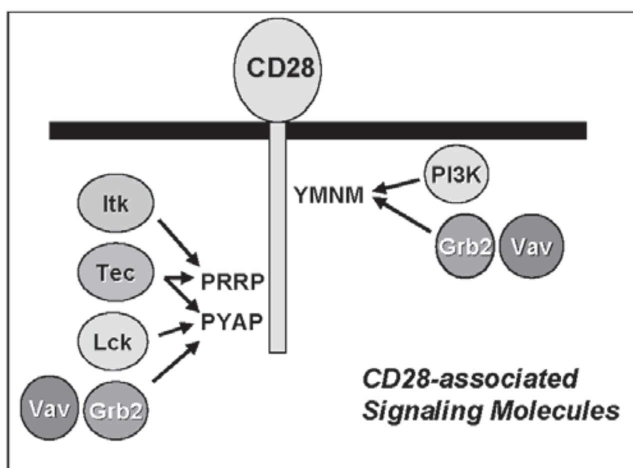

Figure 2. Proteins that inducibly associate with the CD28 cytoplasmic domain.

Previous studies have shown that Lck is the most TCR-proximal kinase activated by TCR ligation. If CD28 augments Lck activity via the PXXP motif, perhaps the select signaling effects mediated by CD28 costimulation occurs as a result of CD28 ligand-dependent compartmentalization of kinase-substrate interactions or by the CD28-dependent activation of inhibitors of specific signaling pathways. The latter effect may contribute to regulating the activation of the ERK MAP kinase pathway. Although TCR signals can stimulate Ras-dependent activation of the ERK MAPK pathway, CD28 coengagement further enhances the ERK response.[53] In addition to activating Ras, TCR signals also activate Rap1, a small G protein that functions as a Ras inhibitor. It was reported recently that CD28-dependent activation of Lck results in the activation of Rap1 GAP, a GTPase activating protein that inactivates Rap1 by catalyzing Rap1-GTP to Rap1-GDP.[54] Thus, CD28 signals antagonize a TCR-dependent negative regulator of the Ras—ERK MAPK pathway, resulting in augmented ERK activity. T cell hyporesponsiveness (anergy) has been associated with defects in ERK activity, and anergic cells have been reported to have high levels of Rap1.[55] CD28-dependent regulation of Rap1 activity provides a mechanism whereby costimulatory signals can protect T cells from anergy.

Signaling Events Regulated by Binding to the CD28 YMNM Motif

There are four tyrosine residues in the CD28 cytoplasmic domain. Although there have been reports that several of these tyrosines can be inducibly phosphorylated,[56,57] there is general agreement that the Tyr in the Src homology domain 2 (SH2) binding motif (Y^{173}MNM) is inducibly phosphorylated by Src family kinases, Lck and Fyn, after CD28 crosslinking. In 1994 the first report was published showing a physical interaction between class 1 phosphatidylinositol 3-kinase (PI3K) and CD28.[41] Since then multiple reports evaluating the effects of this association have produced conflicting results. PI3K phosphorylates phosphatidylinositol, phosphatidylinosital 4-phosphate, and phosphatidylinosital 4,5 bisphosphate generating PI 3-P, PI 3,4-P_2 and PI 3,4,5-P_3, respectively. The association of PI3K with CD28 brings PI3K to the plasma membrane where it can interact with its substrates. These phospholipid substrates can alter a variety of biological responses by regulating signaling intermediates including protein kinase C (isoforms δ, ε, θ and ζ), AKT (protein kinase B) and calcium.[58] Some of the analyses of CD28-PI3K interactions were done in Jurkat

T cells, which fail to express PTEN (a phosphatase that removes D-3 phosphate of the inositol ring) and, therefore, do not appropriately metabolize PI3K substrates.[59] Even with the caveat that Jurkat may not be an appropriate model to study PI3K-mediated signaling responses, there is no consensus about the role of PI3K-mediated pathways on CD28-mediated costimulatory effects. Disrupting CD28-PI3K interactions has been reported to inhibit IL-2 production[49,60-63] while other reports have failed to observe this inhibition.[64-68] Interestingly, transgenic mice that express a CD28 receptor with Y^{173} mutated have been reported to exhibit altered IL-2 responses[68] while another analysis of similar mutant mice reported no defects in IL-2 responses or proliferation but observed defects in Bcl-X_L expression.[69] ICOS does not have the SH3 PXXP binding motifs but does have an SH2 binding motif (YMFM). Since ICOS has been reported to not efficiently regulate IL-2 gene transcription, one might anticipate that the SH2 binding motif is not responsible for the CD28-mediated regulation of IL-2.

Vav consists of three family members (Vav-1, Vav-2, Vav-3), which are expressed in T lymphocytes. Although the role of Vav-1 has been extensively studied in T lymphocytes, Vav-$2^{-/-}$ mice exhibit no T cell defects[70] and Vav-3 knockouts have not been reported. Vav proteins are guanosine nucleotide exchange factors (GEF) for Rho and Rac family proteins and also function as adaptor molecules. Vav-1 has a pleckstrin homology (PH) domain, a calponin-homology domain, an acidic region, a Dbl-homology domain, a zinc finger domain, two SH3 domains and one SH2 domain (reviewed in ref. 71). The GEF activity of Vav-1 is initiated by Lck-mediated tyrosine phosphorylation (Y^{174}), which alters the Vav-1 configuration and releases an autoinhibitory loop.[72] Although either TCR or CD28 signals can stimulate Vav-1 tyrosine phosphorylation, coengagement of TCR and CD28 synergistically increases Vav-1 phosphorylation.[73] Phosphatidylinositol-3,4,5-triphosphate, a product of PI3K, also regulates Vav GEF activity. The activity of Vav-1 as a GDP/GTP exchange factor regulates the activation and plasma membrane localization of Rho/Rac molecules, which in turn regulate actin polymerization and signaling cascades including JNK, p21-activated kinase (PAK), NF-κB and PI-4,5-kinase (PIP5 kinase).[74] The SH2 domain of Grb2 binds simultaneously to the phosphorylated tyrosine in the CD28 cytoplasmic domain while the Grb2 SH3 domain binds to the Vav-1 N-terminal PXXP motif.[75] A Vav-1 SH2 domain also can bind to the inducibly phosphorylated (by Zap70) adaptor SPL-76, which is bound to phosphorylated LAT via Gads. Through these interactions a multi-molecular signaling complex is formed consisting of TCR, Zap70, CD28, LAT, PLCγ1 and other associated signaling molecules within lipid rafts.

Multiple biological roles have been associated with Vav-1-mediated GEF activity and adaptor functions. Analyses of Vav overexpression in cell lines and loss of function Vav-$1^{-/-}$ mice have demonstrated that Vav-1 regulates TCR-initiated calcium fluxes, activation of the extracellular signal-regulated mitogen-activated protein kinase (ERK MAPK) pathway, and the activation of the NF-AT and NF-κB transcription factors.[76-78] The altered calcium fluxes in Vav-$1^{-/-}$ T cells occur as a result of having impaired activity of phospholipase C-γ1 (PLC-γ1) and subsequent production of inositol triphosphate (IP_3). Although PLC-γ1 phosphorylation was normal in these cells, the defect in PLC-γ1 activity may be due to alterations in the availability of the PLC-γ1 substrate, PIP_2.[79] Vav-1 may regulate the levels of PIP_2 by altering Rac-1-mediated regulation of PIP-5 kinase. Vav may be able to regulate calcium fluxes independently of its GEF activity because constitutively active Rho, Rac and Cdc42 cannot stimulate calcium fluxes.[80] As will be discussed below, the mechanisms by which CD28 regulates calcium fluxes is controversial in that CD28 has been reported to directly regulate PLCγ phosphorylation. Although NF-AT transcriptional activity is regulated by the calcium-dependent phosphatase, calcineurin, it also can be coregulated in some promoters by an adjacent AP-1 transcription factor. Vav-1 may alter AP-1 by regulating the Ras-dependent ERK MAPK pathway as a result of stimulating a PLC-γ1-dependent activation of the diacylglycerol-dependent exchange factor Ras GRP[81] and by the Rac1-dependent activation of the JNK pathway.[75]

Several recent papers provide evidence for a CD28-mediated enhancement of Vav-1 association with SLP-76, resulting in enhanced NF-AT activity and Rac1-dependent cytoskeleton organization.[82,37] Although PLC-γ1 association with LAT is unaffected, the tyrosine phosphorylation of PLC-γ1 was reduced in a comparison of wild-type CD28 vs a mutant CD28 receptor in which the C-terminal 30 amino acids were deleted and were co-engaged with TCR. Thus, CD28 signals may increase NF-AT activity by increasing PLCγ1 phosphorylation. Similarly, SLP-76 phosphorylation was also augmented by CD28 signals. In contrast, CD28 costimulation did not alter TCR-induced ZAP-70 activation and LAT phosphorylation. NF-AT activation was restored in CD28 mutant T cells that overexpress Itk, and CD28 engagement triggers tyrosine phosphorylation and kinase activation of Itk. A model was proposed in which CD28-dependent signals activate Itk, which in turn phosphorylates PLC-γ1 after it associates with LAT. CD28 signaling also contributes to enhanced phosphorylation of SLP-76 and Vav-1 (and increases its GEF activity), thereby augmenting the association of SLP-76 with Vav-1. This model has several discrepancies with previous studies. One study reported that CD28 costimulation of IL-2 expression and proliferation were reduced but not absent in Vav-1$^{-/-}$ mice.[78] In addition, the tyrosine phosphorylation of PLC-γ1, LAT and SLP-76 were normal in Vav-1$^{-/-}$ T cells. The potential role of Vav-3 in regulating the phenotype of Vav-1$^{-/-}$ mice, however, was not evaluated. Vav-1 association with SLP-76 also was reported to be not essential for IL-2 transcription.[83] Itk$^{-/-}$ mice not only respond to CD28 costimulation but exhibit an increased IL-2 response to TCR stimulation, indicating that Itk may negatively regulate CD28 costimulation. As discussed previously, it remains possible that Tec can also contribute to the putative role of Itk in this model but the mechanism of this activity needs to be elucidated. At least some of the discrepancies in these reports also may reflect redundancy in CD28 costimulatory signaling effects. By enhancing the inducible phosphorylation or associations of signaling components in different T cell subsets, CD28 signals may be able to affect multiple nodules in the TCR signaling cascades within a population of T cells. Quantitative differences in specific responses also could be affected by the state of differentiation or activation of the T cells being assayed.

Another biological role that has been attributed to Vav is the regulation of NF-kB activity.[78] NF-κB is a transcription factor that regulates a variety of genes that participate in the T lymphocyte activation program.[84] The IL-2 promoter contains a NF-κB binding site as well as a CD28RE site, which binds both NF-κB and AP-1 transcription factors[85] or perhaps NFAT/NF-κB and AP-1.[86] These transcription factors are synergistically regulated by coengagement of TCR and CD28.[87,88] NF-κB is sequestered in the cytosol as a complex associated with members of the IκB protein family and NF-κB is translocated to the nucleus after the IκBs are inducibly phosphorylated and subsequently degraded. The AP-1 transcription factor, which consists of homo- or heterodimers of Jun and Fos family members, can be regulated at both transcriptional and postranscriptional levels. However, JNK, which phosphorylates and activates c-Jun, is also regulated synergistically by TCR and CD28 signals.[89] Within the past several years multiple papers have identified signaling molecules that participate in regulating NF-κB activation resulting from coengagement of TCR and CD28. Coengagement of TCR and CD28 in Vav-1$^{-/-}$ T cells resulted in no degradation of IκB-α and no NF-κB activity,[78] implicating Vav-1 as a requisite component in the activation of NF-κB. Multiple reports have shown a necessary role for the calcium-independent isoform of protein kinase C, PKC-θ, in the TCR/CD28 activation of NF-κB and components of the AP-1 complex.[90-93] Following TCR engagement, PKC-θ translocates to the TCR-enriched SMAC at the contact site between the APC and T cell.[27] Unlike other PKC isoforms, PKC-θ membrane localization is not dependent on calcium and/or diacylglycerol signals. Although the mechanism by which PKC-θ is inducibly translocated to the synapse is incompletely understood, both intact Vav and PI3K activity are required.[94-96] PKC-θ has been reported to be constitutively associated with Vav-1

and to dissociate upon stimulation through TCR and CD28.[97,98] Although Vav is located upstream of PKC-θ in this signaling cascade,[93,94] there are conflicting reports about the signals needed to initiate PKC-θ translocation. Bi et al[95] reported that Lck but not Zap-70 activity was needed, while Herndon et al[16] reported that both ZAP-70 and SLP-76 were required. Vav-1 forms a stable complex with SLP-76 as a result of the Vav-1 SH2 domain binding to Y^{113} and Y^{128} in the SLP-76 N-terminal region, resulting in enhanced activation of NF-AT, Rac and Cdc42.[97,98] Mutation of the Vav-1 binding site on SLP-76 was defective in restoring NF-κB activity in SLP-76 deficient Jurkat cells,[16] suggesting that ZAP-70-mediated phosphorylation of SLP-76 and subsequent Vav-1 binding is needed for a TCR/CD28-mediated NF-κB response.

Multiple reports have demonstrated that Vav-$1^{-/-}$ DP thymocytes have defects in positive and negative selection.[96,99-101] TCR transgenic Vav-$1^{-/-}$ DP thymocytes fail to up-regulate early activation markers (CD69, CD5) but have normal TCR-induced tyrosine phosphorylation, and activation of MAPK, JNK and NF-κB.[96] The Vav-$1^{-/-}$ thymocytes exhibit defects in calcium mobilization and cytoskeleton reorganization. Although engagement of TCR + CD28 stimulates activation and differentiation of naïve T cells, these signals stimulate activation (CD69 expression) and apoptosis in DP thymocytes. The TCR/CD28-dependent apoptotic response is defective in Vav-$1^{-/-}$ DP thymocytes and could be rescued by PMA but not calcium ionophore treatment. Since PKCθ is the only PKC isoform that physically associates with Vav-1 in thymocytes and T lymphocytes, the authors suggested that Vav may regulate thymocyte apoptosis via PKCθ. PKCθ clearly regulates distinct events in T cells and in thymocytes. In contrast to the NF-κB defect in PKC$\theta^{-/-}$ T lymphocytes, DP thymocytes from PCK$\theta^{-/-}$ mice have normal TCR-initiated NF-κB responses. PKC-$\theta^{-/-}$ mice also do not exhibit alterations in either positive or negative selection.[92] If the TCR/CD28-initiated Vav signals are needed to initiate the thymocyte activation program before apoptosis is initiated, the observed defects in apoptosis result from inhibition of activation and not from blocking of apoptotic signals.

The identity of the components in the PKC-θ-dependent signaling pathway is also controversial. Koshnan et al[102] reported that PKC-θ physically associates with the IKK complex, which consists of IKKα, IKKβ and IKKγ and is responsible for the stimulus-induced phosphorylation of IkBs. The TCR/CD28-dependent localization of PKC-θ and the IKK complex in the lipid rafts provides a direct pathway whereby PKCθ can activate the kinases responsible for regulating NF-κB nuclear localization

Another report implicated an intermediary signaling role for the mitogen-activated protein kinase kinase kinases (MAP3K) family member mixed-lineage kinase-3 (MLK3), which is located upstream of the IKK complex in the TCR/CD28-mediated activation of NF-κB and JNK.[103] However, although the PKC-$\theta^{-/-}$ T cells exhibit complete loss of NF-κB responses, there was no defect in TCR/CD28-activation of JNK.[94] Tpl-2 (Cot) is another MAP3K family member that has been associated with TCR/CD28-mediated activation of NF-κB.[104] Expression of a kinase-deficient Tpl-2 inhibited TCR/CD28-initiated but not TNFα-initiated activation of NF-κB in Jurkat cells. In contrast, expression of a kinase-deficient NF-κB-inducing kinase (NIK) inhibited both the TCR/CD28 and TNFα pathways as well as the Tpl-2-mediated activation of NF-κB. Although PKC-θ selectively induced the induction of IKKβ, Tpl-2 and NIK have been reported to induce both IKKα and IKKβ.[91] Thus the authors suggest that since TCR/CD28 signals can activate both IKKα and IKKβ, there must be multiple signaling cascades leading to TCR/CD28-initiated NF-κB activation. As discussed previously, this model is not consistent with the observations made with the PKC-θ deficient mice in that T cells from these animals did not produce a TCR/CD28-initiated NF-κB response.

Akt also has been identified as a TCR/CD28 signaling intermediate that is capable of regulating the CD28RE in the IL-2 promoter.[105] Akt is a downstream target of PI3K and is upstream of the kinases in the MAP3K family, which include MLK3, Tpl-2 (Cot) and MKK1.

Expression of constitutively active Akt in T lymphocytes from CD28 deficient mice restored TCR-induced production of IL-2. Engagement of either TCR or CD28 activated Akt (although no synergistic response was observed from coengagement), resulting in NF-κB activation. However, in normal T lymphocytes engagement of TCR activates NF-κB (suboptimal levels) while engagement of CD28 alone does not.

The synergistic activation of NF-κB by coengagement of TCR and CD28 and the subsequent regulation of the IL-2 promoter maybe an example of the value of the sustained signaling response mediated by CD28-enhanced raft events. NF-κB transcription factor is comprised of multiple homo- or hetero-dimeric proteins. As in most other NF-κB responses, TCR/CD28 engagement stimulates an acute NF-κB nuclear localization response (20 to 60 minutes) that consists of RelA-containing NF-κB complexes. However, the IL-2 promoter selectively binds c-Rel-containing NF-κB complexes,[106] which translocate to the nucleus during a prolonged (14 hour) NF-κB response. TCR/CD28-mediated NF-kB responses induce c-Rel expression and IkBα expression, resulting in the production of c-Rel-IκBα complexes in the cytosol (c-Rel and IκBα are NF-κB-responsive genes)[88]. Signals from TCR/CD28 selectively degrade IκBα that is associated with c-Rel, resulting in prolonged c-Rel nuclear translocation.

CD28-Associated Phosphatases

Most of the CD28-regulated signaling cascades described above rely on the inducible phosphorylation of proteins either at tyrosine or serine/threonine residues. These signaling pathways are in turn negatively regulated by phosphatases. Two reports have identified phosphatases that bind to the cytoplasmic domain of CD28 in yeast two hybrid screen assays. Chuang et al[107] showed that the serine/threonine phosphatase PP2A constitutively associates with the CD28 and CTLA-4 cytoplasmic domains by binding to the residues adjacent to the YMNM motif (YVKM in CTLA-4). This CD28-PP2A association was detectable in Jurkat cells and was negatively regulated by phosphorylation of the YMNM motif by Lck in an in vitro assay. However, ligand-dependent dissociation of PP2A from CD28 was not shown. Since PP2A has been reported to regulate the ERK MAPK pathway, the authors suggested that the CD28 costimulation might prevent PP2A-mediated dephosphorylation of ERK that occurs from TCR stimulation alone. Marti et al[108] reported that MAP kinase phosphatase-6 (MKP6) associates constitutively with residues adjacent to the C-terminal tyrosine (Y^{200}) in the CD28 cytoplasmic domain. Importantly, they did not detect this association in human peripheral blood T cells. The association was detectable in Jurkat cells that over-expressed MKP6 and the association was not altered by CD28 crosslinking. The authors suggested that the CD28-MKP6 association might facilitate the localization of MKP6 to the membrane where it could interact with its MAPK substrates. Additional studies are needed to show ligand-dependent alterations of phosphatase functions in normal lymphocytes.

Model of CD28 Signaling

Any model that tries to explain CD28-initiated signaling cascades is constrained by the large amount of conflicting data developed from analyses of cell lines and different T lymphocyte populations. The analysis of the CTLA-4 crystal structure shows the receptor dimer can bind two bivalent B7 molecules, such that when CTLA-4 is full ligated, an extended concatemer (…-B7-CTLA-4-B7-B7-CTLA-4-B7…) is be formed.[109] This unique ligand-induced receptor oligomerization may contribute to the signaling effects initiated by CTLA-4 and other CD28 receptor family members. The ligand-dependent concatemerization of multiple CD28 receptors and their subsequent association with signaling intermediates in the TCR signaling complex may significantly contribute to augmenting TCR-initiated signals. It seems unlikely that the only function of CD28 is to enhance the activation of TCR-proximal kinases. Multiple reports have identified unique contributions of CD28 to the TCR-initiated activation response.

The costimulatory effects of CD28 occur in two overlapping phases. During the initial acute response, CD28 signals initiate a cytoskeletal reorganization that enhances the association of signaling components that comprise the TCR signaling complex. The synergistic interactions of signals from CD28 and TCR also regulate a series of prolonged responses, exemplified by NF-kB transactivation that can continue for ten hours or more. Coengagement of TCR and CD28 stimulates the phosphorylation of CD28 $Y^{173}$by Lck or Fyn, providing a docking site for proteins with SH2-containing binding sites (Fig. 3). Grb2 binds to CD28 by associating with both the SH2 binding domain and the SH3 PXXP binding domain. Vav proteins bind to CD28 via Grb2, and TCR and CD28 signals, which phosphorylate Vav at Y^{174} releasing an autoinhibitory loop, synergistically activate the GEF activity of Vav. The Vav GEF activity stimulates Rho/Rac –dependent actin cytoskeletal reorganization. This activation of Vav likely plays a major role in the rapid reorganization of TCR polypeptides and signaling components into lipid rafts, which coalesce at the synapse. The cytoskeletal reorganization facilitates the development of a functional TCR multisubunit complex that amplifies and prolongs T cell responses. However, the structural organization of the TCR complexes within the synapse in different T lymphocyte subsets needs additional study.

PI3K physically associates with CD28 via the SH2 binding domain on the p85 polypeptide, bringing PI3K to the membrane where it can interact with its phospholipid substrates. Although PI3K substrates may activate Vav, it is also likely that Vav activates PI3K. In either case, the potential roles of PI3K phospholipid substrates in regulating IL-2 gene transcription are very controversial. It is likely that the CD28 SH3 binding motif plays a significant role in regulating IL-2 gene transcription. This SH3 binding domain can associate with Lck, Tec and Itk kinases as well as potentially function as a bridge to other signaling molecules via the Grb2 adaptor. CD28-association with Itk results in its activation, which in turn phosphorylates and activates PLCγ1. Although Itk also may function as a negative regulator of CD28-dependent signals, the mechanism by which this occurs is uncharacterized. Similarly, the mechanism by which Tec may regulate IL-2 production is incompletely characterized. The adaptor function of Vav also contributes to the assembly of the TCR signaling complex via association with SLP-76 and its interaction with LAT. Vav is constitutively associated with PKC-θ and TCR/CD28 signals stimulate the dissociation of this complex, resulting in PKC-θ translocation to the synapse. Vav-regulated activation of PKC-θ controls the nuclear localization of NF-κB in T cells, although the signaling intermediates that link PKC-θ with the IKK complex remain controversial. Additional CD28-derived signals also may regulate NF-kB activity.

It is likely that CD28 plays distinct roles in the activation response of T cells at different stages of differentiation. Clearly the differences in both the signaling events and biological effects mediated by CD28 costimulation of TCR-activated DP thymocytes and naïve T cells are striking. These different costimulatory effects in distinct T cell subsets may contribute to the apparently contradictory results observed in analyses of certain CD28-regulated signaling pathways. T cell lines such as Jurkat have made very significant contributions to our understanding of TCR signaling. These cells have been useful because they are easy to grow, are phenotypically homogenous (within a lab) and are easy to transfect with exogenous genes. However, Jurkat cells have unique signaling defects and their activation program may not be representative of different T lymphocyte subsets. Thus, it will be important to examine CD28 signaling cascades in normal T cell populations at different stages of differentiation. The development of new transient transfection technologies[110] and retroviral transduction technologies for analyses in purified T lymphocytes or bone marrow transplants[111] makes the introduction of genes encoding altered signaling components feasible for studies in normal T cells. Gene over-expression analyses have significant limitations in that they provide information about what can happen rather than necessarily what does happen under physiological conditions.

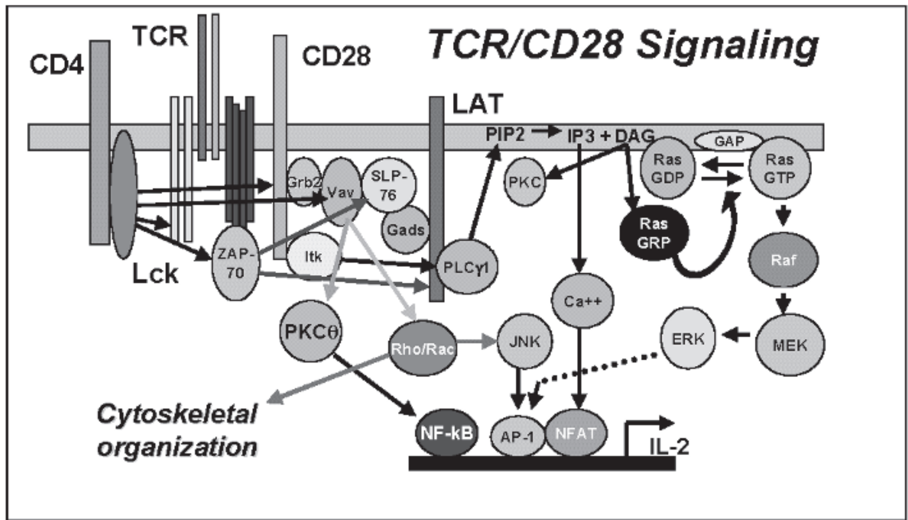

Figure 3. Intracellular signaling cascades associated with coengagement of TCR and CD28 receptors.

However such studies can be very useful as a rapid screen of the effects of signaling intermediates that can be evaluated further with gene knock-out/in approaches.

CTLA-4 Signaling

The interaction of CTLA-4 (CD152) with its natural ligands B7-1 (CD80) and B7-2 (CD86) is an essential pathway for negative regulation of T cell activation.[112-115] It is, therefore, self-evident that a detailed understanding of the molecular mechanisms underlying CTLA-4-mediated negative regulation would represent an important advance in immunologic science. Interestingly, studies of the intracellular signaling events that accompany CTLA-4 engagement have failed to generate a unifying mechanistic model and the area remains a source of controversy. The current level of understanding is, perhaps, best illustrated by two apparently paradoxical observations:

a. The intracytoplasmic (and presumably "signaling") domain of CTLA-4 has been conserved in evolution to a remarkably high degree indicating essential functions for this entire portion of the molecule.[116-118]
b. Mutant forms of CTLA-4 that lack specific key residues or portions of the intracytoplasmic domain remain capable of mediating negative regulation when expressed in T-cells.[119-124]

These observations suggest that in vivo ligation of CTLA-4 by B7-1 or B7-2 may regulate T cell intracellular events by two or more distinct mechanisms. They also raise the possibility that different T cell sub-populations may harness the regulatory capacity of CTLA-4 through different molecular pathways. This latter consideration should be borne strongly in mind when attempting to assimilate the (sometimes conflicting) data from experiments carried out in diverse cell populations and using a variety of ligating agents. Focusing on that portion of the literature dealing specifically with the molecular events associated with CTLA-4 engagement, this review will summarize evidence that individual portions of the protein contribute separate important elements to its signaling profile. In addition to examining data pertaining to the activity of conventional "signaling cascades", emphasis will be placed on studies that have examined intracellular protein compartmentalization and molecular events occurring at the T cell—Antigen Presenting Cell (APC) interface.

The Extracellular Domain: Structural Features of CTLA-4 Interactions with B7-1 and B7-2

The realization that CD28 and CTLA-4 share ligands but mediate opposing effects raised the possibility that the inhibitory function of CTLA-4 simply represents competition for ligand binding. The fact that CTLA-4 is inducible upon T cell activation rather than constitutively expressed like CD28 is compatible with a model whereby the time-period for CD28 to freely interact with its ligands is limited by the appearance of a competitor. Investigation of the stoichiometry and binding affinities of the two receptors for B7-1 and B7-2 added further support to this model.[125] Both CD28 and CTLA-4 are expressed as homodimers with a single disulphide bond in the extracellular domain. Each homodimer binds two molecules of B7-1 or B7-2.[126,127] Kinetic studies comparing the binding characteristics of CD28 and CTLA-4 to their ligands demonstrated rapid "off rates" for both receptors suggesting a propensity for brief ligation events – a characteristic feature of TCR-related intercellular interactions.[128] However, CTLA-4 exhibited strikingly more rapid "on rates" and, for this reason, behaves as a high avidity receptor in comparison to CD28.[129] Clearly this profile would allow CTLA-4 to claim the lion's share of ligand occupancy in the presence of limiting amounts of B7-1/B7-2. In addition, CTLA-4 has been found to have somewhat different binding kinetics to B7-1 and B7-2. Most notably, its dissociation from B7-2 is 4- to 8-fold more rapid than from B7-1.[124,130] Using surface plasmon resonance Greene et al[129] have calculated that binding of CTLA-4 is between 2500- and 270-fold stronger than CD28 for B7-1 and between 570- to 70-fold stronger for B7-2. The high avidity of CTLA-4 has been harnessed experimentally and clinically to block CD28 co-stimulation in the form of a soluble fusion protein (CTLA-4Ig).[112]

Competitive inhibition of CD28 engagement by B7-1 and B7-2 cannot fully account for the negative regulatory effects of CTLA-4. In the first place multiple in vitro and in vivo studies have shown that combined ligation of TCR, CD28 and CTLA-4 by non-cross-reactive monoclonal antibodies results in attenuated T cell activation when compared to ligation of TCR and CD28. Furthermore, the stimulation of pre-activated T cells through the TCR alone can be effectively inhibited by CTLA-4 engagement in the absence of a CD28 signal.[131,132] Nonetheless, two recent studies serve to confirm that competition for ligand between CTLA-4 and CD28 does indeed play a significant physiological role. Carreno et al measured IL-2 production by a panel of Jurkat transfectants using beads coated with anti-CD3 mAb in combination with either anti-CTLA-4 mAb or with B7-1Ig.[122] They found that ligation of a CTLA-4 mutant lacking all but the proximal two intracytoplasmic residues resulted in IL-2 inhibition only when the shared ligand (B7-1) was present. Masteller et al found that the lethal lymphoproliferative phenotype of the CTLA-4 null mouse could be substantially relieved by transgenic expression of a similar "tailless" CTLA-4 construct.[123] These animals demonstrated age-dependent T cell activation in lymph node and spleen but were not found to have lymphocytic tissue infiltration. Animals transgenic for wild-type or tyrosine mutant CTLA-4 had complete phenotype reversal. Thus, current data would indicate that competition for B7-1/B7-2 binding represents a bona fide mechanism for CTLA-4-mediated T cell inhibition but is not essential for it to occur.

The Transmembrane and Proximal Intracytoplasmic Domain: Interactions with the Proximal TCR Signaling Apparatus

In order for CTLA-4 to inhibit T cell activation it is necessary for it to be localized at the point of TCR engagement. As discussed in greater detail below, TCR ligation results both in enhanced expression of CTLA-4 and in the targeted retention of CTLA-4 at the T cell/APC interface.[133] Experimental systems in which TCR and CTLA-4 ligands are expressed on separate cell populations or beads have resulted in unchanged or even enhanced activation

events.[120,132,134] In contrast, CD28 remains capable of delivering a costimulatory signal when engaged "in trans" to the TCR. Interestingly, co-ligation of both CD28 and CTLA-4 "in trans" to the TCR did not result in inhibition.[135] These observations suggest that a physical proximity to molecular events associated with the proximal TCR signaling apparatus is an important if not essential element of CTLA-4 function.

It is therefore of relevance that CTLA-4 has been shown to be physically associated with the TCRζ chain in activated T cells as well as in non T cell transfectants.[136] The binding was enhanced by co-expression of the tyrosine kinase Lck even when tyrosine residues in the CTLA-4 intracytoplasmic domain were mutated to phenylalanine. The finding implies that phosphorylation of TCRζ itself (or an as yet unidentified intermediary protein) induces a physical interaction between this component of the TCR signaling apparatus and CTLA-4. The portion of the CTLA-4 intracytoplasmic domain that is involved in this interaction has not been delineated but may be separate from other motifs (see following section) for which binding partners have been identified. In this regard, Nakaseko et al have observed that a truncated CTLA-4 protein containing only the proximal 7 amino acids of the intracytoplasmic tail remained capable of inhibiting ERK2 activation and IL-2 secretion when expressed in a T cell clone.[119] In contrast, truncated mutants retaining only the proximal 3 or 6 residues failed to inhibit when expressed in Jurkat cells by Carreno et al[122] and by Cinek et al[120] Although, these studies should be interpreted with caution, they raise the possibility that a discreet portion of the intracytoplasmic domain of CTLA-4 participates in an inducible interaction with TCRζ and contributes to the inhibition of primary TCR signaling events. Such an interaction could facilitate the recruitment of negative regulatory proteins bound to more distal portions of the CTLA-4 molecule (e.g., a phosphatase – see below) or, as speculated by Nakaseto et al, could also form the basis of an independent inhibitory mechanism.[119]

The concepts discussed so far might seem to place the functional importance of the intact intracytoplasmic domain in doubt. It should be pointed out, however, that few studies have examined the molecular mechanisms of CTLA-4 function in vivo or under co-ligation conditions other than immobilized antibody/ligand. Furthermore, the use of mutant proteins may simultaneously enhance and reduce the contribution of individual mechanisms. A good example of this is the effect of mutations of the Y201 residue. On the one hand this mutation results in increased surface expression of CTLA-4 (see below) and may increase its ability to compete for ligand and to directly interact with TCRζ. On the other hand the recruitment of negative regulatory components bound to the Y^{201}VKM motif may be deficient resulting in diminishment of other mechanisms of action. In fact, Schneider et al have recently demonstrated that functional differences between wild type CTLA-4 and a Y201F mutant could be observed using soluble but not immobilized antibody.[124] Thus, both the experimental strategy as well as the cell population used for a given study may result in exaggerated emphasis or de-emphasis of specific molecular pathways.

The Intracytoplasmic Domain: Protein-Protein Interactions That Regulate Intracellular Trafficking and Signaling Modules

Analysis of the intracytoplasmic domain of CTLA-4 reveals the presence of potential binding motifs for protein-protein interaction. These include two tyrosine-containing sequences (Y^{201}VKM and Y^{218}FIP) suggesting binding to SH2 domains as well as a proline-rich sequence (PPTEP) with potential to bind to SH3 domains. A number of binding partners have been identified and additional interacting proteins likely exist. Table 1 provides a summary of the proteins that have been shown to interact with the intracytoplasmic domain of CTLA-4 and their possible functional significance. This area has produced both novel insights into the intracellular regulation of CTLA-4 and conflicting findings regarding the functional importance of individual binding partners. The existing data can be summarized under four headings:

Trafficking Proteins

Initial studies of the localization of CTLA-4 revealed that, unlike CD28, the protein was predominantly expressed intracellularly rather than on the cell surface.[133,137,138] Furthermore, as shown by Linsley et al, CTLA-4 undergoes shuttling to and from the cell surface in secretory granules and endocytic vesicles.[133] In this same study, T cell activation by an APC or an anti-CD3-coated coverslip resulted in focal accumulation of CTLA-4 at the point of TCR engagement. The intracellular localization of CD152 was shown to be linked to the peptide motif TTGVYVKMPPT within the intracytoplasmic domain.[137] A number of groups subsequently demonstrated that a portion of this sequence (GVYVKM) interacts with the AP50 (μ_2) subunit of the AP-2 clathrin-associated adaptor protein complex.[139-142] As the AP-2 complex is involved in the endocytosis of surface proteins contained in clathrin-coated pits, the discovery of this interaction provided a mechanistic explanation for the cycling of CTLA-4 from the cell surface. The interaction with AP50 was shown to depend upon the tyrosine residue (Y201) but was also eliminated by phosphorylation of this residue. Thus phosphorylation of Y201 by one or more kinases located at the point of TCR engagement was recognized as a likely mechanism for the focal surface accumulation of CTLA-4 at the T-cell/APC interface. In further support of this model, CTLA-4 mutants in which Y201 is absent or mutated exhibit enhanced surface localization when expressed in a variety of T cell lines.[118-120,123,137,141,143]

Schneider et al identified an interaction between the GVYVKM motif and a separate clathrin-associated trafficking complex—AP-1.[144] Association with AP-1 was also dependent on the unphosphorylated Y201 residue. In contrast to the interaction with the AP-2 complex, however, the association with AP-1 occurred predominantly in the Golgi compartment rather than at the cell surface and was linked to the transfer of CTLA-4 to lysosomes. The interaction was enhanced when CTLA-4 expression was increased and the authors proposed that the AP-1 complex maintains intracellular CTLA-4 homeostasis by shuttling excess protein to a site of degradation. Interestingly, Vieira has identified a homology between this same region of CTLA-4 and the HIV/SIV Nef proteins that are also involved in negative regulation of T cell activation and are important for AIDS pathogenesis.[145] As is the case with CTLA-4, this region of the Nef proteins has been implicated in clathrin-dependent endocytic trafficking. Furthermore, this portion of the Nef molecule has been shown to mediate down-regulation of CD4 surface expression. The finding raises the possibility that CTLA-4 may also mediate some of its effects intracellularly by interfering with the trafficking of signaling components.

It is clear from all of these studies that regulation of intracellular trafficking of CTLA-4 represents an important function for the portion of the CTLA-4 intracytoplasmic domain surrounding the Y201 residue and that this trafficking can be modified to favor surface expression by phosphorylation of Y201. Interestingly, Nakaseko et al observed that mutation of both Y201 and Y218 resulted in even higher surface expression than Y201 mutation alone perhaps suggesting an additional role for the more distal tyrosine in intracellular trafficking.[119]

Tyrosine Kinases

CTLA-4 has been shown to be both a substrate and a binding partner of certain tyrosine kinases. The identification of tyrosine phosphorylation as an important regulating event for intracellular trafficking of CTLA-4 has led to the testing of a variety of T-cell kinases for the ability to phosphorylate the Y201 and Y218 residues. A number of groups have convincingly shown that the Src family kinases Lck, Fyn, and Lyn can phosphorylate both Y201 and Y218.[140,143,146] Furthermore, Lck and Fyn have been found to bind directly to the CTLA-4 intracytoplasmic tail. This interaction was not prevented by mutation of the tyrosine residues suggesting that it is non-phosphorylation dependent.[136,143,146] The exact binding site for Lck and Fyn to CTLA-4 remains unclear although Miyatake et al showed, for Fyn, that it is partly

Table 1. Proteins associated with the intracytoplasmic domain of CTLA-4

Name	Type of Protein	CTLA-4 Binding Region	Possible Role in CTLA-4 Negative Regulation
AP50 (μ2)	Subunit of AP-2 Clathrin-associated complex	GVYVKM (Y201 Unphosphorylated)	Removal of unphosphorylated of CTLA-4 from cell surface
AP-1 Complex	Clathrin-associated complex	GVYVKM	Trafficking of CTLA-4 from Golgi to Lysosomal Compartment
TCRζ	ITAM-containing TCR-associated protein	Unknown	Target of CTLA-4 associated phosphatase
Lck / Fyn	Src family Tyrosine Kinases	Unknown	Phosphorylation of Y218 Modified CTLA-4 at Y201 and phosphorylation of TCR-associated proteins
Rlk (Txk)	Tec family Tyrosine Kinase	Unknown	Phosphorylation of CTLA-4 at Y201. Modified phosphorylation of TCR-associated proteins
JAK 2	Non-receptor Tyrosine Kinase	PPTEP	Phosphorylation of CTLA-4 at Y201. Modified phosphorylation of T cell signaling proteins
SHP-2 (SYP, PTP-1D)	Tyrosine Phosphatase	YVKM (Y201 Phosphorylated or ? Unphosphorylated)	Dephosphorylation of TCRζ and ? other TCR-associated signaling proteins
PP2A	Serine / Threonine Phosphatase	YVKM	Unknown
PI3K p85	Lipid Kinase Subunit	YVKM (Y201 Phosphorylated)	Modification of TCR / CD28-associated signaling events

dependent upon a SH2 domain.[146] The role of the Syk family kinase Zap70 is less clear. While three groups found that Zap 70 is incapable of inducing phosphorylation of CTLA-4 in co-transfection systems,[143,146,147] Bajora et al found that phosphorylation of CTLA-4 in Jurkat cells required expression of both Lck and Zap 70.[121] Schneider et al have reported that the Tek family kinase Txk (Rlk) preferentially phosphorylates Y201 when co-expressed with CTLA-4 in COS 7 cells.[148] This finding may, however, reflect an indirect effect as the two proteins were not found to be physically associated and, in a cell-free system, Ellis et al found recombinant Txk to be unable to phosphorylate the CTLA-4 intracytoplasmic domain.[149] Finally, Chikuma et al have shown that another kinase, JAK 2 (but not the related JAK 1), preferentially phosphorylates the Y201 residue in 293 cell transfectants as well as in a T cell line.[150] In addition, their experiments demonstrated that CTLA-4 and JAK 2 interact at the proline rich (PPTEP) motif of the CTLA-4 protein.

In summary, CTLA-4 may serve as a substrate for a number of tyrosine kinases that are involved in TCR and, perhaps, CD28 signaling. It appears that the two tyrosine residues of the intracytoplasmic tail may be phosphorylated separately or together depending on the individual kinase. As described previously, phosphorylation of CTLA-4 favors its accumulation within the TCR signaling complex and, as such, would allow it to effectively compete with CD28 for B7 ligands. CTLA-4 phosphorylation may also, however, allow for the recruitment of additional binding partners that act to modify signals emanating from the TCR or from co-stimulatory receptors. A further possibility, that binding of CTLA-4 to TCR-associated kinases results in their sequestration from the signaling complex, is unproven but worthy of consideration.

Phosphatases

One of the first interactions to be discovered for the intracytoplasmic domain of CTLA-4 was with the phosphatase SHP-2 (also called SYP and PTP-1D).[151] This interaction has been convincingly identified by a number of groups in activated T cells, T cell lines, and non-T cell transfectants and has also been mapped to the Y^{201}VKM motif. Although the interaction is facilitated by phosphorylation of Y^{201} it has also been found to occur when this residue is mutated to phenylalanine.[136] The inducible binding of CTLA-4 to SHP-2 fits with a well-accepted paradigm for co-inhibitory receptors—the recruitment of a phosphatase to a signaling complex that functions to abort or attenuate phosphorylation-dependent signaling events.[113] Evidence that this mechanism is operative for CTLA-4 following TCR stimulation has been presented by Marenègre et al, and by Lee et al[151,136] In the latter study, the association of CTLA-4 and SHP-2 was enhanced by co-expression of Lck. Furthermore, in the presence of SHP-2, the CTLA-4-associated TCRζ was predominantly in a dephosphorylated form. Thus a model has been proposed whereby TCR activation results in phosphorylation of CTLA-4 by Lck (or another kinase) which is followed by surface retention of CTLA-4 at the TCR/APC interface, physical association with TCRζ, and recruitment of SHP-2 resulting in dephosphorylation of the TCRζ ITAM motifs. This model, while compelling, has not met with universal acceptance. In other experimental systems the interaction between SHP-2 and CTLA-4 has been found to be absent or unnecessary for signaling inhibition.[119,124,147] Gajewski et al have recently shown that the amount of SHP-2 associated with CTLA-4 is comparable in naive CD8 T cells, which are unaffected by CTLA-4 ligation, and pre-activated CD8 T-cells, which are inhibited by CTLA-4 ligation.[152] In addition, some investigators have not observed hypophosphorylation of TCRζ or other components of the proximal TCR signaling apparatus upon CTLA-4 ligation.[124,153] Finally, the finding that SHP-2 plays a positive regulatory role in growth factor signaling pathways has also cast doubt on the validity of this model although it is certainly possible that SHP-2 participates in both positive and negative regulation of intracellular signaling. On balance, it is reasonable to conclude from all of these studies that the interaction between CTLA-4 and SHP-2 occurs in activated T cells and is, to some degree, regulated by tyrosine kinases that are associated with both the TCR and with CTLA-4 itself. The impact of this association on signaling events may, in fact, vary significantly among different T cell populations. It is probable that dynamic complexes of CTLA-4, SHP-2, Lck (or Fyn), TCRζ and additional proteins form at the T cell/APC synapse and have the potential to modify the activity of multiple downstream signaling cascades. Finally, Chuang et al have found that both CD28 and CTLA-4 interact with the catalytic subunit of a serine/threonine phosphatase PP2A.[107] For CTLA-4 this interaction occurred at the YVKM motif. The functional importance of this interaction remains to be determined.

Lipid Kinases

A phosphorylation-dependent interaction between the YVKM motif of CTLA-4 and the p85 subunit of Phosphoinositol-3 Kinase (PI3K) has also been described.[154] The association is similar to that which occurs between p85 and the YMNM motif of CD28. Furthermore, as with CD28, PI3K co-immunoprecipitated with CTLA-4 has been found to be catalytically active.[154] The functional importance of this interaction in both CD28-mediated co-stimulation and CTLA-4-mediated negative regulation is poorly understood and probably varies between different T cell populations.[155] It may be dependent upon other proteins that are associated with the intracytoplasmic domains of the two receptors as well as upon the presence of functionally discreet intracellular pools of PI3K. An indicator of the complexity of this pathway is provided by Zell et al[156] These investigators found that substitution of YMNM with YVKM in the intracytoplasmic domain of CD28 preserved the interaction with catalytically active PI3K but uncoupled CD28 ligation from a specific functional outcome—the upregulation of β1-integrins. Whether CD28 and CTLA-4 compete for binding to the same intracellular pool of PI3K and preferentially direct its activity towards positive or negative regulatory signaling remains speculative. Because the binding site for p85 on the intracytoplasmic domain of CTLA-4 overlaps with that of SHP-2, AP50, PP2A, and, probably, other signaling proteins, it will be a considerable challenge to identify the precise role of this lipid kinase in CTLA-4-mediated T cell inhibition.

Is There Evidence for an Independent Negative Signal?

Two key questions are frequently raised regarding the signaling mechanism of CTLA-4. The first of these is whether CTLA-4 preferentially antagonizes “Signal 1” (i.e., TCR signaling) or “Signal 2” (i.e., CD28 signaling). The second is whether CTLA-4 negative regulation can be entirely explained by inhibition of the signaling complex assembled at the TCR/APC interface or whether it initiates one or more independent “negative signaling pathways”.

An additional perspective on the first of these issues can be derived from studies examining the influence of CTLA-4 engagement on the activity of individual signaling pathways that are induced by TCR ligation in the presence or absence of CD28 co-stimulation. Inhibition of ERK2 activation has been demonstrated in pre-activated T cells and T cell clones upon co-cross-linkage of TCR and CTLA-4 with or without CD28 ligation.[153,119] In addition, Calvo et al observed inhibition by CTLA-4 of CD28-dependent JNK activation in pre-activated T cells.[153] A number of groups have examined the effect of CTLA-4 ligation on nuclear translocation of key transcription factors families including NF-AT, AP-1, and NF-κB.[134,157,158] These studies have demonstrated that CTLA-4 inhibits nuclear translocation and DNA binding of components of each of these families in the context of TCR and CD28 co-ligation. Although the observed inhibition of NF-κB and JNK pathways has been interpreted as indicating that CTLA-4 preferentially “targets” CD28-mediated signals,[157] it is probably not valid to consider these signaling pathways as being independent of the TCR. Indeed, in the study of Fraser et al, both NF-κB-mediated reporter gene transcription and AP-1 nuclear DNA binding could be demonstrated following TCR engagement alone and were inhibited by CTLA-4 co-ligation. Recently, Martin et al have demonstrated that CTLA-4 ligation results in inhibition of lipid raft translocation to the cell surface of resting human T cells in the context of TCR + CD28 stimulation.[159] As the experiments were carried out with antibody-coated beads the finding was clearly not due to competition between CD28 and CTLA-4 for ligand. Given the findings of Viola et al[15] and others that the induced aggregation of lipid rafts on the cell surface appears to be a specialized feature of CD28 co-stimulation it is tempting to speculate that CTLA-4 directly inhibits a unique CD28-mediated signal. Significantly, however, Martin et al also observed inhibition of lipid raft translocation in the context of a TCR signal alone.[159] Therefore,

even this inhibitory effect of CTLA-4 cannot be clearly distanced from the primary signaling events of TCR ligation.

Regarding the second issue, the available data on the impact of CTLA-4 ligation on intracellular signaling cascades does not give strong evidence for the existence of an independent "negative signaling pathway". Only one reported study appears to place the primary inhibitory signal from CTLA-4 "downstream" of proximal TCR signaling events in a normal T cell population. In this study by Calvo et al inhibition of ERK2 and JNK activation was observed in the absence of reduced TCRζ and Zap70 phosphorylation.[153] These findings are in contrast to those of other groups.[136] Given what we currently know of the binding characteristics of CTLA-4 with its natural ligands as well as its intracellular trafficking patterns and the protein interactions of its intracytoplasmic domain, it is difficult to escape the conclusion that the effects of CTLA-4 on individual signaling cascades primarily reflect manipulations applied to the molecular complex assembled at the point of TCR engagement.

It is, however, worth noting that CTLA-4 engagement may not uniformly inhibit signaling events emanating from the T cell/APC interface. Evidence for this includes the observations that the up-regulation of Bcl-X_L and the stabilization of IL-2 mRNA that occur following co-ligation of TCR and CD28 are unaffected by CTLA-4 ligation.[119,160,161] Furthermore, a number of reports suggest that CTLA-4 may activate certain signaling pathways such as those resulting in TGFβ1 production[162-165] and in Fas-independent apoptosis.[166,167] The fact that these findings have not been reproduced in all experimental systems (e.g., Sullivan et al, [168]) supports a conclusion that CTLA-4 signaling mechanisms are not fixed but depend upon the phenotypic and functional state of the T cell in which it is expressed. Finally, it is worth considering that no binding partners have been identified to date for the Y^{218}FIP motif in the distal portion of the CTLA-4 intracytoplasmic tail despite the fact that this region of the molecule is also highly conserved and undergoes inducible phosphorylation. It is certainly possible that this motif mediates separate signaling interactions or modifies those related to more proximally bound molecules. There is clearly much more to learn about the influence of CTLA-4 engagement on intracellular signaling. One of the primary challenges in unraveling the molecular mechanisms of CTLA-4 function will be to link observations on individual signaling cascades in physiologically-relevant T cell sub-populations with the actions of a growing number of confirmed or putative binding partners of the CTLA-4 intracytoplasmic tail.

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Chapter 3

Costimulatory Molecules in T Cell Development, Activation and Effector Function:

Similar Activity, Opposite Consequences

Yang Liu, Jian Xin Gao, Xue-Feng Bai, Xingluo Liu, Huiming Zhang and Pan Zheng

Abstract

Costimulatory molecules were initially dubbed as the second signal determining the fate of naïve T cells after they were engaged by MHC:peptide complex. Accumulating evidence supports the notion that costimulatory molecules play important roles in T cell development, activation and effector function. Here we propose that although costimulatory molecules increase the efficacy of T cell receptor signaling throughout the T cell lifespan, blocking the costimulatory molecules during development and activation can lead to, respectively, autoimmunity and immune suppression. This hypothesis provides the simplest interpretation for the paradoxical observations that targeted mutation, or perinatal blockade of B7 and their receptors, leads to enhanced autoimmunity.

Conceptual Evolution and Molecular Basis of the Second Signal for T Cell Activation

The "two-signal" theory for lymphocyte activation was originally proposed by Bretscher and Cohn[1] to address the perceived defects of the Lederberg hypothesis[2] for immune tolerance as a function of maturation of the putative immune competent cells. In their original form, Bretscher and Cohn suggested that activation and inactivation lymphocytes depended, respectively, on whether one or two antigen-specific events had taken place. In pursuing the cellular basis of the second signal, Lafferty and colleagues uncovered the activity associated with leukocytes, perhaps in the form of secreted factors, as the source of the second signal, which they termed costimuli.[3-5] Jenkins and Schwartz[6]developed a defined in vitro system to demonstrate that the antigenic peptide, presented by fixed antigen-presenting cells, induces T cells clonal anergy, which is characterized by defective proliferation and IL-2 production in response to restimulation in vivo.

The general acceptance of antigen-nonspecific costimulatory molecules as the second-signal marked the first major paradigm shift in the concept of the "second signal". The significance of

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this paradigm shift can be better appreciated in the context of the caveat it created: how do antigen-nonspecific events control the specificity of self-nonself discrimination? Janeway proposed that the immune system does not necessarily discriminate between self and nonself.[7,8] Rather, he suggested that it discriminates non-infectious self and infectious nonself based on pattern recognition by the innate immune response.[7,8] The first critical support for the hypothesis came from a study by Liu and Janeway,[9] who reported that products from yeast, bacteria and viruses can induce costimulatory activity on the antigen-presenting cells. A definitive link, between the innate immune response and the adaptive immune response, emerged when Medzhitov, Preston-Hurburt and Janeway[10] reported that triggering of mammalian homologue of drosophila Toll led to rapid induction of costimulatory molecules. With the identification of various mammalian Toll-like molecules that recognize a variety of microbial products,[11-15] it is generally accepted that such recognition is important for the innate immunity and its subsequent adaptive immunity.[8,16]

Instead of infection, Matzinger has suggested that tissue injury, or "danger" is the initiating event of adaptive immunity.[17] On the surface, it is difficult to judge the difference between the "danger" hypothesis and the Janeway postulate of infectious nonself, as infection is generally associated with some form of tissue injury. However, if one takes the position that selective pressure is what drives the evolution of the immune system, it is easy to envisage that infection is likely a driving force.

Multiplicity of Costimulatory Molecules

With the use of liposome containing TCR ligand alone, Quill and Schwartz revealed that the costimulatory activity for Th1 cells could not be replaced by soluble cytokines known at that time.[18] This inspired a period of rapid growth in the early 1990's for identification of costimulatory molecules.[19] Building on several early observations, including the idea that anti-CD28 mAb promoted T cell activation by somewhat biochemically distinct mechanisms,[20,21] Allison and Jenkins independently reported that CD28 was the primary costimulatory receptor, as anti-CD28 mAb could prevent induction of T cell clonal anergy and promote IL-2 production.[22,23] The role of CD28 for T cell costimulation was strengthened when Linsley et al reported that B7/BB1 (now called B7-1 or CD80) is a CD28 ligand[24] and has potent costimulatory activity for T cell activation. The innovative approaches [25-28] of tumor immunotherapy targeted at the B7 family of costimulatory molecules substantially expanded the horizon of studies on T cell costimulation.

However, the significant, though not completely normal, immunity in mice with targeted mutations of the CD28 gene underscores the point that it is unlikely that CD28 is the essential costimulatory molecule as it has been dubbed.[29] In this regard, it should be noted that multiple costimulatory molecules on antigen-presenting cells, such as CD24,[30] CD48,[31] 4-1BBL,[32] ICAM-,1,[33] LIGHT,[34] MHC class II-associated invariant chain[35] and others on non-professional APC, such as VCAM-1[36] were shown to have potent costimulatory activity in both in vivo and in vitro studies. CD24 becomes essential for immune response in CD28-deficient mice.[37,38] Moreover, a host of new B7 family members have been identified experimentally,[37-44] and even more are predicted by analysis of the human genome.[45]

Expanding the Horizon of T Cell Costimulation: From Activation to Effector Function

T cells are activated in the lymphoid organ.[46-48] Once activated, T cells gain the ability to migrate into target tissues to exert the effector function.[49,50] Since costimulatory molecules were identified on the basis of their ability to promote T cell activation, less emphasis was placed on their potential role in promoting the effector function of T cells. Almost 10 years

ago, Lanier and colleagues reported that expression of B7-1 on the target cells promoted antibody-redirected cytolysis in vitro.[51,52] Our analysis of anti-tumor CTL response provided the first compelling in vivo evidence for a critical role for B7-1 in the effector function of anti-tumor immunity .[27] Briefly, when mice were challenged with both B7-1$^+$ and B7-1$^-$ tumor cells with the same antigen, B7-1$^+$tumor cells were preferentially rejected. While a critical role for B7-1 at the effector phase has been extended to other models,[53,54] the role for B7-1 in the rejection of the P1A-expressing tumors is perhaps the most extensively characterized.[55,56] For example, of all three lineages of tumors expressing the tumor antigen P1A, J558, P815 and Meth A, P1A-specific transgenic T cells preferentially rejected the B7-1-transfected tumors, regardless of whether B7-1$^+$ and B7-1$^-$ cells were injected at the separate sites or as a mixture.[55,56] In addition, in mice that had rejected the B7-1$^+$tumor cells, a small number of tumor recurrence was noted, analysis of the recurrent tumor cells revealed that down-regulation of B7-1 alone was sufficient to allow tumor escape of pre-existing anti-tumor immunity.[57] Moreover, for large established tumors, expression of B7-1 on the tumor cells increased efficacy of tumor therapy.[55,56]

The importance of T cell costimulation at the effector phase has also been demonstrated in several models of autoimmunity. When T cells from diabetes NOD mice were adoptively transferred to young pre-diabetic NOD mice that transgenically express B7-1 on some, but not all, islet cells, the first wave of cells to be destroyed were the B7-1$^+$islet cells.[58] Similarly, in the EAE model, it has been reported that if the recipient mice have targeted mutations of B7-1 and B7-2, pathogenic T cells can only induce a mild EAE.[59]

One of the difficulties in generalizing a role for B7-1 and B7-2 at the effector phase of T cell function is that B7-1 and B7-2 are primarily expressed on hematopoietic cells. Yet CTL are capable of clearing viruses from non-hematopoietic tissues, such as the brain, liver and lung. Recently, the B7 family has been extended substantially. Unlike B7-1 and B7-2, many of the new B7 family members appear to have been transcribed in the non-hematopoietic tissues.[39-44] This has raised an interesting issue as to whether some of these family members may regulate the effector function of CTL. We chose B7h (also called B7RP- and B7-H2) to test this notion as its receptor ICOS is known to be expressed on activated T cells. Using adoptive transfer of T cells specific for the tumor antigen P1A, we showed that expression of B7H substantially increased tumor susceptibility to the effector function of T cells.[60] Since expression of B7H can be regulated by inflammatory cytokines such as tumor necrosis factor (TNF) and interferon gamma (IFNγ),[42] it would be of great interest to determine whether inflammatory stimuli may normally regulate the effector function of T cells, as they did in the induction of T cell response by promoting antigen-presentation by the dendritic cells.

Extending the role for costimulators to the effector phase of T cell response can be significant for at least two reasons. First, it suggests that the effector phase of T cell immunity can be as tightly regulated as that for the induction. At present, such regulation is poorly understood. Second, modulating the effector function of autoreactive or tumor-reactive T cells provides new windows of opportunity for immunotherapy. Since patients with autoimmune disease have pre-existing autoreactive T cells, approaches targeting these cells will be valuable. On the other hand, recent studies have revealed that cancer patients also have substantial numbers of cells specific for cancer associated antigens,[61,62] re-invigorating the effector function of these cells by providing local costimulation that may help to utilize these untapped resources.

Costimulatory Molecules in T Cell Development

The role for costimulatory molecules in T cell development remains an issue of considerable debate. The T cell repertoire is shaped in the thymus by two processes. Positive selection promotes survival of T cells capable of recognizing antigenic peptides presented by the self-MHC

molelcules,[63] while negative selection helps to remove high avidity self-reactive T cells.[64] Positive selection involves lower avidity interaction than does negative selection,[65] which in turn requires lower avidity interaction than T cell activation.[66] While there is no information regarding the contribution of costimulatory molecules to positive selection, accumulating evidence supports a significant role for costimulation. For instance, CD40 is expressed on thymic epithelial cells.[67] Correspondingly, mice with targeted mutations of the CD40 ligand show defective clonal deletion of viral superantigen-reactive T cells.[64] Similarly, costimulatory molecule LIGHT was found to be critical for clonal deletion in vivo.[68] The contribution of B7-1/2:CD28/CTLA4 interaction in the negative selection of T cell repertoire in the thymus has been studied extensively.

The B7-1/B7-2:CD28/CTLA4 receptor ligand pair is present at significant levels in the thymus. Thus, $CD4^+CD8^+$ thymocytes, which are comprised of cells undergoing both positive and negative selection, have the highest level of CD28.[69] Although CTLA4 is not constitutively expressed on the surface of the majority of thymocytes, it is induced upon engagement of the TCR/CD3 complex in vivo.[70] Two groups have confirmed expression of B7-1/B7-2 in the medulla epithelial cells.[71,72] Expression of B7-1 and B7-2 was convincingly demonstrated on purified DC from the thymus.[73] Expression of B7-1 on thymic dendritic cells was also shown by immunohistochemistry.[74] Since both thymic dendritic cells and medulla epithelial cells have been demonstrated to induce negative selection,[75,76] the expression pattern of the B7-1/2:CD28/CTLA4 ligand pair is consistent with a role for this interaction in negative selection of T cells.

The first evidence for a potential role of CD28 in T cell clonal deletion was reported by Punt et al,[77] who showed that agonistic anti-CD28 mAbs promote death of thymocytes in vitro. In thymic organ culture, anti-B7-1/2 mAbs prevent clonal deletion of transgenic T cells.[78] Three lines of evidence are consistent with a role for CD28 in T cell clonal deletion in vivo. First, CD28(-/-) mice have substantially larger thymi than the wild-type mice, and are more resistant to anti-CD3 induced death of thymocytes in vivo.[79] Second, when bacterial superantigen and antigenic peptides were used to induce Vβ-specific clonal deletion, CD28(-/-) thymocytes were significantly less sensitive to the clonal deletion.[80,81] Third, Li and Page reported that deletion of Vβ-expressing T cells was less efficient among the CD28(-/-) mice.[82] Similarly, anti-CTLA4 mAb was found to block anti-CD3 induced cell death in vivo.[70] The role for B7-1 and B7-2 in T cell negative selection has not been systematically analyzed. However, a recent study indicated that transgenic expression of B7-1 on the thymic epithelial cells selectively reduces the number of mature CD4 T cells.[83]

The perinatal period is the most critical for the generation of T cell repertoire. We have recently analyzed the effect of perinatal blockade of B7-1 and B7-2 on negative selection of T cells.[107] We found that perinatal blockade of B7-1 and B7-2 substantially reduced clonal deletion of VSAg-specific T cells that express Vβ3,5,11 and 12. Moreover, in transgenic mice that express tumor antigen P1A and its specific TCR, perinatal blockade prevented T cell clonal deletion. These results demonstrate that B7-1 and B7-2 play major roles in clonal deletion, at least during the perinatal period.

Interestingly, T cells rescued by perinatal blockade of B7-1/2 were highly pathogenic in the immune deficient host.[107] For instance, thymocytes isolated from the anti-B7 treated syngeneic mice induced lethal multiple organ inflammation. Moreover, the VSAg-specific T cells rescued by anti-B7 mAbs were pathogenic in syngeneic mice expressing the endogenous murine mammary tumor viruses. Our results reveal an important function of B7 expression in the thymus in promoting T cell clonal deletion and in preventing the development of pathogenic autoreactive T cells.

Negative Costimulation? Paradigm Revisited

One of the most surprising findings in the area of T cell costimulation was that mice with a targeted mutation of the CTLA4 gene develop lethal lymphoproliferative diseases.[84-86] This finding was generally regarded as the definitive evidence for the hypothesis that CTLA4 is a negative regulator for T cell activation.[87] However, it has recently been reported that a targeted mutation of CD28 exacerbated autoimmune diabetes in NOD mice.[88] Similarly, targeted mutation of B7-2 leads to peripheral polyneuropathy in NOD mice.[89] Thus, although the phenotype of CTLA4(-/-) mice is more severe, mutation of the CTLA4 gene is not unique among the components of the B7:CD28/CTLA4 family in enhancing autoimmune disease.

The second main challenge to the notion of negative costimulation by CTLA4 is that many in vivo experiments have failed to reveal increased functionality of naïve CTLA4(-/-) T cells. For instance, CTLA4(-/-) transgenic T cells mount a normal response to viral infection [90]. Similarly, naïve CTLA4(-/-) T cells have comparable proliferation in vivo and in vitro, although the recall T cell response has been reported to be enhanced if T cells are deficient for the CTLA4 gene.[91,92] Interestingly, the autoimmune phenotype of CTLA4(-/-) T cells is absent in chimera mice consisting of both CTLA4(-/-) and CTLA4(+/+) bone marrow[93]. These results not only argue against an intrinsic negative regulatory function of the CTLA4 gene, but also provide chimera mice with polyclonal T cell repertoire to determine whether CTLA4(-/-) T cells preferentially expand in response to infections. In several models tested, CTLA4(-/-) failed to show any advantage in response to infection by both viruses and bacteria.[94] On the other hand, others and we have demonstrated that CD28(-/-) T cells respond positively to costimulation by B7-1/2.[95,96] While Mandelbrot et al[97] have suggested that receptors other than CD28 and CLTA4 are responsible for CD28-independent costimulation, we have argued that B7-CTLA4 interaction can provide positive costimulation for T cell activation.[98] This is because the function of B7-1 can be blocked by the Fab fragment of the anti-CTLA4 mAb. Moreover, mutant B7-1 molecules promote T cell activation in vivo and in vitro. In addition to a lack of reproducible evidence that B7-CTLA4 interaction indeed inhibits T cell activation, the hypothesis[99] that CTLA4 inhibits T cell response by bringing in SHP-2 to TCR signaling complex has been challenged on two fronts. First, the inhibitory effect of anti-CTLA4 mAb is independent of its cytoplasmic domain that mediates CTLA4:SHP-2 association.[100] Second, in both TCR and EGF models, association to SHP-2 enhances, rather than inhibits, receptor signaling.[101,102] As such, an association with a phosphatase does not necessarily imply a negative regulatory function.

B71/2 Mediated Costimulation in T Cell Activation and Development: Similar Activity, Opposite Consequences

The significant contribution of B7-1/2:CTLA4/CD28 in negative selection, as reviewed above, provided a simple explanation for the paradoxical autoimmune phenotype in mice with targeted mutations of CD28, B7-2 or CTLA4. In brief, we propose that B7-1/2 and B7-2-mediated costimulation is the life-long companion of the T cell receptor. By reducing the threshold of TCR signaling, costimulatory molecules would help to increase the efficacy of T cell responses, although the nature of T cell response is determined by the program of T cells at the time the costimulation is delivered. For instance, removing costimulation during T cell development will reduce the efficiency of clonal deletion, hence allowing the accumulation of autoreactive T cells. On the other hand, blocking B7 during the induction and effector phase of an immune response would decrease the efficacy of immunity. For mice with a germ-line mutation of a given component of B7-1/B7-2: CD28/CTLA4, the ultimate consequences will be determined by the relative importance of the component to clonal deletion and activation.

Obviously, this hypothesis is a major departure from the traditional view that TCR:MHC/peptide interaction determines the specificity of immunity while costimulation determines the fate of T cells. While the hypothesis remains to be substantiated, several lines of evidence are consistent with it. Thus, agonistic anti-CD28 mAb reduces the number of engaged TCR necessary for activation of T cells.[103,104] Conversely, an increase in either the amount or duration of antigen helps to bypass the need for CD28.[105] Moreover, biochemical signals delivered by B7 and CD28 that are distinct from those delivered by TCR remain to be identified.[106] Also, our recent results indicated that autoreactive T cells rescued by perinatal blockade of B7-1 and B7-2 are highly pathogenic in an immune deficient host,[107] while germ-line mutations of CD28, B7-2 and CTLA4 result in enhanced autoimmunity.[85,86,88,89] Our hypothesis provides the simplest explanation for these observations and is clearly testable by inducible deletion of genes at different stages of a T cell's life span.

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Chapter 4

Negative Costimulatory Functions of B7-H1

Haidong Dong, Hideto Tamura, Shengdian Wang and Lieping Chen

Introduction

Costimulatory molecules B7-1 and B7-2 belong to an emerging family of the immunoglobulin (Ig) superfamily and share ~25% identical amino acid sequences concentrated in the Ig V- and Ig C-like extracellular domains while profound differences are in cytoplasmic domains.[1-3] Despite this limited homology, B7-1 and B7-2 have a similar tertiary structure and costimulatory function.[4-6] In the presence of antigen signal, the engagement of CD28 by either specific antibodies or its natural ligands of B7-1 and B7-2 promotes antigen-specific T cell proliferation, enhances production of cytokines, and induces differentiation of effecter T cells.[1-3] Signaling through homologous CTLA-4 counter-receptor of B7-1 and B7-2 on activated T cells, however, may deliver a negative signal that inhibits T cell proliferation, IL-2 production, IL-2 receptor expression and cell cycle progression.[7-9]

Recent studies indicated that new members of the B7-CD28 family may also participate in the regulation of cellular and humoral immune responses.[10,11] B7-H1 is the third member of molecules in the B7 family[12], and is capable of binding to PD-1.[13] Recent studies indicate that B7-H1 ligation promotes T cell responses in both priming and effector phases. In addition, it is possible that a non-PD-1 receptor on T cells is involved in these responses. In this chapter, we will summarize recent studies in B7-H1 and will discuss potential functions of B7-H1 in evasion of tumor immunity and in the progression of autoimmune diseases.

Molecular Structure and Expression of B7-H1

Human B7-H1

By searching molecules sharing homology with the IgV and IgC domains of B7-1 and B7-2 among the human cDNA expressed sequence tags (EST) in NCBI database, we identified a new gene designated as B7-H1 (B7 homologue-1).[12] B7-H1 was subsequently shown to bind PD-1,[13] and also called PD-L1. Human B7-H1 gene is localized on human chromosome 9p24 and the sequence encoding extracellular domain of human B7-H1 shares higher homology to B7-1 (20% amino acid identity) compared to B7-2 (15%). The open reading frame of the B7-H1 gene encodes a putative type I transmembrane protein of 290 amino acids consisting of a Ig V-like domain, a Ig C-like domain, a hydrophobic transmembrane domain and a cytoplasmic tail of 30 amino acids.[4-6] Four structural cysteines that are apparently involved in forming the disulfide bonds of the Ig V and Ig C domains,[14] are well conserved in the B7-H1 molecule. Interestingly, the intracellular domain of B7-H1 also consists of one potential site for protein kinase C (PKC) phosphorylation. In addition, the intracellular domain of B7-H1 shares

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16% identity with that of B7-2 (only 6% with B7-1), which has been shown to deliver a “reverse” signal to B cells after being triggered with specific monoclonal antibodies (mAb).[15] It is thus possible that B7-H1 could also transmit signals. In fact, we have recently demonstrated that ligation of B7-H1 by immobilized PD-1 or mAb against B7-H1 in the presence of anti-CD3 resulted in the activation of naïve T cells and apoptosis of activated T cells.[16] These findings will be discussed later in this chapter.

Northern blot analysis revealed that the distribution of B7-H1 mRNA was abundant in heart, skeletal muscle, placenta and lung, but was weak in thymus, spleen, kidney and liver. The B7-H1 mRNA was not detectable in brain, colon, small intestine and peripheral blood mononuclear cells.[12] Prominent mRNA transcript is 4.1 kb, and a 7.2-kb transcript is also detectable in some human tissues,[12] while a smaller transcript of 1.8 kb could also be observed.[17,13] Thus, existence of B7-H1 isotype could not be excluded at this time. At mRNA levels, B7-H1 could be rapidly induced during the maturation of human blood-derived dendritic cells as well as in human karatinocytes and endothelial cells treated with phorbol ester and/or IFN-γ.[13,17] In FACS analysis, freshly isolated T and B cells express negligible levels of B7-H1 while 16% of $CD14^+$ monocytes constitutively express B7-H1. After activation, 30% of $CD3^+$ T cells and 90% of $CD14^+$ express B7-H1, but only 6% of $CD19^+$ B cells express B7-H1, even after activation by cell surface Ig cross-linking, which induce detectable levels of B7-H1 mRNA.[12,13] Up to 43% of human monocyte original dendritic cells express B7-H1, which could be up-regulated to 95% after activation (Dong, unpublished data). By immunohistochemistry, the expression of human B7-H1 protein was limited to the macrophage-like cells, such as Kuppfer cells in liver, macrophages in lung and histocytes in the paracortical region of tonsil,[18] albeit B7-H1 has a broad distribution at the mRNA level. There is no expression of B7-H1 protein in the parenchyma of liver, lung, pancreas, uterus, kidney, colon, breast, muscles and lymphocytes in tonsil. In addition, B7-H1 protein expression is also found on a broad range of human cancer tissues.[18] The significance of this broad expression pattern of B7-H1 will be further discussed in this review. It should be noted that high expression of B7-H1 both at mRNA and protein levels was found in $CD3^+$, $CD14^+$ or $CD19^+$ cells from the patients with HIV-infection.[19] In addition, an inverse correlation was detected between B7-H1 expression and $CD4^+$ cell counts, whereas up-regulation of B7-H1 was positively associated with HIV plasma viremia, suggesting a role for B7-H1 in the control of $CD4^+$ T cell numbers.

Mouse B7-H1

The mouse B7-H1 gene encodes a putative type I transmembrane protein of 290 amino acids and has 69% overall amino acid homology with human B7-H1.[20] Like human B7-H1, the protein of mB7-H1 consists of an Ig V-like extracellular domain, an Ig C-like domain, a hydrophobic transmembrane domain and a cytoplasmic tail. Mouse B7-H1 shares overall homology to mB7-1 (20%), mB7-2 (14%), and mB7-H2 (19%).[21] Immunoprecipitation revealed a single molecule of 43 kDa from the lysate of B7-H1 transfectant.[22] RNA analysis demonstrated that mB7-H1 mRNA was also abundant in heart, spleen, lung, skeletal muscle, and liver,[20] an expression pattern similar to human B7-H1 mRNA. Negligible expression of B7-H1 mRNA was observed in pancreas and testis.

Resting $CD3^+$ T cells did not express B7-H1 in FACS assay using independently generated mAb from two laboratories.[20,23] These mAb, however, detected low level expression of B7-H1 in a small fraction of B220+ B cells and Mac-1+ macrophages.[20] A recent study using a MIH6 mAb showed that resting T cells, B cells and macrophages constitutively express B7-H1,[22] a result different from two previously published studies.[20,23] At this time, it is unknown whether MIH6 binds to a distinct epitope although all these mAb appears to block the binding of B7-H1 to PD-1 receptor. Activation of T cells, B cells, macrophages and dendritic cells greatly

increased the expression of B7-H1 on the cell surface.[20,22] Similar to human B7-H1, mouse B7-H1 is also an inducible cell surface molecule in mouse lymphocytes and antigen presenting cells (APC). In mouse bone marrow cells, B7-H1 was found on the majority of pre-B cells and myeloid cells.[23] In the thymus, the most immature CD4/CD8-double negative (DN), but not double positive (DP) thymocytes also expressed B7-H1, whereas mature CD4 or CD8 –single positive (SP) cell population only marginally express B7-H1. Several mouse leukemic cell lines also constitutively express B7-H1 on their surface.[23]

Regulation of B7-H1 Expression

Broad mRNA distribution but limited protein expression on cell surface supports post-transcription control of B7-H1. As IFN-γ is a potent inducer of cell surface expression of B7-H1 in monocytes, dendritic cells and karatinocytes, one can predict that functions of B7-H1 in tissues are largely regulated by the local cytokine milieu. In fact, the promoter region of human B7-H1 contains several IFN-γ responsive elements.[17] Given the fact that B7-H1 expression is up-regulated in HIV-infected human monocytes, T cell or B cells, and increased expression of B7-H1 could be reversed by the antiviral therapy,[19] it raises the possibility that HIV may evade protective immunity by inducing B7-H1.

In addition to regulation by cytokines, B7-H1 gene expression may also be regulated by *whn* gene product.[24] The *whn*-coded protein WHN is a winged-helix transcription factor that controls the differentiation of thymic epithelial cells, and the disruption of the *whn* gene recapitulates the phenotype of the athymic nude mouse.[24] To identify the target genes of *whn*, RNA samples from wild type and nude (*whn*-/-) thymic rudiment were analyzed by in situ hybridization and RT-PCR. It was found that B7-H1 is one of the five epithelial original genes that are present in wild type, but absent in nude samples.[24] It seems that the expression of B7-H1 is under the control of *whn*, at least at the early development stages of thymic epithelial cells. However, this data should be interpreted with caution as the B7-H1 is a broad distributed gene,[12] and could be controlled by multiple transcription factors in different tissues.

Counter-Receptor of B7-H1

The SQDxxxELY motif in the Ig V-like domain is required for B7-1 and B7-2 to bind their counter receptor CD28 and CTLA-4.[5] Both human and mouse B7-H1 do not contain the similar motif like SQDxxxELY, a result consistent with lack of binding of B7-H1 transfected cells to CTLA-4Ig nor ICOSIg fusion protein. Furthermore, B7-H1Ig did not bind to Jurkat cells despite their constitutive expression of CD28.[12] These results suggest that B7-H1 is not the ligand for CD28, CTLA-4 and ICOS. The receptor for B7-H1, however, is a more difficult issue to address. At this time, experimental data support there are at least two receptors on T cells.

PD-1

B7-H1 was found to bind PD-1,[13] an inducible molecule on T and B cells, supporting PD-1 as a receptor for B7-H1. We would refer to Chapter 5 in this volume for detailed analysis of PD-1 molecule.

Evidence for a Non-PD-1 Receptor

B7-H1 was first identified as a costimulatory molecule with moderate capacity (in comparison with CD28 signaling) to stimulate proliferation of T cells.[12] Different from B7-1 and B7-2, B7-H1 ligation appears to selectively induce IL-10 secretion in both human and mouse T cells.[12,20] We found that soluble PD-1Ig did not block B7-H1Ig-mediated costimulation of resting human T cells in vitro.[12,18] In addition, our recent studies demonstrated that B7-H1Ig

is capable of costimulating proliferation and cytokine secretion of T cells from PD-1$^{-/-}$ mice. We have recently demonstrated that, by molecular modeling and site-directed mutagenesis, B7-H1 mutants with complete loss of binding capacity to PD-1 are still capable of costimulating T cell responses.[25] In a study using human T cell clones, B7-H1Ig was able to bind and induce apoptosis in PD-1 negative human T cells.[18] Taken together, these results support costimulatory and apoptotic functions of B7-H1 that could be uncoupled from the binding of PD-1.

Immunological Functions of B7-H1

Costimulation and Inhibition of T Cell Responses

Even though the binding receptor of B7-H1 was found only on activated T cells, it is intriguing to notice that B7-H1Ig, when immobilized together with anti-CD3, enhanced proliferation of resting T cells.[12] Similarly, mouse B7-H1 mediated the costimulation of mouse spleen T cell growth as either immobilized fusion protein or cell surface form.[20] Furthermore, B7-H1Ig induced the proliferation of both CD28$^{-/-}$ and CD28$^{+/+}$ T cells in a similar degree in the presence of anti-CD3, supporting that B7-H1 costimulates T cell growth in a CD28-independent fashion. With the same concentration of B7-H1Ig and anti-CD3, the proliferation of CD4^{+} T cells was up 10-fold and the proliferation of CD8^{+} T cells was only enhanced 2-3 fold.[20] Thus, B7-H1 ligation preferentially costimulates the growth of CD4^{+} T cells.

In contrast to costimulating the resting T cells in their response to TCR signals, B7-H1 down-regulated the activated T cell response. In the prolonged incubation (>72 hrs) with B7-H1Ig in the presence of optimal anti-CD3 signal, the proliferation and cytokine production of both human and mouse T cells were inhibited rather than enhanced.[18,26] This result is consistent with an earlier observation demonstrating that ligation of bead-coated B7-H1 inhibited T cell proliferation.[13] The inhibition mediated by B7-H1 in T cell response could be reversed by the addition of anti-CD28 in CD4^{+} cells, but not in CD8^{+} cells.[27]

The mechanism of B7-H1-mediated inhibitory function of T cells is not yet clear. Freeman and colleagues showed that inhibition by B7-H1 could be abrogated in PD-1$^{-/-}$ deficient mouse T cells, suggesting that B7-H1 conveys this signal via its interaction with PD-1.[13] A cell cycle arrest was observed in pre-activated T cells following the engagement of B7-H1.[26] Our studies, however, showed that T cell inhibition induced by immobilized B7-H1Ig was largely caused by rapid increase of T cell apoptosis in 48-72 hr of culture in the presence of a strong anti-CD3 signal and this effect could not be blocked by the addition of PD-1Ig.[18] In this process, activated T cells expressed high levels of both Fas and Fas ligand (FasL), as well as a high level of IL-10. Inclusion of anti-FasL or anti-IL-10 monoclonal antibodies (mAbs) partially inhibited the apoptosis. B7-H1 mediated inhibition could also be caused by IL-10, which is a potent attenuator of T cell responses. It was reported that immature dendritic cells (DC) stimulated the production of IL-10 from regulatory T cells that could induce anergy status of T cells, and the production of IL-10 could be blocked by anti-B7-H1 mAb.[28] It is possible that B7-H1 costimulated a subset of T cells that has intrinsic capacity to secrete IL-10. The production of IL-10 in the process of DC-T cell interaction may also facilitate the down-regulation of T cell response in the presence of mature DC. This notion is consistent with an emerging concept that unless the DCs are activated or "licensed", the stimulation of T cells by DCs—even leading to proliferation—will, by default, lead to tolerance.[29] It is thus important to point out that costimulation of T cell proliferation may not always lead to enhanced immunity and the outcome of costimulation is dependent on the nature of cytokines and probably on functional subsets to be costimulated.

Regulation of Humoral Immune Response

To further dissect the function of B7-H1 in vivo, we used a model in which the mice were immunized with TNP-conjugated KLH. In this system, both the T helper cell and B cell responses could be monitored. T cells from both spleens and lymph nodes of the immunized mice proliferated vigorously to KLH in a dose-dependent fashion. Administration of mB7-H1Ig to the immunized mice amplified the proliferate responses.[20] B7-H1Ig treatment also increased the TNP-specific IgG2a antibody without significant changes on other Ig components. This effect is different from that treated by mB7-1Ig since other IgG components, including IgG1 and IgG2b, were also increased.

Several potential mechanisms may be responsible for the enhancement of antigen-specific IgG2a antibody response upon B7-H1Ig administration. B7-H1 costimulation of T cells leads to secretion of IL-10 that prompted antibody production in various systems.[30] In addition, IFN-γ can facilitate the Ig class switching to IgG2a.[31] IL-10 has been found to enhance IgG1 and IgG3 isotypes together with anti-CD40 mAb.[32,33] In B7-H1-costimulated T cells, however, both IL-10 release and CD40L up-regulation were observed while there is no significant IgG1 and IgG3 elevation in B7-H1Ig-treated mice.[20] The possibility that injection of B7-H1Ig temporarily block the binding of endogenous B7-H1 to PD-1 could also not be entirely excluded in this system. Therefore, there is no simple explanation for selective increase of IgG2a antibody in the presence of B7-H1 costimulation. IgG class switching is regulated by multiple cytokines and accessory molecules, and the end result is likely to be a balance of these interactions.

Regulation of Antigen-Specific T Cell Response

Costimulation mediated by B7-1 and B7-2 plays a crucial role in the induction of CTLs. The expression of B7-1 and B7-2 on tumor cells such as P815 enhances MHC class I-restricted CTL responses against tumor antigens and leads to regression of tumor in immunocompetent mice.[34,35] In contrast, the expression of B7-H1 on P815 cells has only negligible effect on the generation of CTLs against P815 in vitro and in vivo.[20] The failure of B7-H1 to stimulate CTL response may be largely due to a lack of activated T cells, which could express the receptors for B7-H1. In addition, it is known that the majority of responsive cells after P815 injection are $CD8^+$ T cells, which are much less responsive to B7-H1 than $CD4^+$ T cells. In sharp contrast, co-expression of B7-H1 abrogated tumor-inhibitory effect of B7-1 and led to an outgrowth of otherwise regressive tumor.[18] This observation supports the notion that B7-H1 down-regulates activated T cells and inhibits tumor immunity. The mechanism of B7-H1 inhibitory effect will be discussed in the next section.

Effect of Tumor-Associated B7-H1 in Immune Evasion

B7-H1 was originally identified in an EST clone of human ovary tumor[12] and subsequent studies demonstrated the expression of B7-H1 in a panel of human tumor lines derived from different tissue origins using an anti-B7-H1 mAb. B7-H1 immunoreactivity was detected in 4/9 lung carcinomas and 1/3 ovarian carcinoma, but not in 4 colon/duodenum, 5 leukemia, 2 choriocarcinoma and 6 melanoma cell lines.[18] Recently, other groups also confirmed the expression of B7-H1 on a panel of mouse tumor cells lines.[22,36] The majority of B7-H1 negative tumor lines, however, can be up-regulated to express B7-H1 upon treatment with IFN-γ.[18] More importantly, immunohistochemistry analysis demonstrated B7-H1 immunoreactivity in a majority of freshly isolated human carcinomas of lung, ovarian and colon as well as melanoma.[18,37] B7-H1 protein could be detected in both the plasma membrane and cytoplasm of cancer cells. These findings support an inducible pattern of B7-H1 on tumor cells.

Tumor-Associated B7-H1 Increased Apoptosis of Activated T Cells

The role of tumor-associated B7-H1 in the stimulation of apoptosis of activated T cells was first discovered by in vitro tumor-T cell co-culture system. M15 is a cytolytic T cell line that recognizes an HLA-A2 restricted epitope of the gp100 tumor antigens.[38] When M15 CTL were co-cultured with B7-H1-transfected 624mel cell (B7-H1/624mel cell), a human melanoma cell line expressing high levels of gp100 antigen, the apoptosis of M15 CTL cells increased significantly compared with that of mock/624mel.[18] In addition, B7-H1/624mel melanoma cells could survive in the co-culture with M15 CTL, while nearly all mock-transfected 624mel cells were eliminated. There was no different between mock/624mel and B7-H1/624mel cells in their susceptibility to lysis by M15 CTL in 4hr standard^{51} Cr release assay, and no growth advantage in vitro of B7-H1/624mel cells over mock/624mel cells.[18] Taken together with increased apoptosis of M15 CTL, we conclude that tumor-associated B7-H1 rendered the tumor cells survival advantage by counter-attacking the killer cells. Similarly, a human breast tumor line (HBL-100), which constitutively expresses B7-H1, also enhanced the apoptosis of another CTL cell line that recognized the CEA antigen on the HBL-100 cells.[18]

We also employed a T cell adoptive transfer model to examine the consequence of activated T cells after countering B7-H1 on tumor cells. In this model, 2C T cell receptor (TCR) transgenic T cells, which recognize a p2Ca peptide in the context of L^d MHC class I molecule on the P815 tumor line,[39] were activated in vitro and infused to reconstitute RAG-1$^{-/-}$ mice. Exposure of activated 2C T cells to mock/P815 induced a rapid increase of T cells and elimination of tumor cells. In contrast, transfusion of activated 2C T cells into the mice harboring B7-H1/P815 cells led to increased apoptosis of 2C T cells and rapid growth of tumor cells. Importantly, administration of a neutralizing 10B5 mAb to mouse B7-H1 inhibited the growth of B7-H1/P815 in vivo.[18] These results support a role of tumor-associated B7-H1 in the evasion of tumor immunity.

Our studies may help to address the dilemma in cancer immunotherapy that cancer vaccines are quite effective in preventing the initial growth of a tumor, but usually fail to induce regression of established tumors.[40] Current strategy for cancer vaccination is focusing on enhancing efficacy of antigen processing and presentation. Our results indicate that, even using highly effective T cells that are elicited by cancer vaccine, confrontation of such T cells with negative signal like B7-H1 in tumor sites could eliminate the effect of activated T cells. Therefore, Blocking of B7-H1 should offer a novel approach to improve effectiveness of cancer vaccines.

Strategies to Augment Tumor Immunity by Manipulation of B7-H1 Pathway

Direct implication from the study of B7-H1 is that blockade of B7-H1 pathway should be a part of the strategy to enhance efficacy of active immune responses. As discussed above, B7-H1 may be involved in the downregulation of cell-mediated immune responses in priming state by inducing inhibitory cytokines such as IL-10. In addition, engagement of fully activated T cells by B7-H1 may lead to increased programmed cell death. Theoretically, blockade of B7-H1 pathway could be used in conjunction with all active and passive immunotherapy aiming to stimulate activation of T cells. For example, tumor antigen-based vaccinations are undergoing extensive testing in human clinical trials.[41,42] In addition, adoptive transfer of in vitro activated T cells, which were isolated from cancer patients, has also been used for adoptive immunotherapy of advanced human cancers.[43] Blockade of B7-H1 by neutralizing mAb thus provides an attractive approach to use in conjunction with other immunotherapy approaches to enhance and sustain T cell immunity against cancers. In fact, B7-H1 blockade regimen in control of tumor growth in vivo was also confirmed in mouse P815 tumor model in a recent report.[36]

Reverse Signaling of B7-H1 to Regulate T Cell Responses

Similar to B7-1 and B7-2, expression of B7-H1 was only found on activated T cells but not, if any, on resting T cells.[12,44] Further study demonstrated that B7-H1 expression could be detected on the majority (64-78%) of $CD4^+$ T cells and $CD45RO^+$ memory T cells (62-84%) but in lower levels of $CD8^+$ T cells (32-45%).[16] The expression of B7-H1 on T cells is often accompanied by other activation markers such as OX40, CD95 or CD25 (Dong et al, unpublished data).

Triggering of B7-H1 Costimulates $CD4^+$ T Cell Responses

The functional role of B7-H1 on the $CD4^+$ T cells was first examined in resting $CD4^+$ T cells by the ligation of anti-B7-H1 mAb or PD-1Ig. In the presence of suboptimal doses of an anti-CD3 mAb, signal through B7-H1 induced a significant proliferation of purified human $CD4^+$ T cells in vitro.[16] Ligation of B7-H1 by anti-B7-H1 mAb or PD-1Ig also increased the expression of CD25 on $CD4^+$ T cells, and promoted $CD4^+$ T cells in the S/G_2 phase.[16] These results support a role for T cell-associated B7-H1 on the activation of $CD4^+$ T cells.

Using the human cytokine cDNA expression array, we found that B7-H1 triggering increased mRNA expression of IL-1α/β, IL-6, IL-10, IL-15, IL-16, and IFN-γ during the first 24 hrs of culture (Dong, unpublished data). The expression of IL-1α, IL-6, IL-10 and TNF-α increased up to 1.2-3.7 fold even after 72 hrs stimulation, while the expression of IL-3, IL-5, IL-9, TNF-α/β declined after 48 hrs. By ELISA, we confirmed that B7-H1 triggering gradually increased the production of IL-10 from 24 to 72 hours of stimulation, and a small increase of IFN-γ secretion was also observed.[16]

Triggering of B7-H1 Promoted the Apoptosis of Activated T Cells

To examine the effect of B7-H1 mAb on activated $CD4^+$ T cells, we employed an in vitro culture system in which optimal doses of anti-CD3 mAb can drive T cell proliferation without additional costimulation. In this setting, B7-H1 mAb significantly increased apoptosis of $CD4^+$ T cells.[16] Similarly, immobilized PD-1Ig also increased apoptosis of activated T cells. TCR signaling appears to be critical for the induction of apoptosis because neither soluble nor immobilized mAb to B7-H1 or PD-1Ig alone had the effect. In a different setting, $CD4^+$ T cells were pre-activated by optimal doses of anti-CD3 mAb for 48 hrs, in which all $CD4^+$ T cells expressed high levels of B7-H1, and were further stimulated with B7-H1 mAb. Similarly, pre-activated T cells also had increased apoptosis after exposure to a co-ligation of immobilized anti-CD3/B7-H1 mAb, but not to ligation of B7-H1 mAb alone (Dong, unpublished data). Our results support that B7-H1 mAb in the presence of strong TCR signaling promotes apoptosis of activated $CD4^+$ T cells.

To define the mechanism of the apoptotic effect, we used cDNA array to analyze mRNA expression of apoptosis-related genes in $CD4^+$ T cells after B7-H1 trigging. Up to 72 hrs following ligation with anti-CD3/B7-H1 mAb, transcription of caspase-10 and caspase-3 genes increased reproducibly.[16] Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was also up-regulated. Elevated TRAIL was confirmed in anti-CD3/B7-H1 mAb stimulated T cells by staining with anti-TRAIL antibody in FACS analysis. Significant increases of active caspase-3 protein were also detected at 48 and 72 hrs after stimulation. Neither B7-H1 mAb nor anti-CD3 (at suboptimal doses) alone stimulated these changes. Therefore, B7-H1-mediated apoptosis is involved, at least in part, by activation of caspase pathway. In addition to caspase pathway, B7-H1 mAb stimulated secretion of IL-10 from activated $CD4^+$ T cells. Inclusion of anti-IL-10 mAb significantly reduced the amount of apoptosis induced by anti-CD3/B7-H1 mAb. In contrast, neutralizing antibodies against Fas ligand and IL-2 had no effect. These results suggest that IL-10 is partially involved in the induction of apoptosis by B7-H1 mAb triggering.

Although our results support that triggering of T cell-associated B7-H1 transmits functional signaling, the nature of this signaling is unknown. The cytoplasmic domain of B7-H1 does not contain obvious structural elements that are directly relevant to signaling for cell activation or death.[45,46] It is possible that adapter proteins might play a role in transmitting B7-H1 signal to T cells. It is also possible that multiple signaling pathways are involved since B7-H1 signaling leads to diverse outcomes including costimulation of T cell growth, secretion of IL-10, up-regulation of caspases and TRAIL leading to increased apoptosis of activated T cells. Our data thus add a new insight into the emerging picture of the bi-directional signaling role of B7 family members, as indicated also in B7-1/2 and B7-DC on human B cells or dendritic cells.[15,47,48] The reverse signaling function of B7-H1 on T cells thus may provide additional fine tuning for T-T cells or T-APC interactions.

B7-H1 Autoantibodies and Systemic Autoimmune Diseases

In systemic autoimmune diseases such as rheumatoid arthritis (RA), a majority of peripheral $CD4^+$ T cells are in activated status.[49,50] These T cells release more IL-10 and have increased apoptosis than cells from normal individuals.[51] We reported recently the existence of autoantibodies against B7-H1 in RA patients.[16] Sera or plasma from the patients were examined by a specific sandwich ELISA using plates coated with purified B7-H1Ig containing human B7-H1 extracellular portion and mouse IgG2a Fc. Autoantibodies binding to B7-H1 were detected by anti-human IgG mAb. Significantly increased levels of IgG autoantibodies to B7-H1 were found in 18/63 (29%) RA patients. More importantly, 89% of RA patients in the B7-H1 autoantibody positive group were in their active disease phase, while significantly less (56%) RA patients in anti-B7-H1 negative group demonstrated disease activity,[16] suggesting an association between increased B7-H1 autoantibodies and disease activity. Furthermore, B7-H1 autoantibodies demonstrated a role for costimulation of human $CD4^+$ T cells when cross-linked in the presence of anti-CD3 in vitro.[16] The costimulatory activity of the autoantibodies could be blocked by the pre-addition of soluble B7-H1Ig, but not by PD-1Ig. These results suggested that the autoantibodies to B7-H1 might be involved in autoimmune disease progress by a direct effect on T cells.

The presence of B7-H1 autoantibodies may disrupt the regulatory network and result in the aberrant outcome of T cell responses as observed in human autoimmune diseases. The in vitro effects of B7-H1 autoantibody ligation were analogous to those observed with immobilized PD-1Ig, suggesting a possible role of PD-1 as a ligand to trigger T cell-associated B7-H1 in aberrant $CD4^+$ T cell activation in RA patients. In addition, B7-H1 autoantibodies may also interfere with the interaction between B7-H1 and PD-1, leading to increased activation of T cells and autoimmune diseases. It is worth noting here that PD-1 deficient mice spontaneously develop systemic lupus-like autoimmune diseases characterized by immune complex deposits in the renal parenchyma of aged B6 mice.[52] Our results thus support a possible involvement of B7-H1 autoantibodies in the pathogenesis of RA and possibly other systemic autoimmune diseases.

Conclusion

We are just starting to understand a complicated receptor-ligand system in which at least two receptors (PD-1 and non-PD-1 receptors) and two ligands (B7-H1 and B7-DC[53]) are involved. Ligation of B7-H1 to receptor(s) on T and B cells has profound effects in the induction and generation of both cell-mediated and humoral immunity, and the regulation of T cell responses by B7-H1 appears to be at priming and effect phases. The expression of B7-H1 in human tumor cells and its role in promoting apoptosis of effector T cells suggests a new mechanism for immune evasion and provides a new target for manipulation. Further studies are needed to understand the molecular mechanisms responsible for the reverse signals through

B7-H1 on activated T cells, as well as the role of this new signaling mechanism in the pathogenesis of systemic autoimmune diseases.

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Chapter 5

PD-1:PD-1 Ligand Pathway

Gordon J. Freeman and Arlene H. Sharpe

Introduction

The PD-1:PD-1 ligand pathway is a new pathway within the B7:CD28 superfamily. This pathway consists of the PD-1 receptor and its two ligands PD-L1 (B7-H1) and PD-L2 (B7-DC).[1-4] This chapter focuses on our current understanding of this recently discovered pathway. First, PD-1 structure and expression will be detailed. The important negative immunoregulatory role for PD-1, as revealed by the PD-1 deficient ($^{-/-}$) mouse, will then be discussed. Finally, studies examining the structure, expression and functions of the PD-1 ligands will be summarized.

Structure and Expression of the PD-1 Receptor

The structure of PD-1 is similar to that of CD28, CTLA4 and ICOS, and includes a single IgV extracellular domain, transmembrane domain and a cytoplasmic domain with tyrosine signaling motifs.[5-7] The extracellular domain of PD-1 is only weakly related to CD28 and CTLA4 with 21 and 16% amino acid identity, respectively, but has a somewhat higher homology to TCR-α and Ig-kappa. PD-1 lacks the MYPPPY or FDPPPF motif found in CD28, CTLA4, and ICOS. PD-1 also does not have the 3 additional, non-Ig consensus, cysteines found in CD28, CTLA4, and ICOS that allow these structures to be homodimeric and also have an extra internal disulfide bond. PD-1 also lacks the intracellular targeting motifs that control cell surface expression of CTLA4.[8] The cytoplasmic domain of PD-1 has two tyrosines, one of which constitutes an immunoreceptor tyrosine-based inhibitory motif (ITIM), V/IxYxxL. Human and murine PD-1 share 59% amino acid identities and are located on chromosome 2q37.3 and 1D, respectively.[5] The CD28/CTLA4/ICOS cluster is on the same chromosome, approximately 25 cM centromeric .

PD-1 was isolated as a gene whose expression was upregulated in two cell lines undergoing two different types of programmed cell death, the T cell hybridoma 2B4.11 in which death was induced by ionomycin/phorbol ester stimulation and a lymphoid/myeloid progenitor line, LyD9, in which death was due to IL-3 deprivation.[7] PD-1 mRNA expression was low or absent in these healthy cell lines but strongly upregulated when these cells underwent programmed cell death (thus, the name PD-1 for Programmed Death 1). 2B4.11 cells expressed moderate levels of cell surface PD-1 protein and PD-1 was upregulated by anti-CD3 or phorbol ester/ionomycin but not dexamethasone. LyD9 did not express PD-1 and IL-3 deprivation induced strong mRNA expression but only modestly upregulated cell surface expression.

In normal murine tissues PD-1 mRNA is detected only in thymus.[7] PD-1 is expressed in the thymus primarily on CD4^{-}CD8^{-} (DN) T cells.[9-11] PD-1 expression on the αβ T cell lineage is transiently induced at low levels late in the transition phase from DN to DP and expression

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is lost after the transition to DP.[9] γδ DN thymocytes express high levels of PD-1 and NK-T cells express low levels of PD-1.[9] In vivo anti-CD3 injection upregulates PD-1 mRNA expression in the thymus and spleen. PD-1 protein is not present on unstimulated B cells but is upregulated by anti-IgM crosslinking but not by LPS.[11] PD-1 is also expressed on activated myeloid cells.[11] PD-1 expression is not detected in unstimulated T cells, but is induced in murine and human $CD4^+$ and $CD8^+$ T cells by mitogen or anti-CD3 activation and in Jurkat cells by phorbol ester.[6,7,11] Thus, the expression of PD-1 is much broader than the expression of other CD28 family members, which is restricted to T cells.

Although cloned from cells undergoing programmed cell death, PD-1 expression does not correlate with programmed cell death. The PD-1 protein on Jurkat cells is tyrosine phosphorylated following activation, but expression correlates with G1 arrest, not programmed cell death.[6] Neither PD-1 crosslinking by mAb or PD-1 ligands nor PD-1 transfection into cells induces programmed cell death.[2,11,12] Thus, PD-1 expression appears to correlate with cell activation.

Gene chip analyses have shown that PD-1 mRNA is highly expressed in $CD4^+CD25^+$ regulatory T cells and anergic T cells.[13,14] Consistent with this, in vitro studies of TCR transgenic T cell activation have indicated that a weak TCR plus costimulatory signal induced higher PD-1 expression on activated T cells than a strong TCR plus costimulatory signal.[2] Some reciprocal interplay of the PD-1 and CD28 pathways was evident since CD28 expression was higher with a strong TCR plus costimulatory signal.

Function of PD-1

$PD-1^{-/-}$ mice have given insight into PD-1 function in vivo. $PD-1^{-/-}$ mice exhibit an autoimmune-like phenotype, indicating a role for PD-1 as a negative regulator of immune responses. These mice were generated by deletion of the transmembrane and cytoplasmic domain exons.[15] On the C57Bl/6 background $PD-1^{-/-}$ mice develop a late onset, progressive arthritis and lupus-like glomerulonephritis associated with high levels of IgG3 deposition.[16] Mice carrying the PD-1 mutation plus the lpr (fas gene) mutation exhibit more rapid disease progression and most of these mice exhibit symptoms by six months of age. In contrast, on the Balb/c background $PD-1^{-/-}$ mice develop a fatal dilated cardiomyopathy with an earlier onset, and 50% of the mice are dead by 20 weeks of age.[17] The $PD-1^{-/-}$ hearts do not exhibit significant mononuclear infiltrates but have IgG and complement deposited around the cardiomyocytes accompanied by scattered degeneration of cardiomyocytes. All affected mice develop high titers of an autoantibody that binds to a heart-specific 33 kD protein on the surface of cardiomyocytes but do not have other common autoantibodies. $PD-1^{-/-}$ $RAG-2^{-/-}$ Balb/c mice do not develop this phenotype, indicating the involvement of T and/or B cells. $PD-1^{-/-}$ mice have increased serum levels of IgG3 and lesser increases in IgA and IgG2b. $PD-1^{-/-}$ mice develop moderate splenomegaly with markedly increased numbers of myeloid cells and moderately increased B cells. $PD-1^{-/-}$ B cell responses to anti-IgM crosslinking are enhanced, as compared to wild-type B cells, but $PD-1^{-/-}$ T cell responses to anti-CD3 are comparable to wild-type T cells.[15] Myeloid cells from $PD-1^{-/-}$ mice have an augmented response to GM-CSF.[15] The phenotype of the PD-1-/- mice suggests that the PD-1:PD-Ligand pathway is important for B cell tolerance, given the marked defects in B cell responses and enhanced antibody production. However, whether these B defects are secondary to impaired T cell tolerance or whether there are defects in both B cell and T cell tolerance remains to be determined.

In a TCR transgenic model, $PD-1^{-/-}$ mice have revealed a role for PD-1 in regulating $CD8^+$ T cell responses.[16] When C57Bl/6 $H-2L^d$ reactive 2C-TCR transgenic mice were bred with $PD-1^{-/-}$ BALB/c mice, the resulting 2C-TCR $PD-1^{-/-}$ mice on the potentially autoreactive $H-2^{b/d}$ background exhibited a dramatic phenotype. In $PD-1^{-/-}$ 2C-TCR $H-2^{b/d}$ mice, the 2C-TCR is negatively selected in the thymus but the few 2C-TCR cells that progress to the

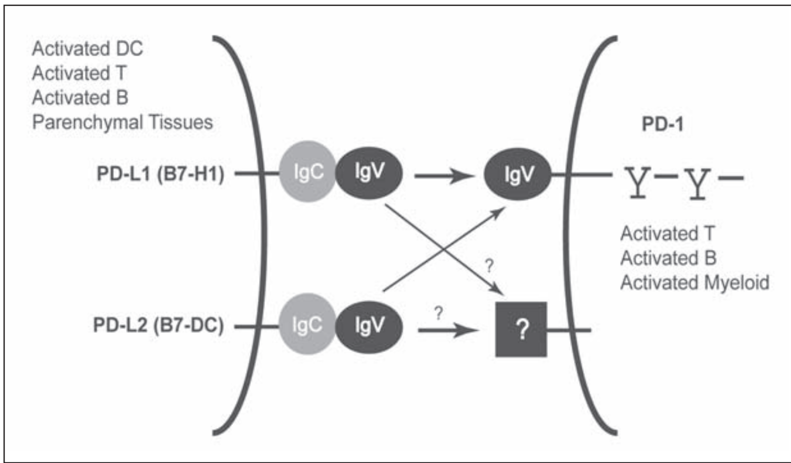

Figure 1. The PD-1:PD-L1(B7-H1)/PD-L2 (B7-DC) pathway. PD-L1 amd PD-L2 are immunoglobulin superfamily members with immunoglobulin-V-like and immunoglobulin-C-like domains. PD-L1 and PD-L2 are both ligands for PD-1 and may also bind to another, as yet to be identified, receptor on T cells (indicated by dotted lines and question marks).

periphery become activated and cause a lethal graft-versus-host-like disease.[9,16] These mice developed skin lesions with inflammatory cell infiltrates in the epidermis and infiltrates in multiple other organs. In contrast, in wild-type 2C-TCR H-$2^{b/d}$ mice, the 2C-TCR is negatively selected in the thymus and only a small number of 2C-TCR are found in the periphery but they neither become activated nor cause disease. In vitro, previously activated 2C-TCR T cells from PD-$1^{-/-}$ C57Bl/6 mice proliferated much more vigorously in response to H-2^d splenocytes than did wild-type 2C-TCR T cells, consistent with a role for PD-1 as a negative regulator of immune responses. These results strongly suggest that potentially autoreactive T cells in the periphery can become activated in the absence of PD-1, and implicate PD-1 in regulating peripheral T cell tolerance.

PD-$1^{-/-}$ mice also exhibit modest defects in thymic selection with an increase in $CD4^+CD8^+$ (DP) T cells and reduced numbers of single positive CD8 T cells.[9] These alterations correlate with the thymic expression of PD-1 predominantly on DN T cells.[9,10] These results suggest a role for PD-1 in central tolerance, probably in setting thresholds for thymocyte activation.

Biochemistry Basis for PD-1 Function

PD-1 transduces a signal when crosslinked along with TCR or BCR, but does not transduce a signal when crosslinked alone, similar to other CD28 family members. In a Jurkat T cell model, co-ligation of TCR and PD-1 led to rapid tyrosine phosphorylation and activation of SHP-2 (src homology 2-domain containing tyrosine phosphatase-2).[2] SHP-2 de-phosphorylates signaling molecules in the membrane proximal TCR signaling cascade, resulting in a diminished signal to the nucleus and reduced cytokine mRNA synthesis. In a B cell model of PD-1 function, chimeric molecules containing the extracellular domain of FcγRIIB were fused to the PD-1 cytoplasmic domain and transfected into a B lymphoma cell line.[18] Co-ligation of the chimeric PD-1 molecule with the BCR inhibited BCR-mediated growth retardation, Ca^{2+} mobilization, tyrosine phosphorylation of effector molecules including Syk, PLCγ2, PI3K, Vav, and ERK1 and ERK2 but not Lyn or Dok. Co-ligation of the chimeric PD-1 molecule with the BCR resulted in tyrosine phosphorylation of the PD-1 cytoplasmic domain and the rapid recruitment and activation of SHP-2, but not SHP-1 or SHIP. Interestingly, it is the

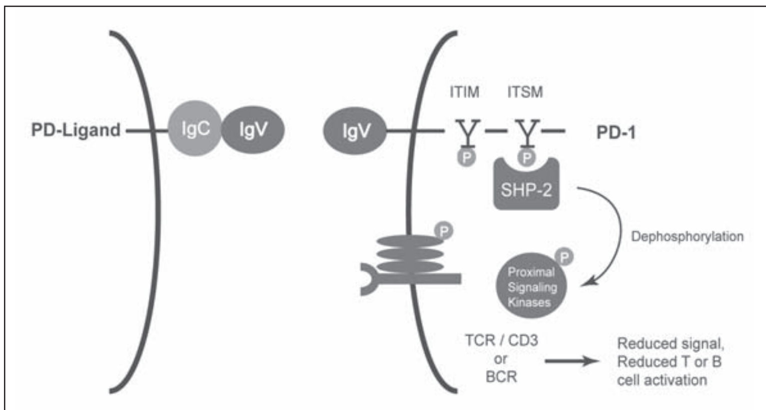

Figure 2. Signaling through PD-1. PD-1 has two tyrosines in its cytoplasmic tail, forming ITIM and ITSM motifs. The ITSM motif is critical for PD-1 mediated signals, since mutation of the ITSM tyrosine but not the ITIM tyrosine abrogates PD-1 mediated inhibitory signals. Co-ligation of TCR and PD-1 can result in tyrosine phosphorylation and activation of SHP-2, leading to dephosphorylation of signaling molecules and decreased cytokine mRNA synthesis. Co-ligation of BCR and PD-1 can inhibit tyrosine phosphorylation of effector molecules.

more COOH-terminal tyrosine and not the ITIM-motif tyrosine that has the major role in mediating the inhibitory signals of PD-1. The ITIM-motif tyrosine is phosphorylated but when it is mutated, this has little effect, and the signals it transmits remain to be determined. SHP-2 recruitment and inhibitory activity are obliterated when the COOH-terminal tyrosine is mutated to phenylalanine.[18] The more COOH-terminal tyrosine of PD-1 matches the recently described immunoreceptor tyrosine-based switch motif (ITSM), TxYxxV/I, first described in CD150 (SLAM).[19] The ITSM motif in CD150 switches association with different phosphatases via the SAP (SH2D1A) adaptor protein. In the presence of SAP, SHIP (SH2-containing inositol phosphatase) preferentially associates with the CD150 cytoplasmic domain but in the absence of SAP, SHP-2 preferentially associates. As CD150 has been reported to deliver both positive and negative signals, this may be relevant to the function of PD-Ligands, which have been reported to deliver either positive or negative signals.

Structure and Expression of PD-1 Ligands

Because the PD-1 structure resembled CD28/CTLA4, it was hypothesized that PD-1 would bind a novel B7 gene family member. Two novel B7 homologues PD-L1 (B7-H1) and PD-L2 (B7-DC) have been identified as ligands for PD-1.[1-4] PD-L1 and PD-L2 bind to PD-1, but not to any additional CD28 family members. Human PD-L1 and PD-L2 are located on chromosome 9p24.2, oriented in the same direction, 42 kb apart, with a similar exon organization.[2,4] Murine and human PD-L1 have 69% amino acid identity, similar to the 70% identity between human and murine PD-L2 and higher than the 45-51% identity between human and murine B7-1 or human and murine B7-2. The five members of the B7 gene family, B7-1, B7-2, ICOS-L, PD-L1, and PD-L2 share 21-27% amino acid identity and a structural organization consisting of a signal sequence, IgV-like, IgC-like, transmembrane and short cytoplasmic tail. The cytoplasmic tail of PD-L1 is well conserved between the murine and human forms. In contrast, the cytoplasmic tail of murine PD-L2 is much shorter than human PD-L2, 5 amino acids versus 30, and represents the end of the transmembrane exon without the use of the two cytoplasmic exons found in human PD-L2.

In contrast to the expression of B7-1 and B7-2, expression of the PD-1 ligands is not restricted to antigen presenting cells. Northern blot analyses detected PD-L1 and PD-L2 in non-lymphoid organs such as heart, placenta, and lung in both humans and mice.[1-3] Both PD-L1 and PD-L2 were expressed in human heart at high levels, but PD-L2 was expressed at a low level in mouse heart. Immunohistochemistry shows that in the human heart PD-L1 is expressed in the endocardium and at lower levels on cardiomyocytes (D. Dorfman and G. Freeman, unpublished). The high levels of PD-L1 and PD-L2 expression in the heart are particularly intriguing in view of the cardiomyopathy that develops in PD-$1^{-/-}$ BALB/c mice,[17] and suggests that PD-1 ligand expression on peripheral tissues may be important for regulating peripheral tolerance and/or B cell and/or T cell activation. In addition, PD-L1 is highly expressed on placental syncytiotrophoblasts, the cells at the maternal/fetal interface, suggesting a role in immune suppression by the fetus (D. Dorfman and G. Freeman, unpublished). Some tissues, such as human pancreas, lung and liver, express PD-L2 but not PD-L1, and human fetal liver expresses PD-L1, but not PD-L2.[2] The expression of PD-L1 and PD-L2 within non-lymphoid tissues suggests that these PD-1 ligands may serve to downregulate self-reactive T or B cell responses in peripheral tissues, and/or may serve to attenuate inflammatory responses at these sites.

PD-L1 is also expressed on endothelial cells. PD-L1 protein is constitutively expressed on a microvascular endothelial cell line derived from microvessels of murine pancreatic islets, and is strongly upregulated on this cell line by IFN-α, β, or γ.[20] In vivo administration of IL-12 upregulates PD-L1 expression on the endothelial cells of all organs tested. This is dependent on IFN-γ since PD-L1 upregulation was abolished in IFN-γ deficient mice although the constitutive level was unchanged.[20]

The expression of PD-L1 and PD-L2 mRNAs also is upregulated in a variety of tumor cell lines.[2] Cell surface expression of PD-L1 has been demonstrated on some human breast cancer cell lines.[2] PD-L1 is also expressed in situ on most lung and breast malignancies and many ovarian tumors (D. Dorfman and G. Freeman, unpublished). PD-L1 and PD-L2 expression on tumors may assist in immune evasion, and suggests a new means by which tumors may attenuate anti-tumor responses.

PD-L1 and PD-L2 mRNA are expressed at low levels in the thymus and on spleen and lymph nodes.[1-3] PD-L1 protein is expressed on thymic epithelial cells (David Dorfman and Gordon Freeman, unpublished). PD-L1 mRNA is not expressed in thymic epithelial cells in nude mice, which are mutant in the winged-helix transcription factor, Whn. PD-L1 was one of 5 genes identified as being deficient in thymic epithelial cells from nude mice and these are candidates for the defect in T cell maturation of the nude mouse.[21] The relatively minor effect on thymic differentiation in PD-1 deficient mice argues against a major role. On human monocytes and dendritic cells, PD-L1 and PD-L2 are upregulated upon activation or IFN-γ treatment.[1-4] Human and murine T cells express PD-L1 following TCR or mitogen stimulation, but PD-L2 is not expressed on human T cells[3] and (J. Brown and G. Freeman, unpublished).

Function of PD-1 Ligands

The functions of PD-L1 or PD-L2 in regulating T cell responses are just beginning to be understood, and this is an area of some controversy. Since PD-1 is upregulated on activated T cells,[11] the functions of PD-L1 and PD-L2 have been examined using previously activated T cells. When previously activated DO11.10 TCR transgenic $CD4^+$ T cells were cultured with peptide and CHO cells expressing PD-L1 or PD-L2, T proliferation and cytokine production were markedly inhibited by CHO cells expressing PD-1 ligands.[2] T cell proliferation and cytokine production were both strongly inhibited when the TCR transgenic T cells were cultured with low concentrations of antigen and CHO transfectants expressing PD-L1 or PD-L2 plus a positive costimulatory signal provided by B7-2. At high antigen concentrations, these

transfectants markedly reduced production of multiple cytokines including IL-2, IL-4, IL-5, IL-10, IL-13 and IFN-γ but did not inhibit T cell proliferation. The PD-1 ligands exert these effects by causing cell cycle arrest in G_O/G_1, but not cell death.[2,12] These studies demonstrate overlapping functions of PD-L1 and PD-L2 and support a role for the PD-L:PD-1 pathway in downregulating T cell responses.

PD-L1 or PD-L2-Ig proteins coupled to beads together with anti-CD3 mAb inhibited T cell proliferation and cytokine production by both resting and previously activated $CD4^+$ and $CD8^+$ T cells and even naïve T cells from cord blood.[1,2,12] This inhibition was not observed when $PD\text{-}1^{-/-}$ T cells were stimulated with anti-CD3 plus PD-L1-Ig, indicating that the inhibitory signal was transduced by PD-1.[1] Using 2C TCR transgenic CD8 T cells and transfectants expressing MHC class I and PD-L1, PD-L1 was found to strongly inhibit CD8 T cell proliferation.[12] The addition of exogenous IL-2 could overcome this inhibition, but a costimulatory signal provided by anti-CD28 mAb could not overcome this inhibition. The latter results contrast with those using $CD4^+$ T cells, where anti-CD28 mAb or B7-2 could overcome the inhibition, probably because of the higher IL-2 production by $CD4^+$ T cells.[1,2,12] Taken together, these results demonstrate that PD-L1 and PD-L2 can antagonize T cell activation when the TCR/CD28 signal is weak or limiting. A strong TCR/CD28 signal predominates over PD-L1 or PD-L2, though cytokine production may be diminished. Down-regulation of growth factor production is probably a major mechanism of PD-1-mediated inhibition.

The inhibitory effects of PD-L1 and PD-L2 transfectants have prompted studies examining the role of this pathway in $CD4^+CD25^+$ regulatory T cell function. $CD4^+CD25^+$ regulatory T cells do not constitutively express PD-L1.[22] $CD4^+CD25^+$ regulatory T cells suppressed $CD4^+CD25^-$ T cell proliferation, IFN-γ, and IL-13 production in response to anti-CD3 plus anti-CD28 and accessory cells. Treatment with a blocking anti-PD-L1 mAb led to an increase in $CD4^+CD25^-$ T cell proliferation, IFN-γ, and IL-13 production.[22] However, the suppressive effect of $CD4^+CD25^+$ regulatory T cells on this proliferation was blocked by anti-PD-L1 mAb only at moderate ratios of regulatory: $CD4^+CD25^-$ cells. When more regulatory cells were added, suppression was restored. These results suggests that the PD-1:PD-L1 pathway may play a role in $CD4^+CD25^+$ regulatory cell function, but that this is not the major inhibitory pathway for these regulatory cells.

However, not all studies support a negative regulatory role for PD-L1 and PD-L2. In three studies these B7 homologues stimulated T cell proliferation.[3,4,23] Chen and colleagues first isolated B7-H1 by identifying three ESTs from an ovarian tumor with homology to B7-1 and B7-2.[3] Resting T cells stimulated with low levels of anti-CD3 and immobilized B7-H1-Ig (PD-L1-Ig) or COS cells transiently transfected with B7-H1 had moderately enhanced proliferation. $CD4^+$ T cells were more strongly stimulated than $CD8^+$ T cells and the stimulation was also observed with $CD28^{-/-}$ T cells.[23] Neither CTL generation nor lysis were enhanced by B7-H1. B7-H1 strongly enhanced IL-10 production, modestly stimulated IFN-γ and GM-CSF production, and had little effect on IL-2 or IL-4 production.[3,23] The increase in IL-10 production was dependent on IL-2. B7-H1-Ig stimulated the rapid expression of CD40L. In vivo, B7-H1-Ig stimulated the production of IgG2a antibody.[23] Tseng et al. isolated B7-DC (PD-L2) by subtractive hybridization of murine bone-marrow derived, GM-CSF cultured DCs with RNA from IFN-γ and LPS stimulated adherent bone marrow-derived M-CSF macrophages.[4] By virtual Northern, B7-DC was strongly expressed in the DC but not the macrophage populations nor in placenta. Stimulation of resting CD4 T cells with immobilized anti-CD3 plus B7-DC-Ig led to markedly enhanced proliferation, indeed, stronger than with B7-1-Ig. IFN-γ production was strongly stimulated but IL-2, IL-4, or IL-10 production was not. Little effect was observed on CD8 T cell proliferation.

The reason for the contradictory results of functional studies with the PD-1 ligands is not clear. The experiments of the Chen and Tseng labs have used resting T cells and observed

stimulation.[3,4,23] In contrast, the experiments of the Freeman, Sharpe, Honjo, and Wood labs have focused on previously activated T cells expressing PD-1 and observed inhibition of T cell responses, however, experiments with resting T cells also show inhibition.[1,2,12,22] Using plastic immobilized anti-CD3 and PD-L1-Ig or PD-L2-Ig, another independent group has examined the effects of PD-1 ligands on T cell activation, and found an inhibitory effect when plastic of low binding capacity (normal tissue culture treated) is used, but a stimulatory effect when plastic of high binding capacity (ELISA plates) is used (S. Suggs and S. Yoshinaga, personal communication).

Summary and Concluding Remarks

The PD-1:PD-1 ligand pathway is the most recently delineated pathway in the CD28: B7 superfamily. The expression of PD-1 is not restricted to T cells, but also expressed on B cells and macrophages. The phenotype of the PD-1-/- mouse supports an important negative regulatory role of PD-1 for T cells, B cells, and macrophages, and suggests a role for PD-1 in regulating T and/or B cell tolerance. The expression of PD-1 ligands is broader than that of B7-1 and B7-2. PD-1 ligands are expressed on lymphoid and nonlymphoid tissues. The expression of PD-1 ligands on nonlymphoid tissues and on tumors suggests a previously unappreciated means by which immune responses, in peripheral tissues or by tumors, may be regulated. The functions of PD-1 ligands are an area of controversy at present, with some studies indicating an inhibitory function, but others showing a stimulatory function.

One possible way to reconcile these contradictory results might be that similar to the B7:CD28/CTLA4 pathway, a second receptor for PD-1 ligands might exist with the capacity to deliver a stimulatory signal like CD28. Arguing against this possibility is the fact that when PD-1$^{-/-}$ T cells are stimulated with PD-L1-Ig and anti-CD3 mAb, no increase in proliferation over that of anti-CD3 alone is observed. No inhibition is observed in PD-1$^{-/-}$ T cells as compared to wild-type T cells. In comparison, CTLA4$^{-/-}$ T cells are more strongly activated by B7 since the CD28 pathway is unopposed. In addition, PD-1 Ligand-Ig does not detectably bind to unstimulated T cells (G. Freeman, unpublished). Resolution of these differences will likely come from experiments using blocking antibodies and knockout mice.

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Chapter 6

The ICOS/B7RP-1 Costimulation Pathway

Steven K. Yoshinaga

Abstract

A novel costimulation pathway homologous to CD28/B7 has recently been discovered and characterized. The Inducible Costimulator (ICOS) is structurally and functionally homologous to CD28. The B7-Related Protein-1 (B7RP-1) is structurally and functionally homologous to B7.1 and B7.2 (B7.1/2), and is the ligand for ICOS. ICOS/B7RP-1 is emerging as a costimulatory pathway for a variety of effector and memory T-cell responses. Th2 responses are particularly sensitive to ICOS/B7RP-1 regulation; however, Th1 and CD8$^+$ activities are also affected by its function. ICOS is not present on naïve T-cells, but is rapidly induced after primary antigen stimulation. B7RP-1 is expressed on a number of different cell types, including professional and non-professional antigen presenting cells (APCs), and may play a role in the establishment of costimulatory environments in peripheral tissues. Manipulation of the pathway results in alterations of immune responses that may be applicable in therapeutic indications as diverse as cancer, infectious and autoimmune diseases, and transplantation. These initial characterizations of the pathway indicate that the regulation of ICOS/B7RP-1 holds promise in the control of immunological disease at the level of effector T-cell responses in peripheral tissues.

Introduction

The therapeutic manipulation of costimulatory proteins offers the promise of inhibiting antigen specific immune responses without the knowledge of the specific antigen.[1] Analyses of the CD28-mediated costimulatory pathway have revealed functions in immunological tolerance, anergy, and memory. Although CD28 regulation has profound effects on immunity, its function appears to reside mainly in the control of primary, but not secondary, immune responses.[1]

In 1999, three groups independently described the receptor, the ligand, or both receptor and ligand, of a novel CD28/B7-related costimulation pathway. Kroczek and colleagues characterized the novel CD28-related molecule, which they called ICOS, for Inducible Costimulator.[2] Sha and colleagues described a unique B7-related protein, which they named B7h, for B7 homologue.[3] We demonstrated that ICOS and B7-related protein-1 (B7RP-1 and B7h are identical) are a receptor/ligand pair that is distinct from the previously characterized CD28/B7 pathway.[4] In contrast to the constitutively expressed CD28, ICOS was induced on naïve T-cells[2,4,5] after primary TCR engagement. Consequently, ICOS was theorized to operate downstream of CD28, perhaps in effector or secondary responses. We initially showed that soluble B7RP-1 enhanced the challenge phase of a murine contact hypersensitivity reaction[4]. Subsequent studies have supported ICOS/B7RP-1 secondary functions in other experimental sce-

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narios; however, the role of ICOS/B7RP-1 in an immune response needs further clarification. This paper reviews the current understanding of this exciting new T-cell costimulatory pathway.

Terminology

The independent discovery of the proteins in the ICOS/B7RP-1 pathway by several groups has resulted in different designations of its components. The Inducible COStimulator (ICOS),[2] has also been called CD28-Related Protein (CRP-1),[4] and Activation Inducible Lymphocyte Immunoregulatory Molecule (AILIM).[5] Generally, the field is using the ICOS designation for the CD28 homologous receptor. The ligand for ICOS, B7-related protein-1 (or B7RP-1),[4] has also been designated as B7h (B7-homologue),[3] ICOSL,[6] LICOS,[7] GL50,[8] and B7-H2.[9] All of these terms are used in the current scientific literature, but for clarity, this review will refer to the ICOS ligand as B7RP-1.

Structures of ICOS and B7RP-1

Murine ICOS shares approximately 19% amino acid (aa) identity with murine CD28, and approximately 14% amino acid identity with murine CTLA-4. This low degree of identity is consistent within members of this family, as murine CD28 and CTLA-4 share only 26% aa identity, and both bind B7.1 and B7.2 (B7.1/2). Although the homology is weak between ICOS and CD28, the cysteines that form the disulfide loops and the putative intermolecular bond for homodimer formation[10] are conserved in ICOS.[4] The programmed cell death-1 (PD-1) protein, another receptor that binds B7-related molecules,[11] lacks this cysteine conservation, thus implying that ICOS may have functions more similar to those of CD28/CTLA-4 than PD-1. Furthermore, the size of the ICOS protein (murine: 200 aa; human:199 aa) and the position of the transmembrane domain relative to the conserved cysteines are similar in ICOS and CD28 (human: 207 aa). Notably, an important motif for B7.1/2 binding that is present in all forms of CD28 and CTLA-4 consists of the amino acid sequence MYPPPY, and is not present in ICOS. At the same position in the protein, ICOS has the aa sequence FDPPPF, which is partially conserved with the MYPPPY motif and may indicate that ICOS binds a similar, but different, ligand as CD28 and CTLA-4. Human ICOS is located on chromosome 2q33-34,[12,13] adjacent to CD28 and CTLA-4, further indicating its close relation to those genes.

Murine B7RP-1 shares approximately 20% amino acid homology with murine B7.1 and B7.2. Similar to the CD28 homologous proteins, the low degree of homology is typical for this family, as murine B7.1 and B7.2 share only ~24% amino acid identity. More importantly, the four cysteines that putatively form the disulfide loops for the two immunoglobulin-like domains are conserved, and the overall size of the protein (murine: 322 aa; human: 302 aa) and the position of the transmembrane domain relative to the conserved cysteines[4] are similar to those of B7.1 (murine: 306 aa). Some other amino acid similarities with B7.1 and B7.2 are evident, particularly in the variable Ig-like domain.[8]

The mouse and human ICOS proteins share a similar degree of homology (approximately 69% amino acid identity) as the mouse and human CD28 proteins (~68%).[4] Likewise, the mouse and human B7RP-1 proteins share a similar degree of homology (~43%) as the mouse and human B7.1 proteins (41%).[14] In spite of this low degree of amino acid identity, the mouse and human ICOS and B7RP-1 proteins cross-interact, revealing the conservation of structural and functional homology. In contrast to the low amino acid identity between the mouse and human B7RP-1 or B7.1, B7-H1 (also called PD-L1), another B7-related protein, shares a significantly greater degree of homology between the mouse and human proteins (~70% amino acid identity),[11] which suggests that B7RP-1 is functionally more similar to B7.1 than B7-H1.

ICOS Expression

Mouse ICOS is expressed on activated CD4$^+$ and CD8$^+$ T-cells, and on resting memory T-cells.[4,15] Murine Th2 effector T-cells express greater amounts of ICOS as compared to Th1 effector T-cells.[13,15] Human ICOS is similarly expressed on activated CD4$^+$ and CD8$^+$ T-cells.[12,14] Recent data indicate that ICOS is also expressed on NK cells (L. Lanier and C. Hunter, unpublished results).

Tissue analyses of human ICOS revealed expression in the medulla of the fetal thymus. In addition, human ICOS is expressed in this region of the thymus from a two-week old.[12] Therefore, ICOS may be involved in early ontogeny and may not be restricted to effector or memory T-cell functions. T-cell zones occasionally express ICOS, indicating potential DC interactions, and its expression in the apical light zones in the germinal centers of tonsils suggests ICOS functions in T-B-cell interactions.[2]

Additionally, experimental models of disease in mice have revealed that the T-cells that infiltrate into diseased tissue express ICOS.[16-19] The expression of ICOS on these putatively pathogenic T-cells may allow us to target functional disease processes specifically.

B7RP-1 Expression

B7RP-1 is widely expressed on a variety of tissues. RNA analyses of mouse tissues revealed B7RP-1 expression in the heart, spleen, lung, kidney, and brain, with lower expression in the thymus, lymph nodes, liver, testis, and skeletal muscle.[3,8] In situ RNA analyses localized the expression of B7RP-1 in the cortex of the lymph nodes, the follicles and marginal zone in the spleen, the follicles in Peyer's patches, and the medulla in the thymus.[4] Human northern analyses indicated B7RP-1 mRNA expression in brain, heart, skeletal muscle, liver, small intestine, colon, placenta, and lung.

B7RP-1 is also expressed on a wide variety of cell types. FACS analyses revealed that B7RP-1 is expressed on murine B-cells and monocytes. Furthermore, the expression of B7RP-1 on murine B-cells and monocytes is up-regulated by TNFα or LPS stimulation.[14] Correspondingly, human B-cells and monocytes increased B7RP-1 expression in response to TNFα and LPS. Dendritic cells express B7RP-1; however, its expression is down-regulated by TNFα and LPS induction. This differential B7RP-1 regulation by TNFα stimulation on B-cells and monocytes relative to dendritic cells suggests that B7RP-1 is more involved in either the downstream effector or secondary T-cell functions, rather than the initiation of a primary response via dendritic cells.

Interestingly, some T-cells also express B7RP-1 on their cell surface.[8,20,21] The expression of B7RP-1 on T-cells is not unique, as other B7 molecules are also expressed on T-cells.[22] The function of B7RP-1 on T-cells needs further analysis, and its presence there may complicate interpretations of experimental results.

B7RP-1 is also expressed on non-immune cells and on a wide variety of tissues. Sha and colleagues initially found B7RP-1 as a protein induced on mouse fibroblasts by TNFα .[3] This group also identified several tissues, namely the testes, kidney, and peritoneum, that expressed B7RP-1 mRNA upon LPS induction. They suggested that the non-lymphoid cells, such as fibroblasts, are the source this expression, since the highly vascularized liver did not show this up-regulation, and B7.2 expression was not induced under the same conditions.

Recently, Wuthrich and colleagues directly demonstrated the presence of B7RP-1 on primary renal tubular epithelial cells (TEC) and renal tubular epithelial cell lines (data not shown, paper under review, will submit with review). Using FACS, northern, RT-PCR, and western analyses, they definitively showed that B7RP-1 was expressed on various renal tubular epithelial cell lines. Immunohistochemical analyses indicated strong constitutive expression on human primary renal tubular epithelial cells in distal tubules, collecting ducts, and urothelium.

This study indicates that epithelial cells may express B7RP-1 to contribute to a localized costimulatory environment. Further analyses are warranted to determine the nature of the ICOS/B7RP-1 pathway in the kidney.

Hematopoeitic progenitors also express B7RP-1, and TNFα and NF-κB appear to regulate this expression.[20] The induction of B7RP-1 is dependent on TNFα, and inhibitors of IκBα phosphorylation prevented the TNFα-mediated B7RP-1 upregulation. Although NF-κB is involved in the TNFα-mediated B7RP-1 up-regulation on hematopoetic progenitors, the IFN-γ-mediated B7RP-1 up-regulation on monocytes did not appear to be dependent on NF-κB.[23] Further analyses are indeed necessary to clarify the regulation of B7RP-1 in different cell types. Lastly, embryonic stem (ES) cells were found to bind soluble ICOS-Fc, thus indicating B7RP-1 expression on those cells.[21] The function of this B7RP-1 expression is unknown; however, the expression of other B7 costimulatory molecules on embryonic stem cells is not without precedent, as B7.1 is expressed on ES cells.[24]

In summary, B7RP-1 is differentially regulated on APCs, and is expressed on fibroblasts, hematoprogenic precursors, embryonic stem cells, and epithelial cells. This wide expression pattern suggests roles in such diverse functions as the ontogeny of many different cell types and the costimulation of effector and secondary immune responses. The expression of B7RP-1 on multiple cell types may allow several B7RP-1 signals to influence the ICOS-expressing T-cells in a localized costimulatory environment (Fig. 1).

Cytokine Regulation by ICOS/B7RP-1

Cytokine regulation by the ICOS/B7RP-1 pathway has been characterized by both in vitro and in vivo methods. In vitro experiments, using purified T-cells stimulated by TCR complex engagement and then costimulated by anti-ICOS antibodies or B7RP-1-Fc, demonstrated that ICOS/B7RP-1 upregulates IFN-γ, IL-4, IL-5, IL-10, IL-13, GM-CSF, and TNFα expression.[2,4,12,14,15]

The initial report describing ICOS showed that anti-ICOS mAb costimulation super-induced IL-10 secretion, as compared to anti-CD28 mAb costimulation.[2] Other experiments using the B7RP-1 ligand as the costimulator revealed that IL-10 is indeed induced by ligand engagement[9,14]; however, not as dramatically as that by ICOS engagement with an antibody. Evidently, experimental protocols may influence the magnitude of the effect in vitro, and may not reflect the quantitative levels of in vivo ICOS/B7RP-1 regulation. Nevertheless, it is clear that ICOS regulates IL-10 production.

The ICOS/B7RP-1 pathway has a small role in IL-2 regulation. IL-2 secretion is slightly up-regulated by B7RP-1 costimulation, particularly when signal 1 (TCR engagement) is weak.[9] Although little IL-2 is secreted upon ICOS engagement, IL-2 appears necessary for the ICOS-mediated costimulation of T-cell proliferation [14]and indeed, is a limiting factor in vitro.[25]

In vitro, IL-2 production was not affected by the ICOS inhibition of a primary response, but was significantly inhibited during antigen re-stimulation.[26] In contrast, CTLA-4-Ig inhibited both primary and secondary responses in vitro. This experiment thus identifies the ICOS/B7RP-1 role in IL-2 production in secondary, but not primary, responses.

IL-2 production in ICOS$^{-/-}$ mice was relatively unaffected in comparison to littermate controls, as reported by McAdam et al.[27] This weak effect on IL-2 is consistent with the minor effect on IL-2 secretion in the in vitro experiments using anti-ICOS antibodies and B7RP-1-Fc.[2,4,9,14] In contrast, Dong et al found significant impairment of in vitro IL-2 production in the T-cells from ICOS-deficient mice.[28] Further studies are needed to reconcile this result with the in vitro and in vivo experiments that show a weak ICOS effect on IL-2 regulation.

Analyses of ICOS-deficient mice have demonstrated strong ICOS-dependent IL-4 and IL-13 production; however, there was little or no indication of an effect on IFN-γ secretion.[27-29]

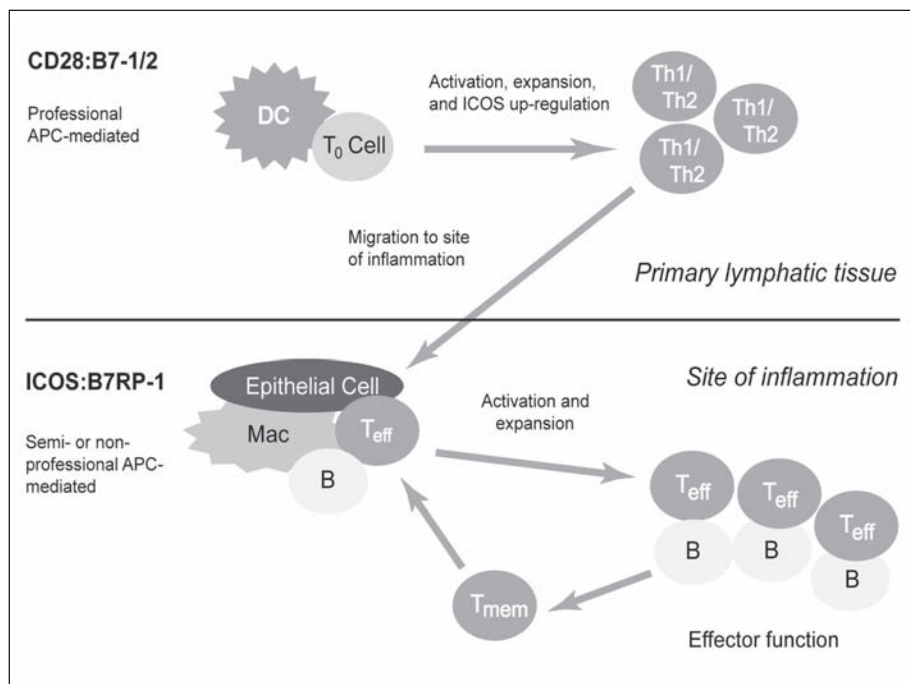

Figure 1. A model of the ICOS/B7RP-1 costimulatory pathway. The CD28-mediated costimulatory pathway is mainly involved in responses of naïve T-cells interacting with dendritic cells (DC), which are professional antigen presenting cells (APC). After antigen activation, ICOS expression is up-regulated on differentiating T-cells, which migrate to the site of inflammation, such as the donor graft or local autoimmune-reactive tissue. These ICOS expressing effector T-cells (T_{eff}) may interact with one or more B7RP-1 expressing cells (Mac: macrophage, B: B-cell, epithelial cell, etc.), each perhaps contributing to the overall costimulatory environment recognized by the T-cell. In addition, the ICOS/B7RP-1 interactions may help promote memory T-cell (T_{mem}) responses.

Although one may speculate that $ICOS^{-/-}$ mice may exhibit an IFN-γ deficiency based on the in vitro ICOS/B7RP-1 costimulation experiments, two gene deletion studies showed no effect on IFN-γ, and one study revealed exacerbated IFN-γ production. These counter-intuitive results suggest that there are other mechanisms controlling IFN-γ expression in these mice.

In a model of immune activation by a bacterial superantigen, an ICOS/B7RP-1 blockade with the ICOS-Fc fusion protein inhibited the secretion of TNFα, IL-10, and IFN-γ, but had no effect on IL-4.[30] ICOS-Fc also inhibited the secretion of IL-4, IL-5, IL-10, and IFN-γ after infection by the nematode, *Nippostrongylus brasiliensis*.[31] In this latter study, ICOS-Fc inhibited both Th1 and Th2 cytokines after infection by LCMV, VSV, or the nematode. Interestingly, the cytotoxic T-cell responses induced by viral infection were not inhibited by the ICOS/B7RP-1 blockade.

The effect of the ICOS/B7RP-1 pathway on cytokine secretion was also assessed in several experimental models of disease. In models of allergic airway disease, an ICOS/B7RP-1 blockade reduced IL-4 and IL-5 production.[26,32] Similarly, IFN-γ secretion was lower after treatment with ICOS/B7RP-1 inhibitors in models of transplantation,[16] multiple sclerosis (EAE),[17] and graft vs host disease (GvHD).[33]

In summary, in vitro and in vivo experiments have demonstrated that the ICOS/B7RP-1 pathway regulates the secretion of both Th1 and Th2 cytokines. The Th1 cytokines IL-2, TNFα, and particularly IFN-γ, are regulated by ICOS/B7RP-1. Th2 cytokines, such as IL-4, IL-5, IL-10, and IL-13, also appear to be regulated by ICOS/B7RP-1. The minor inconsistencies in the data may reflect the inherent differences in the experimental approaches, or possibly, the differential cytokine regulation by ICOS/B7RP-1.

Immunoglobulin Regulation by ICOS/B7RP-1

ICOS significantly affects the development of a humoral response. ICOS was initially found to be involved in T-B cell interactions and immunoglobulin production.[2] The mechanism for this effect may be via the induction of CD40L expression, as ICOS engagement promoted CD40L expression on T-cells.

Three groups independently described the similar phenotype of ICOS-deficient mice. They found that ICOS deficiency led to impaired immunoglobulin production and defective germinal centers in these mice.[27-29,34] In various primary immune challenges, the IgG1, IgG2a, and IgE responses were decreased in the $ICOS^{-/-}$ mice. Of particular interest, a secondary challenge with KLH resulted in severely impaired production of IgG1 and IgG2a from $ICOS^{-/-}$ mice. Furthermore, tertiary re-stimulation with NP-OVA resulted in lowered IgE levels. Therefore, the Th1 (IgG2a) and Th2 (IgG1 and IgE) immunoglobulins and both the primary and recall humoral responses are dependent on the ICOS/B7RP-1 activity. IgM production in these ICOS gene deletion experiments was not affected.

Several other lines of evidence support ICOS/B7RP-1 functions in immunoglobulin regulation. Notably, transgenic mice systemically expressing B7RP-1-Fc developed lymphoid hyperplasia and severe plasmacytosis.[4] Furthermore, in models of autoimmunity, an ICOS/B7RP-1 blockade resulted in normal IgM levels, but impaired IgG1, IgG2a, and IgE immunoglobulin production.[19,26,32,33] Although there are some inconsistencies, the in vitro and in vivo results generally indicate that the ICOS/B7RP-1 pathway is intimately involved in the regulation of immunoglobulin switching to the IgG1, IgG2a, and IgE isotypes.

CD28 and CTLA-4 Regulate, but Are Not Required for, ICOS Function

T-cells from CD28-deficient mice are capable of ICOS-mediated costimulation, thereby indicating that CD28 activity is not required for ICOS stimulation in vitro.[4] In addition, ICOS inhibitors have significant effects in the absence of CD28, indicating CD28-independent ICOS activity[35]. However, the CD28 pathway regulates ICOS expression, and therefore presumably its function, as CD28 activity enhances ICOS expression in vitro.[15] It is intriguing that CTLA-4, a negative regulatory molecule related to CD28, is also a negative regulator of ICOS expression.[25] CTLA-4 is believed to act independently of CD28, although it engages the same ligands. Therefore, the CD28 and CTLA-4 pathways both appear to regulate ICOS expression, but in opposite ways and possibly by different mechanisms. Furthermore, CTLA-4 may also regulate ICOS activity downstream of receptor expression.[25]

The HpH4 HIV Antigen Is ICOS

Janeway and colleagues identified a protein, named H4, using a specific antibody (C398.4A) produced against a mouse T-cell clone.[36] While the gene was not cloned, H4 was later determined to be identical to ICOS.[37] The original C398.4A antibody interacted with both the mouse (H4) and human (hpH4, human putative H4) ICOS proteins, and therefore was used in human studies.[38] Many of these studies on H4 and hpH4 preceded those on ICOS, and their results are consistent with the recent ICOS analyses. H4 or hpH4 was expressed only on

activated T-cells associated with the TCR complex, and the H4 antibody immunoprecipitated a disulfide-linked homodimer of approximately 50-65 kDa. ICOS is a disulfide-linked homodimer of approximately 50-65 kDa.[2,12] In addition, a comparison of the F44 ICOS mAb from Krozcek and colleagues[2] to that of the H4 binding C398.4A mAb revealed similar activities and expression kinetics of their protein antigens. Finally, the C398.4A mAb costimulated the antigen-specific immune responses of a T-cell clone in vitro. Although the costimulation by the antibody was not seen in conjunction with the anti-CD3 antibody engagement and was determined as a soluble mAb (as compared to the F44 mAb, which costimulated when bound to a solid phase), the mAb-mediated costimulation is similar to that of other antibodies to costimulatory proteins.

Interestingly, high ICOS expression is correlated with high levels of T-cell viremia in HIV infected patients.[39] In addition, the reduced expression of ICOS inversely correlates with the activity of HIV protease inhibitors, indicating that ICOS may be useful for monitoring the response to HIV protease inhibitor treatment. Why would HIV prefer ICOS expressing T-cells? One explanation offered by the investigators is that the ICOS pathway is anti-apoptotic, and HIV would prefer a host cell that is long-lived. Consistent with this hypothesis, ICOS engagement by the C398.4A mAb protected against T-cell death mediated by gp120 or anti-FAS mAbs.[39] Indeed, further investigation into the role of ICOS regulation in the HIV lifecycle is warranted.

ICOS and Apoptosis

Other studies, in addition to that described above,[39] have also reported that ICOS may function by inhibiting apoptosis. These preliminary studies utilized different approaches, but were generally consistent. Beier et al showed that ICOS engagement with the F44 anti-ICOS antibody prevented the apoptosis of human T-cells re-stimulated with sub-optimal concentrations of anti-CD3 antibody.[12] Perrin and colleagues took the opposite approach, using the ICOS-Ig fusion protein to inhibit ICOS/B7RP-1 interactions in both naïve and memory T-cells, and found that the ICOS blockade resulted in increased apoptosis of both T-cell populations.[17] Furthermore, they found that the ICOS blockade increased the levels of the pro-apoptotic factor, BAX, and decreased the expression of the anti-apoptotic factor, BCL-2. Taken together, these results are consistent with functions in apoptotic signal inhibition.

The nature of ICOS signaling is beginning to be defined. Coyle and colleagues showed that the internal signaling domain was bound by the intracellular signaling protein, phosphatidylinositol-3 kinase (PI3-K),[13] thus providing evidence that ICOS signaling may be mediated through PI3-K. Since CD28 interacts with PI3-K,[40,41] this result suggests that ICOS and CD28 may share similar signaling components. In contrast, GRB-2 interacts with CD28,[42] but not with ICOS,[13] potentially demonstrating inherent differences between the pathways. Further analyses are needed to define the internal signaling pathways and the downstream genes of ICOS signaling.

B7RP-1-Fc and B7.2-Fc Transgenic Mice Exhibit Intestinal Inflammation

To determine if the B7RP-1 protein regulates important biological functions, we expressed the soluble B7RP-1-Fc fusion protein in mice under the control of a liver specific promoter.[4] The effect of the systemically delivered B7RP-1-Fc fusion protein was complex. At the age of 2-3 months, most of the B7RP-1 expressing transgenic mice developed lymphoid hyperplasia in the lymph nodes, spleens, and intestinal Peyer's patches. This hyperplasia particularly involved the B-cell zones and resulted in plasmacytosis and high levels of circulating immunoglobulins. At the age of 5-6 months, the mice developed an inflammatory bowel disease (IBD)-like

phenotype, consisting of discontinuous epithelial hyperplasia. The proximal and distal colon showed the most severe inflammatory changes, with the small intestine exhibiting milder inflammatory effects. In the colon, large transmural infiltrates and fissuring ulcers, consisting primarily of B- and T- cells, were observed. In addition, the blood, spleen, and mesenteric lymph nodes had increased ratios of $CD8^+$ T cells to $CD4^+$ T cells, in comparison to non-transgenic control mice. The discontinuous inflammatory phenotype, transmural inflammation, and effects in both the colon and the small intestine are more similar to a Crohn's disease phenotype than to ulcerative colitis.

This B7RP-1-Fc transgenic phenotype was fascinating, especially in comparison to that of the transgenic mice expressing soluble B7.2-Fc. The B7.2-Fc transgenic mice developed a far more aggressive IBD-like phenotype, which was more similar to ulcerative colitis than Crohn's disease. This occurred in some animals as early as 4 weeks, and in most animals by 8 weeks. The animals were hunched, and their body weight was on average 30% lower then the non-transgenic controls. The small intestine was mildly thickened, and the colon showed a gross, diffuse thickening along its entire length. Histological examination of the colon revealed marked colonic mucosal hyperplasia, multi-focal crypt abscessation, and pronounced inflammatory cell infiltration (consisting mainly of αβ TCR T-cells and macrophages) into the colonic lamina propria. The mesenteric lymph nodes were enlarged and the spleens of these animals were generally about three-fold larger than those of the control animals. The dissimilar phenotypes of the B7RP-1-Fc and B7.2-Fc transgenic mice may reflect the different roles of the two molecules in the T-cell-mediated immune response. Perhaps B7RP-1-Fc functions only on a previously established response, and such responses require time and other factors to fully mature. B7.2-Fc, on the other hand, likely costimulates the primary response, and therefore may act much earlier to enhance the primary inflammatory reaction.

ICOS/B7RP-1 Therapeutic Modalities

Theoretically, the ICOS/B7RP-1 pathway may be regulated at many steps and modalities. Molecules that engage the ICOS receptor, such as anti-ICOS mAbs and B7RP-1-Fc, have the potential for both activating and inhibiting roles. This is exemplified by the fact that the soluble B7RP-1-Fc protein acts to enhance immune responses in transgenic mice. Molecules that engage the B7RP-1 ligand, such as anti-B7RP-1 mAbs and ICOS-Fc, would likely only inhibit B7RP-1 activity, since it is less likely that engagement of B7RP-1 provides signaling in that direction.

Intracellular signaling molecules could be additional targets for therapeutic modulation. However, modalities that inhibit the receptor/ligand interaction may provide greater specificity as compared to inhibitors of intracellular signaling pathways, since the signaling factors are putatively modular and may be involved in several different signaling pathways.

Anti-Tumor Activities of B7RP-1

Ten years ago, Chen,[43] Allison,[44] and their colleagues found that B7.1, when expressed on tumor cells via gene transfection, promoted an effective immune response against the tumor. These findings were extended to a large number of tumor cells, and generally, "immunogenic" tumors responded preferentially[45] to this type of treatment. Allison and colleagues manipulated this pathway further by using CTLA-4 antibodies to antagonize the negative CTLA-4 activity, and thereby enhanced the anti-tumor immune response.[46] More recently, soluble B7.1/2-Fc fusion proteins have been used to activate the CD28 pathway directly and thus augment the anti-tumor activity.[47-50] Furthermore, non-B7-related costimulatory factors, such as 4-1BB and OX40, have also been implicated in enhancing anti-tumor activity against immunogenic tumors,[51-53] indicating that several different costimulatory pathways are involved in anti-tu-

mor responses. These experiments proved that the manipulation of costimulation is a valid anti-tumor approach.

B7RP-1 also has similar anti-tumor activities in gene transfection experiments and when used as a soluble B7RP-1-Fc fusion protein. Sha and colleagues reported that the ectopic expression of B7RP-1 on SaIN tumor cells enhanced CD4-independent, $CD8^+$ T-cell responses and anti-tumor responses.[54] Furthermore, their experiments implicated B7RP-1 in secondary or recall immune responses. In addition, Liu et al recently demonstrated that J558 tumor cells expressing B7RP-1 promoted tumor specific $CD8^+$ T-cell responses in vivo.[55] Together, these experiments suggest that $CD8^+$ T-cells are the mediators of the B7RP-1 anti-tumor response.

We recently found that the soluble B7RP-1-Fc fusion protein causes rejection or growth inhibition of Meth A, SA-1, and EMT6 tumors when administered in syngeneic mice (manuscript under review). Established Meth A tumors were rejected effectively with a single dose of B7RP-1-Fc; however, the treatment was less effective on larger tumors. Mice that previously rejected tumors by day 30, also rejected a subsequent challenge by the same, but not by different, tumor cells without additional B7RP-1-Fc treatment, indicating a specific, long-lived memory response. Tumor cells believed to be less immunogenic, such as P815 and EL-4 cells, were less responsive to this treatment. However, the EL-4 responsiveness to the B7RP-1-Fc treatment was enhanced by pre-treatment of the mice with cyclophosphamide. As expected, T-cells appeared to be targeted by the B7RP-1-Fc treatment. Thus, the administration of soluble B7RP-1-Fc has therapeutic value in generating or enhancing anti-tumor activity in a clinical setting, and has obvious advantages over the gene transfection approach.

ICOS Blockade in Transplantation

Since ICOS is expressed on activated T-cells, it is perhaps not surprising that Ozkaynak and colleagues found increased ICOS expression on T-cells infiltrating into rejecting tissue in a murine model of cardiac transplantation.[16] In comparison with the pre-transplant tissue, they showed that on day 5 after transplant, ICOS expression is significantly increased in infiltrating mononuclear cells and B7RP-1 expression is slightly increased in infiltrating interstitial dendritic cells and macrophages. Therefore, the up-regulation of ICOS, and to a certain extent B7RP-1, correlates with the rejection process in this model.

The investigators provided compelling evidence for the therapeutic benefit of combining the ICOS/B7RP-1 blockade with other therapeutic regimens in transplant settings. They found that ICOS inhibition using anti-ICOS antibodies, coupled with cyclosporin A immunosuppressive treatment, promoted permanent engraftment of heart tissue in the recipient animals. The observed therapeutic synergy was particularly noteworthy in comparison with the antagonism seen in combining the CTLA-4 or CD40L blockade with calcineurin inhibitors,[56] and thus delineates a difference between these pathways. Furthermore, in a chronic transplant rejection scenario, they showed that ICOS/B7RP-1 inhibition in conjunction with a CD40L blockade resulted in normal histology and prevented transplant arteriosclerosis.[16] These preliminary studies are extremely encouraging; however, in light of the strong constitutive expression of the B7RP-1 protein on kidney epithelial cells, which suggests a suppressive role for ICOS/B7RP-1(Wuthrich and colleagues), it is important to determine whether the ICOS/B7RP-1 pathway functions similarly in other tissues.

ICOS Regulation of Secondary and Recall Responses

The lack of ICOS expression on naïve T-cells initially led to experiments that tested for ICOS/B7RP-1 roles in effector and memory T-cell responses. Indeed, CD28 costimulation appears to be inadequate for T-cell functions downstream of the primary antigen presentation,[1] and therefore, it is important to ascertain whether other immune factors are involved in costimulation downstream of the primary immune response.

Several lines of evidence signify downstream immune functions for ICOS/B7RP-1. Studies of ICOS expression indicate that ICOS is not on resting, naïve T-cells,[2,4] but is expressed on a subset of resting, memory T-cells.[4] Shortly after T-cell activation of naïve T-cells, ICOS expression is up-regulated. Although ICOS is detectable in the lymph nodes, thymus, and other lymphoid organs, ICOS expression on T-cells during an immune response in the peripheral organs is significant.[17-19] B7RP-1 is primarily expressed on B-cells and macrophages, the primary APCs for effector cell function. Dendritic cell (DC) B7RP-1 expression is mainly restricted to immature DCs, and B7RP-1 may not play a role in mature DC function in primary T-cell activation. Consistently, B7RP-1 expression on B-cells and macrophages is also up-regulated during immune activation in a disease model of multiple sclerosis.[17]

In studies of murine contact hypersensitivity, we showed that the B7RP-1-Fc fusion protein augments the challenge response, but has less of an effect on the initial sensitization.[4,57] In contrast, B7.2-Fc functioned better in the sensitization than in the challenge. These were the first in vivo studies to support an ICOS/B7RP-1 function in secondary immune responses.

Several studies of allergic airway inflammation strongly support a role for ICOS/B7RP-1 in secondary responses. Coyle and colleagues showed that immunoglobulin production was decreased in ICOS-Fc treated mice during a secondary response, but not during a primary immune response.[26] Subsequently, they demonstrated that late treatment with an anti-ICOS mAb inhibited OVA-induced airway inflammatory cytokine production, but early treatment had less of an effect.[19] Conversely, the inhibition with CTLA-4-Ig was best with early treatment, and was less effective later. This suggests a temporal order for the CD28 and ICOS pathways, with CD28 acting first and ICOS functioning later in an immune response.

Sperling and colleagues demonstrated that ICOS/B7RP-1 inhibition with ICOS-Fc decreased airway inflammation induced by the *Schistosoma mansoni* soluble egg antigen (SEA).[32] Although the inhibitory effect of ICOS-Fc on airway inflammation was evident in the decreased cellular infiltration and cytokine production in the model, the ex vivo re-stimulation of splenocytes from control or ICOS-Fc treated mice revealed that T-cell priming and the Th2 cytokine profile were unaffected. Intriguingly, the ex vivo re-stimulation of lung Th2 effector cells with SEA was convincingly inhibited by the ICOS-Fc protein. This study is consistent with those of Coyle and colleagues in localizing the ICOS/B7RP-1 function to secondary responses.[19]

EAE Studies Reveal the Promise and the Complexity of ICOS/B7RP-1 Regulation

Three lines of investigation have indicated the importance of ICOS regulation in models of experimental autoimmune encephalitis.[17,18,28] A MOG peptide-induced EAE model in mice lacking ICOS revealed significantly increased disease symptom severity, possibly indicating a negative regulatory role for ICOS in EAE.[28] The investigators suggested that the lack of the negative regulatory cytokine, IL-13, is important in this increased response to the MOG peptides. Coyle and colleagues subsequently demonstrated that ICOS inhibitory mAbs functioned to enhance disease in a PLP-mediated EAE model when administered during the “priming” phase, and to inhibit when added during the “effector” phase of the disease.[18] They showed that the differential activity correlated well with the T-cell infiltration into the diseased tissue, and with the brain expression of several chemokines, chemokine receptors, and cytokines. A correlation with disease was also observed in IFN-γ production and the proliferation of ex vivo splenocyte-specific recall responses. In contrast, the amount of PLP-specific IgG1 antibodies decreased in mice treated with anti-ICOS mAbs during the priming phase of the model, thereby indicating that the exacerbated disease symptoms were not due to an increased specific humoral response.

The investigators suggested that ICOS primarily functions on Th2 cells, and that the inhibition of such a Th2 pathway polarizes to a Th1 immune response. Sperling and Bluestone hypothesized [58] that Th2 responses require a stronger signal, and thus are more sensitive to inhibition, than Th1 responses. In this scenario, the inhibition of Th2 dominant diseases would be more dramatically affected by a factor that regulates both Th1 and Th2 responses. In a Th1-mediated disease, the stronger inhibition of Th2 responses may benefit the Th1 response. Alternatively, the data support the presence of critical regulatory T-cells that can be inhibited during the priming stage of the model with anti-ICOS mAbs. Inhibition of these regulatory T-cells would release a suppressive effect, which would result in an enhanced immune response. In this context, the exaggeration of EAE symptoms in mice lacking ICOS[28] could be interpreted as the loss of activating ICOS function on regulatory T-cells.

A more clinically relevant scenario is one in which the disease is pre-established, and this is where ICOS regulation appears to be most promising. In the PLP EAE model previously mentioned,[18] the disease was inhibited when the anti-ICOS mAb treatment was delayed. Furthermore, Perrin and colleagues reversed the clinical symptoms of an MBP-induced EAE model with the ICOS-Fc fusion protein.[17] Clearly, the initial studies on the therapeutic applications of targeting the ICOS/B7RP-1 pathway are encouraging.

Differential ICOS/B7RP-1 Regulation

Although the ICOS/B7RP-1 pathway generally activates T-cell immune responses, there are several reports of immune inhibition by ICOS/B7RP-1. These counter-intuitive results could be due to the effects on inhibitory cytokines or regulatory T-cells, the unexpected activation by putative "inhibitory" molecules, or combinations of those or other factors. The exacerbation by early treatment with anti-ICOS inhibitory mAbs in the EAE model of multiple sclerosis described in the previous section[18] is reminiscent of the similar results with B7.2 inhibition in EAE [59,60] and of the studies of autoimmune diabetes with B7.1 inhibitors.[61] These differential results using the ICOS/B7RP-1 blockade are similar to studies of the role of IFN-γ in multiple sclerosis (MS) or in EAE models, in which both pro-inflammatory and anti-inflammatory activities were discerned. Although IFN- γ treatment increased the severity of MS in patients,[62] studies with anti-IFN-γ antibodies[63] and in mice lacking IFN- γ[64] showed increased EAE.

The early ICOS antibody treatment aggravation of EAE symptoms in the model of Rottman and colleagues[18] could also be related to the exacerbation of EAE symptoms in mice lacking ICOS. These results may indicate the presence of an ICOS-responsive regulatory T-cell subset that is dominant during the priming phase of the reaction. Alternatively, as the investigators suggested, the significant lack of IL-13 in the ICOS knockout mice could result in enhanced immune responses.[28] These EAE experiments illustrate the complexity of the ICOS-mediated immune response.[65]

Dissimilar results were also seen using an anti-ICOS mAb in acute and chronic models of graft versus host disease (GVHD). Abe and colleagues showed that the same anti-ICOS mAb activated immune responses in an acute GVHD model, but inhibited responses in a chronic model.[33] In contrast, CTLA-4-Ig was inhibitory in both models. This result infers that ICOS, but not CTLA-4, promotes this differential activity. The nature of the ICOS-responsive T-cells in these models should be defined in order to understand the disparate activities of the antibody.

Summary

The present characterization of the ICOS/B7RP-1 costimulatory pathway has identified roles in effector and secondary responses on helper Th1, helper Th2, and cytolytic T-cells. The ICOS activity on those cells is controlled by the B7RP-1 expression on B-cells and macrophages,

and possibly dendritic cells, epithelial cells, and fibroblasts. Although expression studies suggest a role in early T-cell development, the ICOS/B7RP-1 pathway appears to have a significant role in effector and secondary immune responses in peripheral tissues. We need to determine how B7RP-1 expression on various cell types contributes to a costimulatory environment that regulates ICOS-expressing T-cells (Fig. 1).

Of particular interest is the manipulation of the ICOS/B7RP-1 pathway to alleviate disease. We and others found that B7RP-1 enhances cytolytic T-cell responses that result in potent anti-tumor activity. The soluble B7RP-1-Fc fusion protein appears to have immune activating effects in transgenic mice that express the protein systemically; however, it will be important to determine whether there are scenarios where B7RP-1-Fc costimulates unwanted immune responses, thus potentiating autoimmunity. For instance, B7RP-1-Fc could costimulate dominant regulatory T-cell subsets during a particular phase of a disease process and thereby inhibit immune responses.

The blockade of the pathway revealed ICOS/B7RP-1 involvement in experimental models of asthma, multiple sclerosis, and transplantation. These exciting experiments showed that the ICOS blockade inhibited secondary responses, reversed clinical disease symptoms, and facilitated permanent cardiac engraftment. The differential effects of the ICOS/B7RP-1 blockade perhaps illustrate the presence of subsets of ICOS-responsive regulatory T-cells that suppress T-cell functions, either during a certain phase of the disease or within a specific model. Additional studies will define the roles for the ICOS/B7RP-1 pathway on regulatory T-cells. An ambition of immunoregulation is to re-establish tolerance to self-antigens in autoimmune settings and to create tolerance to donor antigens in transplant settings. Although targeting the ICOS/B7RP-1 pathway is compelling, its involvement in immunological tolerance has not been assessed and future experimental efforts should clarify the role of ICOS/B7RP-1 blockade in specific tolerance. The exciting early characterization of this pathway has revealed functions in important therapeutic aspects of the immune response; however, additional studies are required to fully understand the functions of the ICOS/B7RP-1 pathway.

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Chapter 7

Role of ICOS in T Cell Activation

Jeffrey A. Ledbetter, Erik Espling and Martha Hayden-Ledbetter

Introduction

Inducible costimulator (ICOS) was recently identified as a novel member of the CD28 family by Hutloff et al.[1] A monoclonal antibody (mAb), F44, specific for human ICOS was generated by immunization with activated T cells, and was used to isolate the ICOS antigen for immunochemistry studies. From the peptide sequence of ICOS, full length complementary DNA was isolated and found to encode a protein with 24% identity to CD28 and 17% identity to CTLA-4. Like CD28 and CTLA-4, ICOS is expressed as a disulfide-linked dimer with conserved cysteines critical for the formation of the inter-and intra-chain disulfide bonds that stabilize the structure of this molecular family.[2] ICOS is not expressed by resting T cells, but is rapidly induced primarily on CD45 RO memory T cells following T cell activation.[1] Stimulation of T cells with mAb F44 increased T cell proliferation in response to suboptimal activation with anti-CD3 mAb, and increased the production of IL-10, IL-4, IL-5, TNFa, IFNg, and GM-CSF, but did not increase production of IL-2. ICOS costimulation also increased expression of CD25, CD69, and CD154 on activated T cells.[1] Since the initial cloning of ICOS, there has been significant interest in defining the function of ICOS in the adaptive immune response and in identifying the potential for targeting of the ICOS pathway for pharmacological intervention in autoimmune disease and cancer. Figures 1 illustrate the CD28 family and the B7 family of known ligands with some of the key features of their function and regulation indicated.

Interestingly, a T cell activation molecule, termed H4, had been identified several years earlier using a mAb generated in Armenian hamsters.[3] The H4 antibody reacts with activated T cells in both mice and humans, and was selected based upon its ability to enhance proliferation of D10 cells induced by suboptimal concentrations of a clonotypic anti-TCR mAb. H4 was shown to react with ICOS by crossblocking studies with F44 and by reaction with cells transfected with human or mouse ICOS.[4] This group has also shown that ICOS (H4) is expressed by T cells located at the periphery of lymph node germinal centers and paracortical areas, and by large proportions of peripheral blood T cells in acute viral infections, including HIV infection with high levels of viremia.[5,6]

Defective T Cell Help for Isotype Class Switching in ICOS Deficient Mice

Full length complementary DNA clones encoding murine ICOS were isolated[7,8] and ICOS was shown to be encoded by a gene closely linked to the CD28 and CTLA-4 genes on mouse chromosome 1.[8] The three genes also co-localize to human chromosome 2q33 with the

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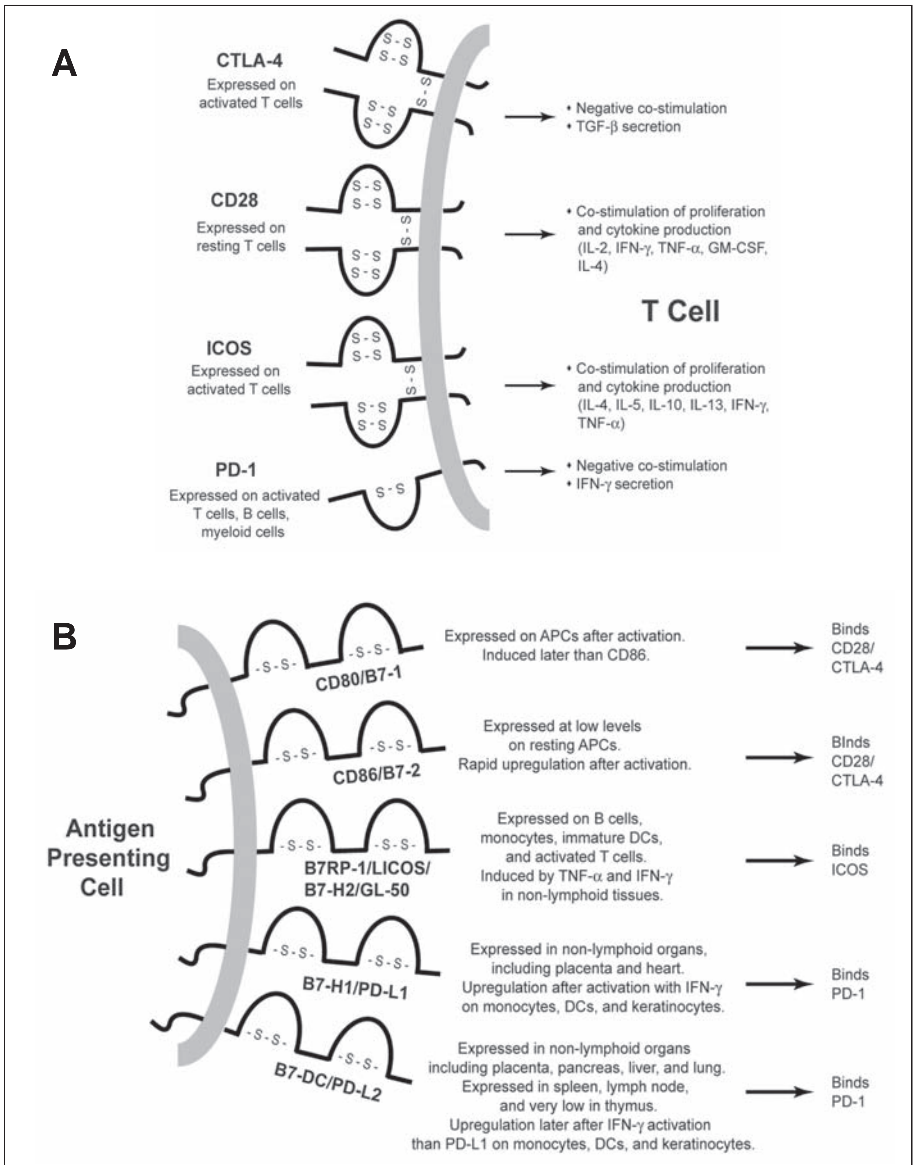

Figure 1A. CD28 Receptor Family. Schematic diagram summarizing the structure and properties of identified members of the CD28 family of T cell receptors. These molecules are all members of the immunoglobulin superfamily of receptors with a single variable-like domain in the extracellular portion of the molecule. CD28, CTLA-4, and ICOS are indicated as homodimeric molecules, while PD-1 lacks the cysteine residues important for mediating dimerization, so is indicated as a monomer in the figure.
Figure 1B. B7 Family Members With Known Ligands. This panel illustrates the structure and summarizes the expression and binding properties for those members of the B7 family of receptors with ligands that have been identified. These molecules contain a single variable and constant like domain in the extracellular region, yet share only 20-30% amino acid identity when compared to one another. The predicted intrachain disulfide bonds are shown. Other members of the B7 family have been identified, but are not included in the figure since their ligands have not yet been defined.

IDDM12 locus, where polymorphisms in the CTLA-4 gene confer risks for the development of type 1 diabetes and lupus.[9-11]

ICOS deficient mice have been created and characterized by three groups.[12-14] $ICOS^{-/-}$ mice exhibit defects in immunoglobulin isotype class switching and germinal center formation after immunization with T-dependent antigens. Serum levels of IgG1 were reduced to about 30% of normal levels in these mice, and production of IgE was defective. The B cells in $ICOS^{-/-}$ mice were able to class switch when stimulated through CD40, indicating that B cells were not intrinsically defective. T cells from $ICOS^{-/-}$ mice failed to produce IL-4 or IL-13 when restimulated in vitro but produced normal levels of IFNγ and IL-10. $ICOS^{-/-}$ mice showed greatly enhanced susceptibility to experimental autoimmune encephalomyelitis, indicating that ICOS signals may help protect against Th1-mediated inflammatory autoimmune diseases. These results indicate that ICOS plays an important role in promoting Th2 differentiation and may be a target for intervention in diseases such as asthma and allergy.

Identification of a Novel ICOS Ligand Related to CD80

ICOS lacks the MYPPPY sequence shared by CD28 and CTLA-4 and important for their binding to CD80 and CD86.[1] Later studies confirmed that ICOS does not bind CD80 or CD86, but binds instead to a novel member of the CD80 family termed B7RP-1 (also called B7h, GL50, or B7-H2).[7,8,15-18] B7RP-1 is expressed by B cells and by monocytes after plastic adherence.[15,17] B7RP-1 expression is increased in nonlymphoid tissues by TNFα and by LPS,[17] and is induced on $CD34^+$ progenitor cells by treatment with GM-CSF plus TNFα prior to the expression of CD80 or CD86.[19]

Since CD80 and CD86 are expressed by chronically activated T cells, we examined whether B7RP-1 was expressed by T cells following activation with anti-CD3 and anti-CD28 mAbs. Figure 2 shows that ICOS-Ig bound to approximately 15% of CD8 T cells and 25% of CD4 T cells on day 4 after stimulation. There was lower expression of CD80 and CD86, detected by specific binding of CTLA4-Ig (8% of CD4 and 10% of CD8 T cells), whereas a control fusion protein (BT3-Ig) bound weakly to non T cells, probably through nonspecific FcR interactions. The binding of ICOS-Ig was highest on day 4 after activation, and returned to baseline levels by day 7 as cell proliferation diminished (data not shown). This result indicates that activated T cells express B7RP-1 and agrees with Ling et al[16] who reported low levels of ICOS-Ig binding to mouse splenic $CD3^+$ T cells. The data also suggests that activated T cells express B7RP-1 prior to expression of CD80 or CD86. It will be important to determine whether B7RP-1 is expressed by T cells in skin lesions of psoriasis and mycosis fungoides where abnormal expression of CD80 has been described.[20]

Role of ICOS in CD4 T Cell Activation

There is significant evidence that ICOS signals are primarily involved in promoting generation of Th2 T cells. First, ICOS costimulation induces preferential production of Th2 cytokines including IL-10 and IL-4 but production of IL-2 was not detected.[1] However, later studies have indicated that ICOS costimulation probably induces a small amount of IL2 production that is rapidly consumed by the proliferating T cells and thus not detected in the supernatant by ELISA.[21] This study also showed that ICOS costimulation increased IL-2 dependent T cell proliferation, but required exogenous IL-2 for sustained growth.[21] ICOS costimulation increased expression of IL-2 receptor (CD25) on activated T cells,[1] providing a likely mechanism for the effects of ICOS on IL-2 dependent T cell proliferation. It is also notable that exogenous IL-2 increased the production of IL-4, IL-10, and IL-13 by T cells costimulated by anti-ICOS.[21]

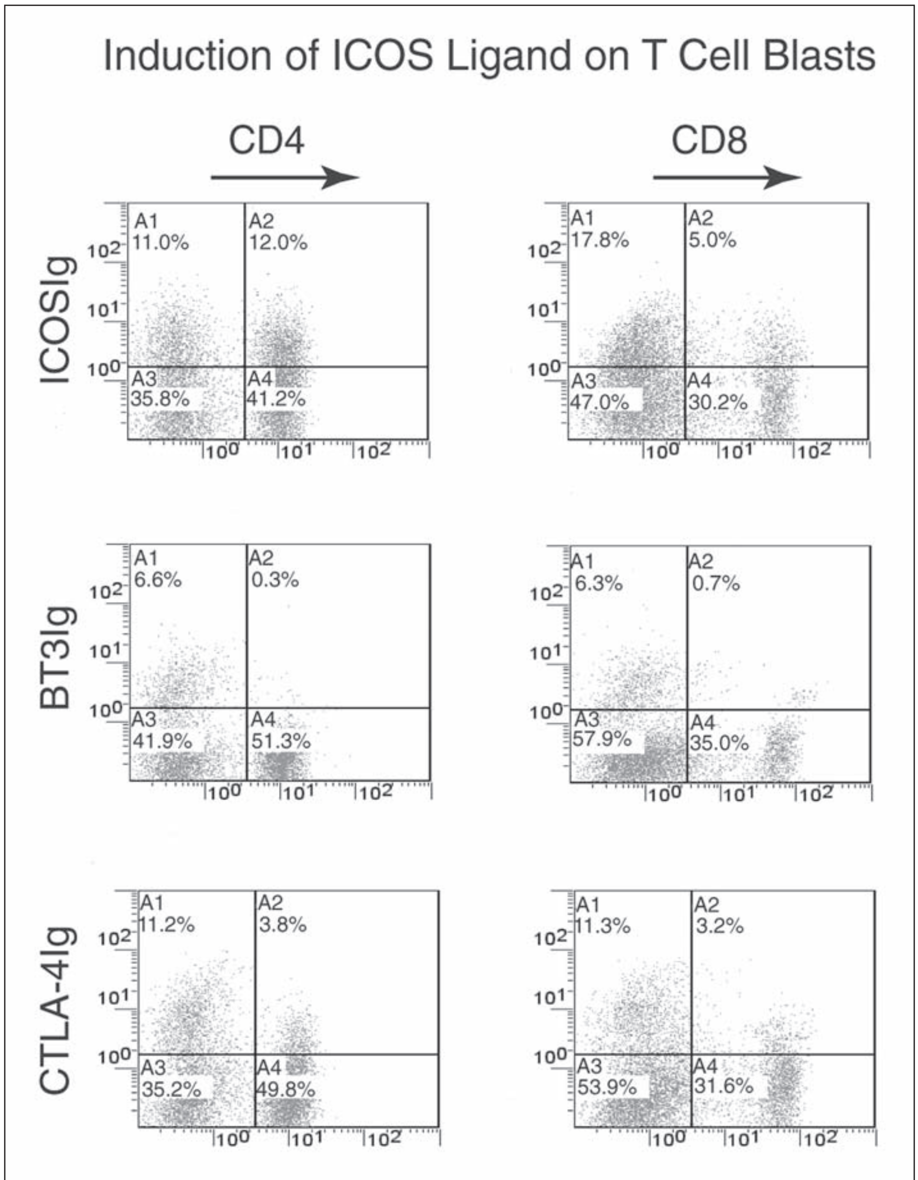

Figure 2. Induction of ICOS Ligand on Activated T Cells. T cells were activated by CD3XCD28 stimulation using magnetic beads (Dynal) conjugated with 64.1 (anti-CD3) and 9.3 (anti-CD28) for four days and cells were analyzed by flow cytometry using two color analysis. Biotin-conjugated ICOSIg, CTLA4Ig, and a BT3Ig control were incubated with cells and binding detected using PE-SA (phycoerythrin-streptavidin, Molecular Probes) at 1:100. FITC-conjugated antibodies for CD4 (G17-2) and CD8 (G10-1) were used to identify T cell subpopulations. Cells were analyzed using a Coulter Epics XL flow cytometer and EXPO analysis software. The figure illustrates the percentage of gated cells in each quadrant.

The phenotype of ICOS deficient mice also supports an essential role of ICOS in generation of Th2 cells. ICOS deficient mice fail to isotype class switch in response to T-dependent antigens, have low levels of serum IgG1, and produce very low levels of IL-4.[12-14] TCR-transgenic T cells differentiated into Th2 cells by addition of IL-4 during antigen stimulation expressed significantly more ICOS than cells differentiated into Th1 by addition of IL-12 and neutralizing antibody to IL-4.[22] Furthermore, blockade of ICOS signals using ICOS-Ig reduced the T cell production of IL-10 and IL-4 in response to antigen, but increased the production of IFN-γ.[22]

Additional support for ICOS function in promoting Th2 differentiation comes from studies of Yoshinaga et al, who showed that transgenic mice expressing a B7RP1-Ig fusion protein exhibit lymphoid hyperplasia and plasmacytosis accompanied by high levels of circulating IgG.[7] They also showed that B7RP-1-Ig when given at the time of sensitization or challenge exacerbates the contact hypersensitivity response to oxazolone. The effect was most dramatic when B7RP-1-Ig was given at the time of challenge, indicating that ICOS has a more prominent role in the effector or secondary phase of T cell activation.[7] In addition, B7RP-1-Ig increased the presence of eosinophils in the lungs of mice sensitized and challenged with OVA to mount an asthamtic reaction.[23] B7RP-1-Ig given in vivo also increased the antibody response to a T-dependent antigen (KLH), primarily increasing IgG2a and IgE responses, while B7-2-Ig did not increase the antibody response.[23] IgG2a production is mediated by a Th1 response,[24] while IgE production is mediated by a Th2 response.[25] Thus stimulation of ICOS with B7RP-1-Ig at the time of antigen priming enhanced both Th1 and Th2 responses.

Other studies show that ICOS is essential for all CD4 responses in CD28-deficient mice, suggesting that ICOS and CD28 serve overlapping roles in stimulation of both Th1 and Th2 responses.[26] For example, ICOS-Ig treatment of CD28-deficient mice infected with *Nippostrongylus brasiliensis* suppressed IFNγ, IL-4, IL-5, and IL-10 production. In addition, CD28-deficient mice infected with choriomeningitis virus produce significant amounts of IFNγ, which is inhibited by treatment with ICOS-Ig.[26] Thus ICOS can costimulate significant production of IFNγ without a requirement for CD28 signals.

Additional evidence for ICOS signals contributing to a Th1 response was provided from cardiac allograft studies where therapy with inhibitory anti-ICOS mAb or with ICOS-Ig prolonged allograft survival by reducing Th1 mediated acute rejection.[27] Inhibition of ICOS in combination with low doses of cyclosporin A induced long term allograft survival without the development of transplant arteriosclerosis. In addition, ICOS blockade in combination with a CD154 mAb to block the CD40 pathway prevented chronic rejection without the need for donor-specific therapy.[27] Inhibition with anti-ICOS in these studies caused a dramatic reduction of IFNγ and IL-10 mRNA expression, and also decreased the expression of multiple chemokines involved in the allograft response.

Some studies have indicated that ICOS costimulation is important for regulation of Th1 mediated autoimmune disease. For example, blockade of ICOS at the time of in vitro priming of autoimmune encephalomyelitis (EAE) transgenic T cells inhibited their ability to induce EAE without preventing their homing to the central nervous system.[28] Production of IFNγ and IL-10 was inhibited by ICOS blockade, while production of IL-2 was not affected. In addition, treatment of mice with ICOS-Ig after paralysis rapidly ameliorated EAE.[28]

These results contrast with those of Rottman et al,[29] who found that inhibition of ICOS at the time of in vivo immunization increased the production of IFNγ and the severity of disease in an EAE model (SJL mice immunized with proteolipid protein). In agreement, McAdam et al[22] also showed that blockade of ICOS with ICOS-Ig increased the production of IFNγ by transgenic T cells stimulated with antigen in vitro, while production of IL-4 and IL-10 was decreased. Inhibition of ICOS at the efferent phase of disease in the SJL EAE model significantly reduced disease and reduced rather than increased production of IFNγ.[29] These studies have led to the hypothesis that ICOS signals are important in the effector phase of the T cell

response, but are less important in the priming phase, where CD28 signals play a more important role. This idea is also supported by the results of Tesciuba et al[30] who showed that ICOS blockade prevented cellular infiltration into lungs and production of IL5 in an allergic airway disease model without prevention of T cell priming and differentiation to a Th2 phenotype.

ICOS Costimulation of CD8 T Cells

An important role for ICOS in costimulation of CD8 T cells was found from experiments studying the immunogenicity of tumor cells that had been transfected to express B7RP-1.[31,32] Both groups found that tumor cells expressing B7RP-1 were rejected by CD8 T cells, and that the mice developed immunity to subsequent challenge with parental tumor cells. CD4 T cells were not required for tumor rejection or development of immunity.[31] Interestingly, tumor cells expressing B7RP-1 were more sensitive to destruction by CTL in vivo, indicating that B7RP-1 increased both clonal expansion of CD8 CTL and tumor killing by CTL.[32] Expression of B7RP-1 by tumor cells increased production of IFNγ and IL-2 by antigen-specific CTL, and dramatically increased the reactivation of primed CTL.[31] These results indicate that ICOS signals are involved in costimulation of resting and activated CD8 T cells, and can promote generation of CTL in the absence of CD4 T cells. The expression of ICOS by $CD3^+$ $CD28^-$ T cells suggests that ICOS could also be important for costimulation of these cells; however, this possibility has not yet been directly examined.

The idea that CD28 costimulation regulates T cell priming, while ICOS costimulation regulates T cell effector function[33] suggests that CD28 and ICOS operate independently of each other. It seems clear that ICOS costimulation does not depend entirely on CD28 signals, since ICOS was essential for all CD4 T cell responses in CD28 deficient mice.[26] However, it also seems clear that CD28 and ICOS are interconnected, since CD28 signals increase expression of ICOS on T cells during activation,[22] and ICOS function is highly dependent upon IL-2 produced following CD28 stimulation.[21] In addition, both CD28 and ICOS costimulation are regulated by CTLA-4 crosslinking.[21]

ICOS is expressed rapidly after T cell activation since it can be detected on the cell surface within 12 hours of stimulation.[1] Our data agree (Figure 3) and show that increased levels of ICOS mRNA can be detected by real-time PCR assays within 4 hours of stimulation of resting human T cells. While CD3 crosslinking alone induced ICOS mRNA expression, crosslinking of both CD3 and CD28 further increased ICOS mRNA expression, while CD154 mRNA levels were not further increased by CD28 costimulation at this early time point. The rapid expression of ICOS suggests that it could participate in T cell priming even though it is not expressed on resting T cells.

One key to the differential function of CD28 and ICOS is likely to be the distinction between the cells and conditions required for expression of their ligands. While CD80 and CD86 are expressed by activated APC and reach high levels on mature DC, B7RP-1 is expressed by resting B cells and monocytes after plastic adherence, but is lost during maturation of DC with LPS.[15,17,18] In addition, B7RP-1 is expressed by $CD34^+$ stem cells and also in non-lymphoid tissues after treatment with TNFα.[17,19] It is thus likely that primary CD4 T cell activation by DC is promoted by costimulation through CD28 without significant signals through ICOS, whereas primary CD4 T cell activation by B cells or monocytes is promoted by both CD28 and ICOS costimulation. After activation, however, effector function and differentiation towards a Th2 phenotype may be promoted by ICOS costimulation during antigen presentation by non-professional APC or even by non-lymphoid cells. Inhibition of ICOS alone is likely to offer significant benefit for Th2 autoimmune inflamatory diseases such as asthma and allergy. In addition, inhibition of ICOS in combination with other inhibitors such as cyclosporine A, anti-CD154, or CTLA4-Ig, is likely to improve therapy for Th1 mediated autoimmune diseases and for prevention of allograft rejection.

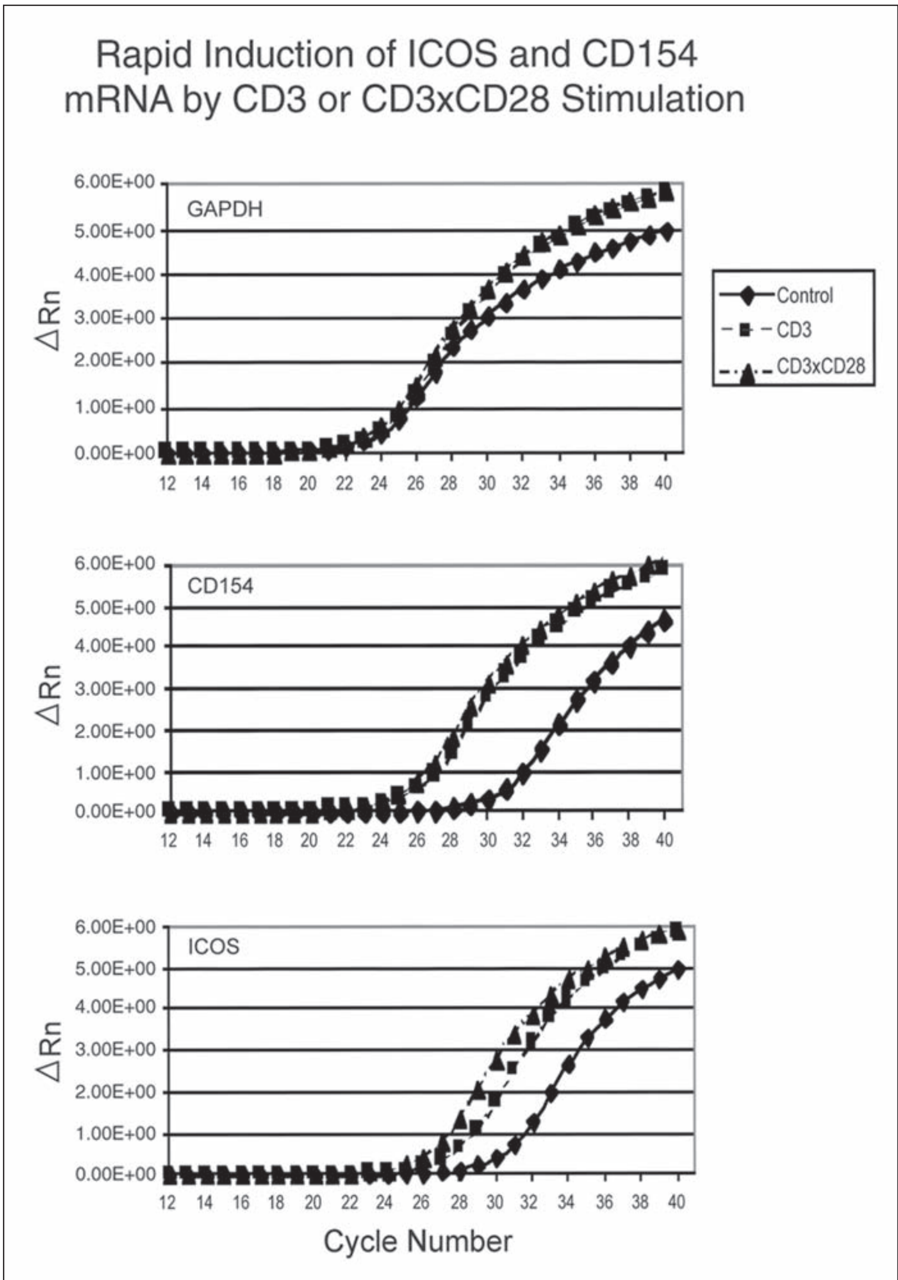

Figure 3. Rapid Induction of ICOS and CD154 mRNA by CD3 or CD3XCD28 Stimulation. Peripheral Blood Mononuclear Cells (PBMC) were stimulated for 4 hours in the presence of immobilized anti-CD3 (64.1) and anti-CD28 (9.3) antibodies (1 μg/ml). Cells were harvested from culture and total RNA prepared using QIAPrep RNA kits. Real time PCR using SYBR green and ABI 7700 TaqMan Analyzer were used in PCR amplification reactions with primer sets specific for inducible molecules, including CD154 and ICOS. GAPDH was amplified as a control.

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Chapter 8

B7-H3

Andrei I. Chapoval and Lieping Chen

Introduction

Although members of the immunoglobulin superfamily (IgS) are involved in many physiological functions including molecular transport, cell adhesion, cytokine receptions, regulation of gene expression, cell migration and others,[1] their function in immune system is a subject of intensive study. It is evident that help from costimulatory molecule(s) is required for optimal T cell activation and functional maturation after engagement of T cell receptor (TCR).[2-4] Our understanding of costimulation is still descriptive and the precise mechanisms of co-signaling are not yet fully understood. Many cell surface molecules including those from the IgS have been reported to have costimulatory function for T cell growth. Whether or not these molecules are simply redundant or are unique in their functions is not yet clear.[5-7] Therefore, one of the important questions in the field is to determine specific functions of each molecule that are not overlapping with others. Here we summarize information about a recently discovered member of B7 family named B7-H3, which specifically induces IFN-γ and leads to differentiation of antigen-specific T cells.[8]

Molecular and in Silico Cloning of B7-H3

A homology search using extracellular regions of published B7 family members[9-16] identified several overlapping clones in the National Center for Biotechnology Information (NCBI) and Human Genome Science Inc. databases. Sequences from these clones were used to isolate B7-like molecules from a human dendritic cells (DC)-derived cDNA library. The nucleic acid sequence was confirmed by analyses of two independent RT-PCR products from the human THP-1 cell line and DC cDNA. This sequence, designated B7-H3, encodes a putative 316 amino acid protein and shares identity with human B7-1 (17%), B7-2 (18%), B7-H2 (19%), B7-H1 (24%), and B7-DC (19%) (See Table 1).

Using Basic Local Alignment Search Tool (BLAST), we identified two nonoverlapping clones in mouse expressed-sequence tag (EST) database homologous to human B7-H3. Both clones are highly homologues to 5' and 3' ends of human B7-H3 respectively, with a gap between them. Primers were designed using obtained sequences and a full-length DNA encoding mouse, B7-H3 was isolated from mouse LN cells cDNA library. Mouse B7-H3 protein has 316 aa, the same as hB7-H3. Murine and human B7-H3 share 81% amino acid identity (Fig. 1).

Using four overlapping sequences homologous to hB7-H3 derived from bovine EST database, we composed a full length of bovine B7-H3. A putative bovine B7-H3 protein (315 aa length) also has a very high amino acid identity to the human and mouse B7-H3 (88% and 82% respectively) (Fig. 1). High conservation of B7-H3 sequence among the species during evolution suggests an important function of this molecule.

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Table 1. Percent identities and similarities among human B7 family members

	B7-1	B7-2	B7h/B7-H2	B7-H1	B7-DC
B7-2	19 (14)[1]				
B7h/B7-H2	18 (15)	17 (17)			
B7-H1	14 (17)	15 (13)	16 (15)		
B7-DC	15 (14)	15 (14)	19 (14)	36 (16)	
B7-H3	17 (14)	18 (17)	19 (16)	24 (14)	19 (18)

1—Percent identities open numbers. Percent similarities numbers in the parentheses

Isoforms of Human B7-H3

By agarose gel electrophoresis analysis of the PCR product amplified from human DC and THP-1 cell cDNA using a set of primers specific for hB7-H3, we found two bands corresponding to ~1200 and ~1900 bp (Fig. 2) suggesting that two forms of hB7-H3 may exist. Using BLAST search of human EST database, we confirmed that mRNA encoding "short" and "long" forms of hB7-H3 is present in various human tissues. Similar to other members of the B7 family, putative hB7-H3 protein obtained by translation of low molecular weight PCR product from DC cDNA contain a signal peptide, IgV-like and IgC-like domains, a hydrophobic transmembrane (TM) region, and intracellular (IC) tail. In contrast, a "long" form of hB7-H3 matching the 1900 bp PCR product contained extra IgV-like and IgC-like domains termed

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hB7-H3      1 MLRRRGSPG-MGVHVGAALGALWFCLTG-ALEVQVPEDPV  38
mB7-H3      1 MLRGWGGPS-VGVCVRTALGVLCLCLTG-AVEVQVSEDPV  38
Bov B7-H3   1 MLCG---PSSTGVSVAPALGVLWFCLTGAEVEVQVPEDPV  37

hB7-H3     39 VALVGTDATLCCSFSPEPGFSLAQLNLIWQLTDTKQLVHS  78
mB7-H3     39 VALVDTDATLRCSFSPEPGFSLAQLNLIWQLTDTKQLVHS  78
Bov B7-H3  38 VALVGTDATLRCSFSTEPGFSLAQLNLIWQLTDTKQLVHS  77

hB7-H3     79 FAEGQDQGSAYANRTALFPDLLAQGNASLRLQRVRVADEG 118
mB7-H3     79 FTEGRDQGSAYSNRTALFPDLLVQGNASLRLQRVRVTDEG 118
Bov B7-H3  78 FAEGRDQGSAYANRTALFPDLLAQGNASLRLQRVRVADEG 117

hB7-H3    119 SFTCFVSIRDFGSAAVSLQVAAPYSKPSMTLEPNKDLRPG 158
mB7-H3    119 SYTCFVSIQDFDSAAVSLQVAAPYSKPSMTLEPNKDLRPG 158
Bov B7-H3 118 SFTCFVSIRDFGSAAVSLQVAAPYSKPSMTLEPNKDLRPG 157

hB7-H3    159 DTVTITCSSYRGYPEAEVFWQDGQGVPLTGNVTTSQMANE 198
mB7-H3    159 NMVTITCSSYQQYPEAEVFWKDGQGVPLTGNVTTSQMANE 198
Bov B7-H3 158 DTVTITCSSYRGYPEAEVLWQDGQGAPLTGNVTTSQMANE 197

hB7-H3    199 QGLFDVHSVLRVVLGANGTYSCLVRNPVLQQDAHGSVTIT 238
mB7-H3    199 RGLFDVHSVLRVVLGANGTYSCLVRNPVLQQDAHGSVTIT 238
Bov B7-H3 198 QGLFDVHSVLRVVLGANGTYSCLVRNPVLQQDAHGSVTIT 237

hB7-H3    239 GQPMTFPPEALWVTVGLSVCLIALLVALAFVCWRKIKQSC 278
mB7-H3    239 GQPLTFPPEALWVTVGLSVCLVVLLVALAFVCWRKIKQSC 278
Bov B7-H3 238 GQPMTFPPEALWVTVGLSICLIALLVALAFVCWRKIKQSC 277

hB7-H3    279 EEENAGAEDQDGEGEGSKTALQPLKHSDSKEDDGQEIA 316
mB7-H3    279 EEENAGAEDQDGEGEGSKTALRPLKPSDNKEDDGQEIA 316
Bov B7-H3 278 HEENAGAEDHDGEGEGSKTALRPLKHSDNKEDDGPEIV 315
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Figure 1. Alignment of putative amino acid sequences human, mouse and bovine B7-H3. Identical amino acid residues are shaded and conserved residues are outlined.

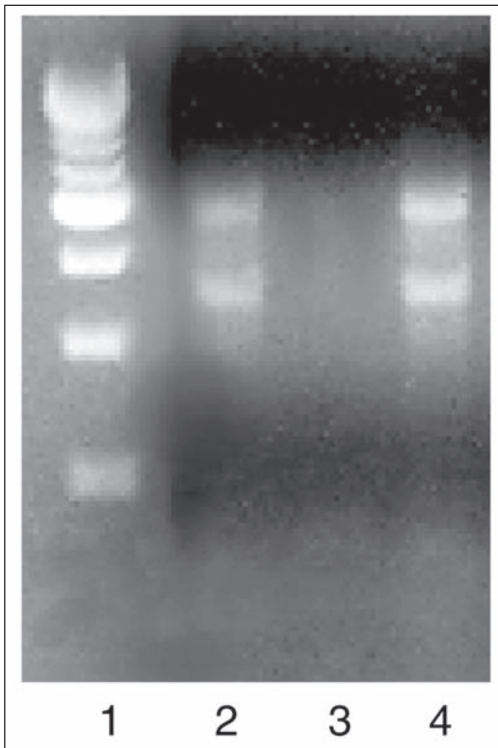

Figure 2. Identification of two hB7-H3 transcripts in THP-1 and DC by RT-PCR analysis. RNA was isolated from THP-1 and DC and converted to cDNA. PCR products from the amplification of cDNA from THP-1 (lane 2) or DC (lane 4) were analyzed by agarose gel electrophoresis. As control water alone (lane 3) was used.

here as IgV' and IgC'. IgV and IgV' shared 95% identity while alignment of IgC and IgC' revealed 98% identity.

Employing SMART, a simple modular architecture research tool (http://smart.embl-heidelberg.de/), we found that there are no proteins with a similar domain organization (IgV-IgC-IgV-IgC-TM-IC) as was found in the "long" form of hB7-H3. However, 537 proteins, including most B7 family members, with the domain organization similar to "short" form hB7-H3 (IgV-IgC-TM-IC) which were found in SMART database. In addition, no mouse EST corresponding to the "long" form of B7-H3 was found in the NCBI database. Thus, the biological significance of the "long" form B7-H3 in human and other species remains to be determined.[17]

B7-H3 Gene Analysis

In a BLAST search of human genome sequence, contig NT_010223 from chromosome 15 was found to contain the entire B7-H3 gene. The identity between B7-H3 mRNA and Chr15 sequences was absolute (E value of 0.0). Based on the Evidence Viewer Data (NCBI) the human B7-H3 gene spans ~1.6 kb and is composed of 7 exons with a similar organization to other B7 family members. Exon 1 of B7-H3 encodes 5'- untranslated region (UTR) and first 26 aa refereed as a signal peptide which is cleaved during protein processing. The second and third exons encode IgV (27-139; 113 aa) and IgC (140-238; 99 aa) domains, respectively. The fourth exon encodes transmembrane domain (239-283; 45 aa). Exon 5 (284-297; 14 aa), exon 6 (298-309; 12 aa) and exon 7 (310-316; 7 aa with stop codon and 3'- UTR) encode three pieces of an intracellular portion of B7-H3.

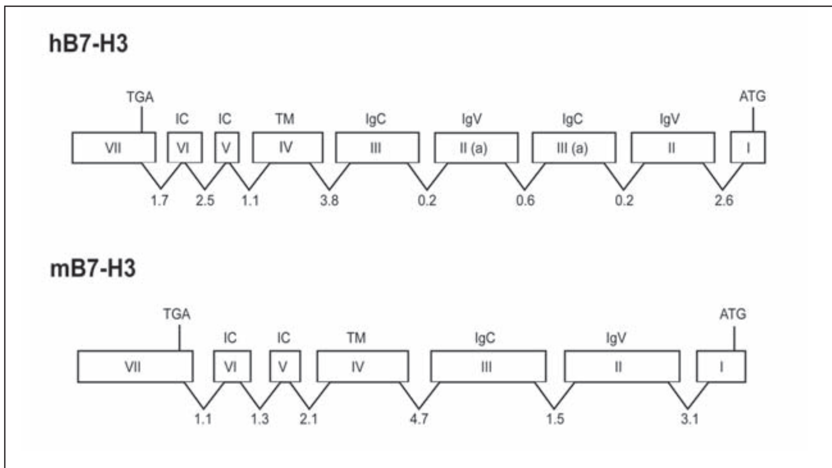

Figure 3. Structure of human and mouse B7-H3 genes. The general structure of the gene is shown as numbered boxes (exons) and connecting lines (introns) with indicated size of introns in kb.

Further analysis of the human genome sequence revealed two additional exons in the B7-H3 gene corresponding to IgV' and IgC' domains found in mRNA encoding "long" form of human B7-H3 (Fig. 3). Alignment of sequences from exons encoding IgV and IgC' with IgV' and IgC including introns demonstrate a very high sequence homology (about 99%) in both exons and introns (Fig. 4). Interestingly the sequence homology does not span outside of the analyzed exon-intron-exon region suggesting limited duplication of the genomic sequence. The presence of the extra IgV and IgC domains in genomic sequence of B7 family members is not unusual. In the draft of human genome we found exons encoding extra IgV and IgC domains in hB7-1 gene (AC074271), however no EST matching "long" form of B7-1 was found in the NCBI database.

Mouse B7-H3 gene located on chromosome 6 was found in the Celera Mouse Genome database. Analysis of the mouse genome sequence showed no presence of exons analogous to extra IgV' and IgC' domains found in the human B7-H3 gene. The gene structure of mouse B7-H3 was comparable with other members of the B7 family (Fig. 3). It was found that a high homology of IgV/IgV' and IgC/IgC' region including introns between them in human B7-H3 genome sequence and the absence of additional exons equivalent to human IgV' and IgC' in mouse genome. This suggests that the duplication of a portion of human gene encoding IgV-IgC parts of B7-H3 occurred relatively recently in evolution.

B7-H3 Tissue Distribution and Expression of B7-H3R on Activated T Cells

As was previously published, northern blot analysis showed that B7-H3 is encoded by a single ~4.1-kb transcript and is expressed at high levels in many human tissues including heart, liver, placenta, prostate, testis, uterus, pancreas, small intestine and colon.[8] Low levels of B7-H3 mRNA were also found in brain, skeletal muscle, kidney and lung. B7-H3 mRNA could also be detected in several lymphoid organs including spleen, lymph nodes, bone marrow, fetal liver and thymus. No B7-H3 mRNA was found in peripheral blood leukocytes (PBL). Several tumor lines, including melanoma G361, cervical adenocarcinoma HeLa S3, chronic myelogenous leukemia K562, lung carcinoma A546 and colorectal adenocarcinoma SW480, also ex-

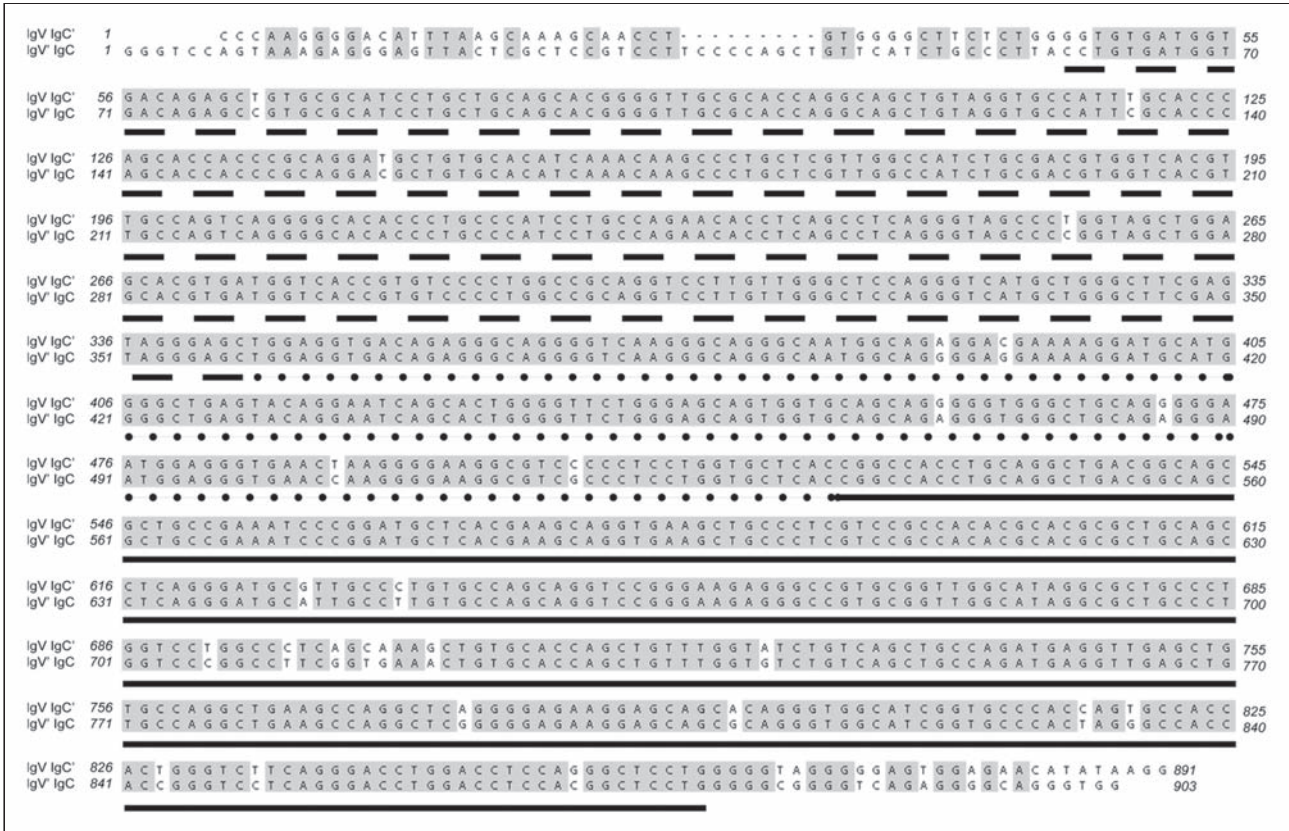

Figure 4. Alignment of IgV-IgC' with IgV'-IgC regions derived from hB7-H3 genome sequence (see Fig.3). Identical nucleotides are shaded. Exons encoding IgV and IgV' domains (solid line), introns (doted line) and exons encoding IgC' and IgC domains (dashed line).

Table 2. Number of hits in EST database and SAGE library searched with the full length of B7 family members genes

	SAGE Library Hits	EST Database Hits
B7-1	21	8
B7-2	16	26
B7-H1	6	21
B7-DC	9	17
B7h/B7-H2	33	54
B7-H3	72	214

pressed B7-H3 mRNA. Broad distribution of mRNA encoding B7-H3 in various tissues including tumors was also confirmed by searching EST database and SAGE library (Table 2).

It was shown by others that B7-H3 mRNA is expressed at high levels in immature DCs and is down-regulated upon maturation or LPS stimulation of DC.[19] However we could not detect B7-H3 protein on the surface of immature DC, monocytes, T cells or B cells. In contrast, incubation of DC with IFN-g or $CD14^+$ monocytes with GM-CSF induced B7-H3 expression.[8] In addition, activation of DC, monocytes and T lymphocytes with a combination of PMA and ionomycin triggered expression of B7-H3. Therefore, B7-H3 is an inducible molecule on the surface of DC, monocytes and T cells.

To determine the expression of a B7-H3 receptor on T cells, we employed the fluorescent spheres to increase binding avidity of receptor and ligand. Briefly, green fluorescent spheres (0.2 mm) precoated with avidin were incubated with biotinelated anti-mouse IgG2a mAb followed by incubation with a fusion protein consisting of the extracellular portion of B7-H3 and the Fc portion of mouse IgG2a. The resulting construct was used for FACS staining. Density plots show staining of resting and activated T cells with CD4/CD8 mAb and B7-H3Ig (Fig. 5). Resting T cells are not stained by B7-H3Ig, while ~25% of PHA activated T cells stained positive with B7-H3Ig. Both CD4 and CD8 cells expressed the same levels of B7-H3 receptor.

None of the tested fusion proteins of known inducible receptors of the B7 family ligands including CTLA4Ig, ICOSIg and PD-1Ig stained B7-H3 transfected 293 cells.[8] This suggests that B7-H3 has a unique receptor that is distinct from CTLA-4, ICOS and PD-1.

B7-H3 Costimulates T Cell Growth in Vitro

Since receptor for B7-H3 was found on both $CD8^+$ and $CD4^+$ T lymphocytes, we tested the ability of B7-H3 to costimulate proliferation of two subsets on T cells. Purified CD8 or CD4 T cells were stimulated by immobilized monoclonal antibodies (mAb) to human CD3 (anti-CD3) in the presence of immobilized control Ig, B7-1Ig or B7-H3Ig. The growth of T cells upon stimulation was determined by [^{3}H]TdR-incorporation after 72 h incubation. B7-H3Ig increased proliferation of CD4 and CD8 cells in the presence of a suboptimal dose (40 ng/ml) of anti-CD3. B7-1Ig universally induced significantly higher T cell proliferation than did B7-H3Ig.[8] In the absence of anti-CD3, neither B7-H3Ig nor B7-1Ig induced proliferation of T cells.

To evaluate the ability of B7-H3 in CTL generation, purified human T cells from healthy donors were stimulated with the melanoma line 624mel transiently transfected with hB7-H3 gene in pcDNA vector or control vector. CTL activity was determined by lysis of ^{51}Cr-labeled 624mel cells. 624mel cells transiently transfected with the hB7-H3 gene express the protein on

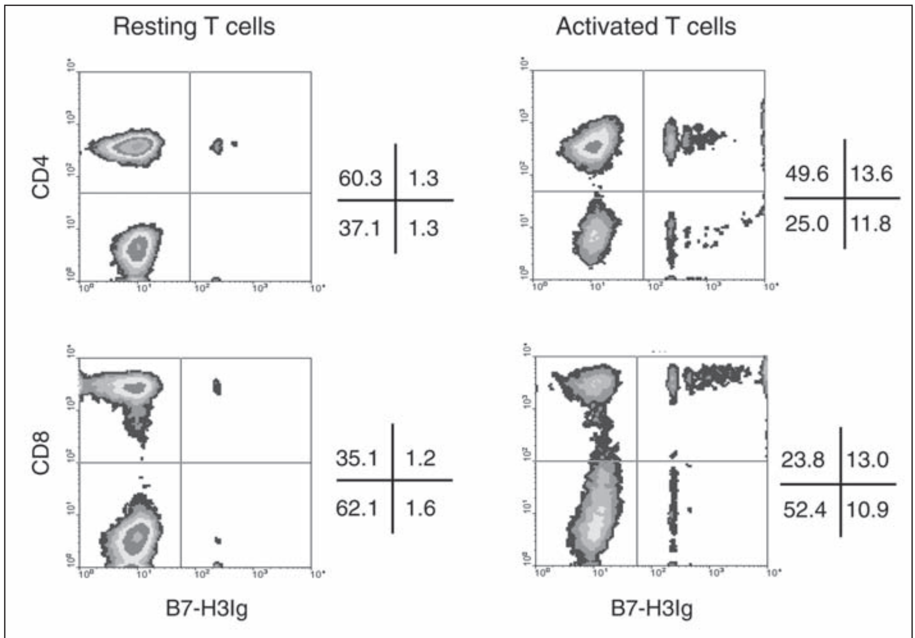

Figure 5. Expression of B7-H3R on activated T cells. T cells were isolated from peripheral blood mononuclear cells of healthy donors and were cultured with PHA (5 μg/ml). At 24 h T cells from cultures were stained with B7-H3-Ig coupled to Green Fluorescent Spheres (Molecular probes) and with anti-CD4-PE or anti-CD8-PE mAb.

cell surface and induced higher CTL activity than that of mock-transfected 624mel cells, presumably against allogeneic antigens on 624mel melanoma cells.[8] Our results demonstrate that B7-H3 can costimulate the growth of both $CD4^+$ and $CD8^+$ T cells and enhance the generation of CTL *in vitro*.

B7-H3 Selectively Increases IFN-γ Production

Previously we investigated how B7-H3 may affect cytokine profile in human T cells after TCR engagement using SuperArray.[8] Total RNA was obtained from T cells cultured with immobilized CD3 and control or B7-H3Ig. RNA was converted to cDNA and labeled with ^{32}P. Labeled cDNA was hybridized to membranes pre-spotted with cytokine gene fragments. Levels of cytokine gene expression were analyzed relevant to housekeeping genes (b-actin and GAPDH) spotted on the membranes. We observed an18 times increase of IFN-γ RNA in T cells cultured with CD3 and B7-H3Ig. We also confirmed the increased production of IFN-γ protein by T cells in the presence of B7-H3.[8]

In the mouse system we compared the effects of B7-1 and B7-H3 on cytokine production by T cells activated with immobilized anti-CD3 mAb. As can be seen on Fig. 6, mB7-1Ig increased production of IL-4, IL-10 and IFN-γ. In contrast mB7-H3Ig moderately increased IL-4 production, did not induce secretion of IL-10 and significantly increased production of IFN-γ. Interestingly, the production of IFN-γ by T lymphocytes cultured in the presence of B7-H3Ig was significantly higher than that in B7-1Ig stimulated T cells. Therefore B7-H3 may play an important role in the regulation of cell-mediated immune responses against infection and cancer by stimulating production of IFN-γ.

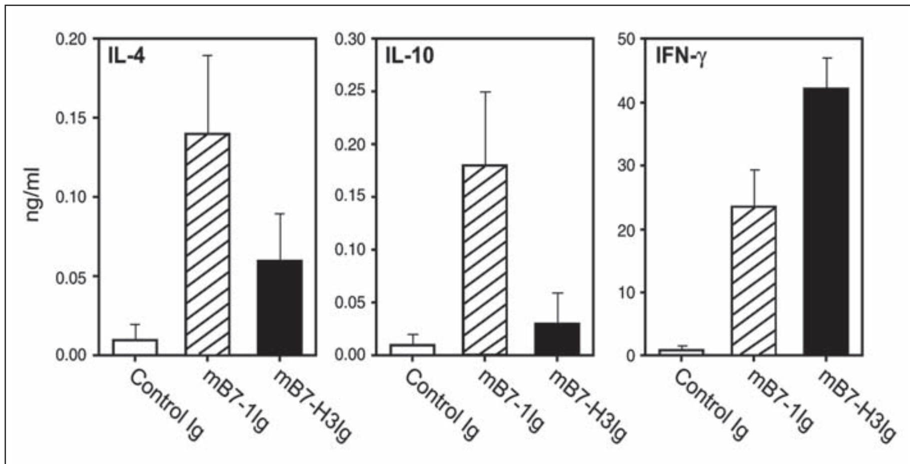

Figure 6. B7-H3 augments the expression of IFN-γ. Nylon wool enriched mouse T cells (5×10^6/well) were cultured in 24-well plates pre-coated with 500 ng/ml anti-CD3 and 10 μg/ml of either control Ig (open) B7-1Ig (hatched) or B7-H3Ig (filled). Supernatants were collected at 48 h. IL-4, IL-10 and IFN-g concentration was measured by ELISA

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Chapter 9

The Role of B7s in Transplantation

Didier A. Mandelbrot, Wayne R. Godfrey, Arlene H. Sharpe and Bruce R. Blazar

Introduction

The manipulation of the B7 family of molecules holds great promise for developing therapies to be used in clinical transplantation. A major goal in the field of transplantation immunology is to induce antigen-specific tolerance to foreign histocompatibility antigens differentially expressed between the graft donor and recipient. In the case of solid organ transplantation, if antigen-specific tolerance were achieved, a recipient's immune system would not reject a graft, but would maintain the ability to respond to all other foreign antigens. In the case of hematopoietic grafts, the immune response of mature T cells in the graft that are reactive with the recipient would be avoided. Tolerance would allow transplantation of solid or hematopoietic organs without incurring the toxicities of the broadly immunosuppressive medications currently used in clinical practice. This chapter will review data from studies in animals and in humans showing that targeting of B7 family molecules may play an important role in the induction of transplant tolerance, both in solid organ and bone marrow transplantation (BMT).

The Absence of B7 Costimulation Renders T Cells Anergic in Vitro

The idea that T cells require two distinct signals for full activation is over 30 years old.[1] The first signal is provided by the interaction between an MHC/peptide complex on an antigen presenting cell (APC) and the T cell receptor (TCR). The second, or costimulatory signal, is provided by accessory molecules.[2] Although many accessory molecules provide important signals for T cell activation, the best characterized costimulatory molecules are in the B7 family. B7 ligands, B7-1 (CD80) and B7-2 (CD86), present on APCs, can bind two homologues which have been identified and shown to be present on T cells: CD28 which provides positive costimulatory signals and CTLA-4 which provides negative costimulatory signals to T cells that have been activated via their antigen-specific TCR. In vitro models have demonstrated that antigen-specific unresponsiveness (termed anergy) can be induced by providing a T cell with an antigenic signal in the absence of B7 costimulation.[3,4] In these models, subsequent exposure of T cells to both TCR and CD28 stimulation is unable to activate the T cell. These initial studies generated great enthusiasm that this approach could be extended to organ and hematopoietic cell transplantation, since in this clinical situation, it is possible to time the exposure of a recipient to foreign antigens to coincide with blockade of B7 costimulation.

The B7-CD28 Family Molecules, edited by Lieping Chen.
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Blockade of B7 Molecules Prolongs Organ Graft Survival

The first demonstration that blockade of B7 molecules could be immunosuppressive in vivo used CTLA4-Ig, a fusion protein consisting of the extracellular domain of CTLA4 fused to the Fc portion of an immunoglobulin. CTLA4-Ig binds both B7-1 and B7-2 with high affinity.[5] Early studies showed that CTLA4-Ig given in vivo could prolong the survival of xenogeneic (human) pancreatic islets in mice[6] and heart allografts in rats.[7] Graft survival was prolonged to a similar degree by using a combination of antibodies to B7-1 and B7-2,[8] suggesting that CTLA4-Ig acts by binding exclusively to B7-1 and B7-2 and not to additional B7 molecules. This was confirmed by in vivo studies using knockout mice lacking B7 ligands and CTLA4. In these studies, mice lacking CTLA4 ($CTLA4^{-/-}$) developed uncontrolled T cell proliferation, due to loss of negative costimulatory signals provided by CTLA4, if either B7-1 or B7-2 was present to interact with CD28. However, if both B7-1 and B7-2 also were absent, T cells did not proliferate. Based upon these in vivo data, B7-1 and B7-2 are the only B7 molecules that act physiologically to costimulate T cells through CD28.[9]

Additional studies in a variety of rodent models with different solid organ allografts demonstrated the broad applicability of B7 blockade as an approach to prolong graft survival. These included kidney transplants in rats[10] and skin transplants in mice.[11] In a few reports, antigen-specific tolerance was shown to be induced by CTLA4-Ig treatment. For example, treated recipients of hearts rejected third party skin grafts, but accepted skin grafts from the original donor strain.[12] However, this result was not widely reproduced. CTLA4-Ig also was shown to be immunosuppressive in non-human primate recipients of kidney [13] and islet transplants,[14] but the effect on graft survival was much less pronounced than in rodent models.

The importance of B7 molecules in allograft rejection as elucidated by CTLA4-Ig fusion protein or anti-B7 monoclonal antibody infusions was confirmed by studies using mice lacking both B7-1 and B7-2 ($B7^{-/-}$). In particular, using $B7^{-/-}$ mice as either recipients or donors of fully MHC mismatched cardiac grafts, it was shown that absence of B7 expression on the recipients allowed indefinite survival of all cardiac allografts.[15] In contrast, the presence or absence of B7 on the donor cardiac allograft had no effect on graft survival. The absence of B7 molecules in the recipient also was shown to prolong the survival of mouse skin grafts, a model in which graft survival is more difficult to achieve.[16] The fact that recipient, and not donor, B7 expression determines cardiac allograft survival has important therapeutic implications. Blocking B7 molecules only in the donor graft, by pre-treating the graft prior to transplantation, had previously been proposed as a therapy that would have no side effects on the recipient, and has some utility in inducing the survival of mouse islet grafts.[17] However, the fact that recipient B7 molecules are sufficient to mediate rejection in the mouse cardiac allografts suggests that B7 blockade of the donor graft would be ineffective in heart transplantation.

Allorecognition Pathways Affected by B7 Costimulation

In contrast to immune responses to infectious organisms, in which antigens are presented only by host APCs, the response to an allograft can be mediated by donor or recipient APCs, termed direct and indirect allorecognition, respectively. Direct allorecognition is defined by presentation of donor graft histocompatibility antigens by donor graft APCs to recipient T cells. These T cells respond to intact foreign MHC/peptide complexes on donor APCs. Alternatively, graft responses can be triggered by the process of indirect allorecognition, defined by presentation of donor histocompatibility antigens by recipient APCs to recipient T cells. These T cells respond to foreign MHC molecules which have been released from donor APCs, taken up and processed by recipient APCs, and presented as allopeptides in the context of recipient MHC. Direct antigen presentation is the dominant mechanism in acute graft rejection.[18] However, mouse models of graft rejection have demonstrated that the indirect pathway can also

mediate acute rejection,[19] and this pathway may in fact be more important for chronic rejection[20]. Nonetheless, the indirect pathway may be required for tolerance induction with B7 blockade. In the addition to the aforementioned cardiac allograft studies in $B7^-$ mice, CTLA4-Ig was found to be ineffective in prolonging allograft survival in MHC deficient recipients which are incapable of mediating allograft rejection via the indirect pathway.[21]

In studies of graft rejection by the indirect pathway, it is assumed that antigen, as well as B7 costimulation, are both provided by recipient APCs. Provision of both antigen and costimulation by the same cell has been termed costimulation in cis, in contrast to costimulation in trans, in which B7 is provided by a different cell from that providing antigen. Just as the direct pathway is more potent than the indirect pathway in an acute alloresponse, costimulation in cis is generally considered to be more potent than in trans.[22] Therefore, the most potent alloresponse is due to direct allorecognition with costimulation in cis (Fig. 1A). To discern the role of other potentially sub-dominant mechanism(s) of graft rejection, studies were performed using $B7^{-/-}$ grafts (to eliminate the contribution of direct allorecognition in cis) or MHC-deficient recipients (to eliminate the contribution of indirect allorecognition). Indirect allorecognition with costimulation in cis likely contributes to the rapid rejection of $B7^{-/-}$ cardiac allografts by wild-type mice, since only recipient cells can express B7[15] (Fig. 1B). Using a model in which graft recipients are MHC-deficient and therefore incapable of indirect presentation, rejection of $B7^{-/-}$ cardiac allografts was mediated by direct allorecognition with costimulation in trans (Fig. 1C). In this model, donor APCs can provide alloantigen, but not B7 costimulation, while recipient APCs can provide B7 costimulation, but not alloantigen presentation.[23] Thus, B7 costimulation provided in trans is sufficient to mediate acute cardiac allograft rejection.

The Role of B7 Molecules in Chronic Graft Rejection

In early studies of experimental and clinical transplantation, before the advent of powerful immunosuppressive regimens, acute rejection was the major barrier to graft survival. However, in recent years, chronic graft rejection has become a more common cause of graft loss than acute rejection in clinical transplantation.[24] As a result, there is great interest in developing therapies, such as B7 blockade, that might prevent chronic rejection. Although the mechanisms underlying chronic rejection are still poorly understood, substantial differences from acute rejection are likely to exist. In particular, the histologic findings are distinct. While acute rejection shows many inflammatory cells and acute tissue destruction, chronic rejection shows thickening of vessel walls and tissue scarring. While recurrent episodes of acute rejection may lead to chronic changes, it is also likely that antigen-independent factors, such as hemodynamic stress, also contribute to chronic rejection.

Studies of CTLA4-Ig in rat models of heart[26] and kidney[26] transplantation demonstrated that in addition to prolonging graft survival and blocking acute rejection, B7 blockade prevents the vascular changes of chronic rejection. In $B7^{-/-}$ mouse recipients of cardiac allografts, long-term surviving grafts show no evidence of chronic rejection.[15] These studies are analogous to studies of kidney transplantation in humans showing that acute rejection is a major predictor of chronic rejection[27]. In addition, delayed administration of CTLA4-Ig can be shown to inhibit chronic rejection in a rat kidney model of experimental chronic allograft rejection.[28] In this model, short-term cyclosporin treatment is used to allow graft survival and the development of the proteinuria and vascular changes of chronic rejection. CTLA4-Ig given at 8 weeks prevented the progression of both functional and histologic signs of chronic rejection.

Role of B7 in Xenotransplantation

The xenoresponse to transplanted organs includes a number of pathways that are distinct from an alloresponse.[29] For example, hyperacute rejection of porcine tissue by humans is mediated by antibodies to foreign carbohydrate residues. However, B7 costimulation of T cells

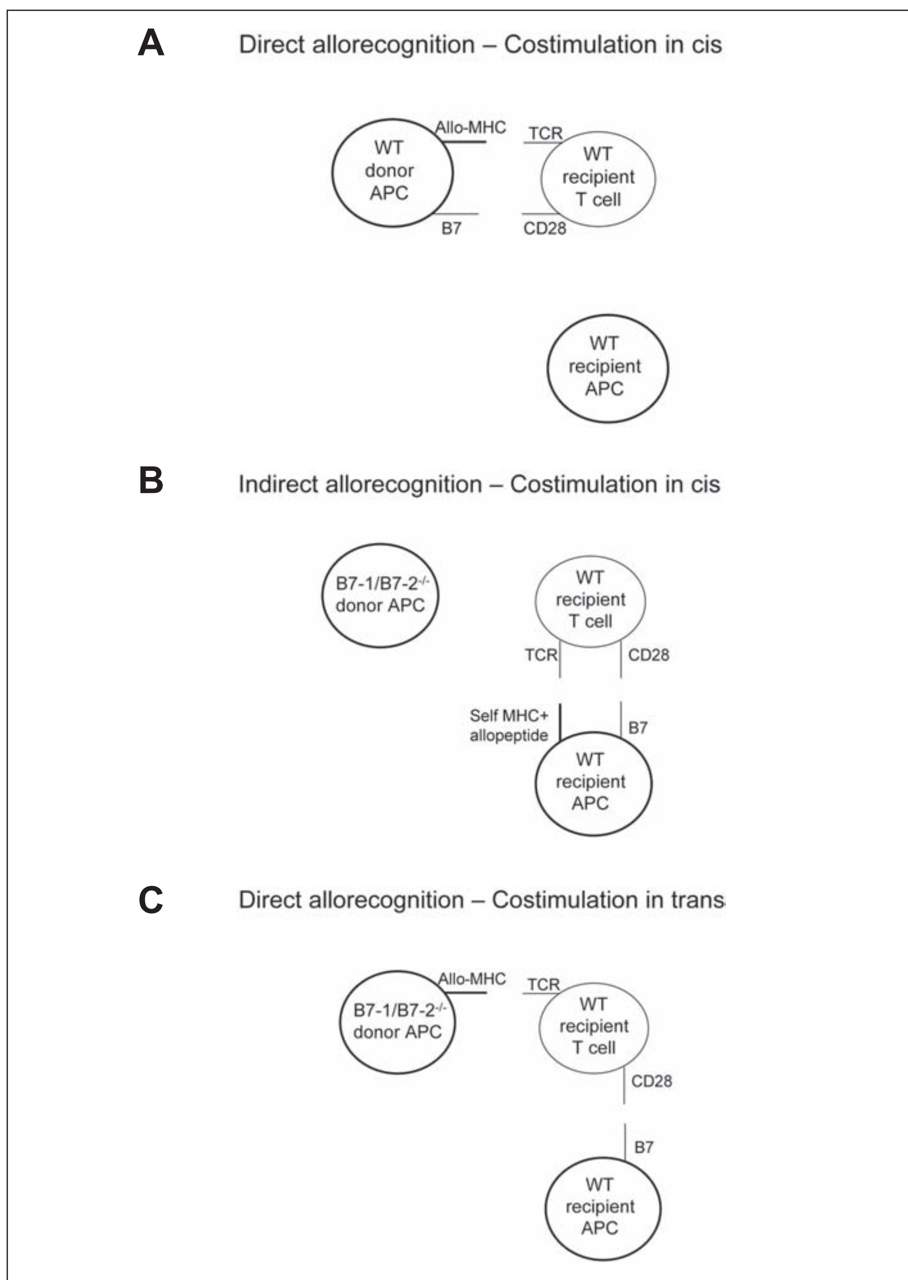

Figure 1. The most potent mechanism of acute rejection is direct allorecognition, with costimulation in cis (A). Two possible mechanisms could mediate the rejection of B7-1/B7-$2^{-/-}$ cardiac allografts by wildtype (WT) mice. In both, B7 costimulation is provided to recipient T cells by recipient APCs, since donor APCs lack B7. In indirect allorecognition with costimulation in cis, the antigenic signal is also provided by recipient APCs, in the form of self MHC/allopeptide complexes (B). In direct allorecognition with costimulation in trans, the antigen is intact allo-MHC on donor cells(C).

appears to be important in xenoresponses as well as alloresponses. In fact, the first report of CTLA4-Ig to prolong graft survival used a model of human islets transplanted to mice.[6] Further studies demonstrated in a rat-to- mouse cardiac xenograft model that CTLA4-Ig[30] or antibodies to B7-1 plus B7-2[31] prolongs survival. To prolong the survival of rat-to-mouse, or pig-to-mouse skin xenografts, simultaneous blockade of both B7 and CD40 pathways was required.[32]

The Role of B7-1 versus B7-2 Mediated Costimulation in Transplantation Responses

Since the discovery that CD28 has two B7 ligands, both the distinct and overlapping roles of B7-1 and B7-2 have been studied in multiple different systems.[33] The B7 molecules can be shown to bind both CTLA4 and CD28, although B7-1 binding occurs with slower on and off kinetics than B7-2.[34] In general, B7-1 and CTLA4 are not detectable on resting cells, but are expressed several days after cell activation.[35] In contrast, CD28 and B7-2 are expressed on resting cells. These patterns of expression are one basis for the hypothesis that B7-1 may preferentially interact with CTLA4 to inhibit immune responses, while B7-2 would interact with CD28 to initiate T cell activation. In addition, the crystal structure of B7-1 suggests that B7-1 may bind CTLA4 more tightly than does B7-2.[36] Whether preferential interaction of B7-1 with CTLA4 is due to binding characteristics, patterns of cell surface expression, or both, has not been determined. Most of the data comparing the two B7 molecules in acute transplant rejection models is consistent with effects predicted by the preferential binding B7-2 to CD28 (providing activating signals) and B7-1 to CTLA4 (providing negative regulatory signals). For example, antibody to B7-2 which would block B7-2/CD28 interactions, prolongs survival of mouse islets, whereas antibody to B7-1, which would block B7-2/CTLA4 interactions, does not prolong islet cell survival.[8] In some studies of mouse cardiac transplants, antibody to B7-2 alone, but not to B7-1, can induce long term graft survival.[37] Most dramatically, in $CD28^{-/-}$ mouse recipients, antibodies to B7-1 versus B7-2 have opposite effects on cardiac allograft survival.[38] Anti-B7-1 shortens survival by blocking the T cell inhibitory signal normally produced by B7-1 binding to CTLA4. In contrast, anti-B7-2 prolongs graft survival, presumably by blocking the interaction between B7-2 and a stimulatory T cell receptor which is not CD28. Such a receptor has been functionally described, but not yet identified, in mice lacking both CD28 and CTLA4[39](see below). Thus, B7-2 appears to be more potent than B7-1 as a positive T cell costimulatory molecule in acute rejection.

Nevertheless, B7-1 does interact with CD28 in vitro,[34] and in vivo, this interaction is strong enough to produce lethal lymphoproliferative disease in mice lacking CTLA4 and B7-2.[9] Possibly because B7-1expression is more prolonged than B7-2 in cardiac allografts, studies of chronic rejection in mice lacking B7-1 or B7-2 suggest that B7-1 is more important than B7-2 in producing chronic vascular changes.[40] Similarly, in a rat cardiac model of chronic rejection, late blockade of B7-1 prevented the development of chronic vasculopathy, although this same blockade was ineffective in preventing acute graft loss.[41]

Combination Approaches to Improve Allograft Survival Based upon B7:CD28/CTLA-4 Blockade

Although B7 blockade alone is highly effective in preventing cardiac and renal allograft rejection in rodents, attempts to extend these studies to more immunogenic organs such as islets,[14] or to non-human primates,[13] have provided less dramatic results. Such studies suggested that B7 blockade alone would be unlikely to be a sufficient immunosuppressive strategy in human transplantation. As a result, numerous studies have examined the role of additional agents in potentiating the effect of B7 blockade, including agents already in clinical use.

One approach which has been widely studied in rodent models is to administer peri-transplant donor specific transfusions (DST) in addition to CTLA4-Ig. These transfusions can be from blood, bone marrow or lymphoid tissue cells. Although the mechanism by which DST has an immunosuppressant effect is poorly characterized, increased provision of MHC antigens appears to be important (D. Mandelbrot: unpublished data). Similarly in humans, blood transfusions can reduce the rate of kidney graft rejection.[42] In rodents, DST plus CTLA4-Ig can in fact be shown to have a synergistic affect in prolonging graft survival,[43] although such an approach has not yet been tested in humans.

Another combination regimen that has been carefully studied is the addition of anti-CD40L antibody to B7 blockade. This regimen has exploited the extensive interactions between these two costimulatory pathways. In particular, CD40 engagement is critical in activating antigen presenting cells, and upregulates expression of B7 molecules.[44] B7 engagement of CD28 in turn upregulates expression of CD40L.[45] Numerous groups have shown that combined blockade of B7 and CD40L are additive and even synergistic in prolonging cardiac and skin graft survival in rodents[e.g., 11]. The co-blockade of the B7:CD28/CTLA-4 and CD40/CD40L pathways also appears superior to either CTLA4-Ig or anti-CD40L in models of chronic rejection such as mouse aortic allografts.[46] This combination was also shown to be more effective than either agent alone in prolonging kidney grafts in non-human primates.[13]

Studies have also been performed to see the effect of combining B7 blockade with immunosuppressive agents currently used in clinical transplantation. Although this approach seems promising, a clear consensus on these combinations is still lacking. Early studies with mouse heart transplants suggested that the effect of CTLA4-Ig and the calcineurin inhibitor cyclosporin are additive.[47] In a mouse skin transplant model, the addition of either cyclosporin or the mTOR (molecular target of rapamycin) inhibitor, sirolimus, further prolonged the graft survival induced by CTLA4-Ig.[48] Finally, in non-human primate renal transplants, the effects of anti-B7 antibodies and cyclosporin were also additive.[49] In contrast, in a mouse heart transplant model, cyclosporin actually inhibited the induction of long-term graft survival by CTLA4-Ig.[50] Furthermore, in a rat model of chronic rejection, the addition of cyclosporin also seemed to abrogate the protective effect of CTLA4-Ig on chronic allograft rejection.[51] This finding may be explained by the fact that cyclosporin inhibits signaling through the TCR, and a sufficiently strong antigenic signal through the TCR may actually be necessary for costimulatory blockade to be effective in inducing tolerance. Because of the many different protocols in clinical use, and differences between various animal models of B7 blockade, further work is required to clarify which combinations are more likely to be beneficial.

CTLA4 Signaling Prolongs Graft Survival

The role of CTLA4 in providing an inhibitory signal to T cells has been extensively reviewed.[52] The importance of the B7:CTLA4 signal, in the absence of any effects on modifying CD28 signaling, has been shown in a model of cardiac transplantation using $CD28^{-/-}$ mice as recipients.[53] In these studies, blockade of B7:CTLA4 interactions using either CTLA4-Ig or anti-CTLA antibody accelerated graft rejection. The idea that B7 signaling through CTLA4 is necessary to induce T cell anergy was first proposed using an in vivo model of mouse immune responses to a nominal antigen.[55] These studies were extended to a variety of transplant models, in which anti-CTLA4 prevented long term graft survival. In one model, rodents underwent thymectomy and were given anti-CD40L antibody and DST as a triple therapy to block rejection of skin grafts.[55] Anti-CTLA4 prevented the induction of long-term graft survival. In a different model in which CTLA4-Ig plus DST was used to block rejection of heart grafts,[36] anti-CTLA4 antibody prevented long-term cardiac allograft survival. Thus the importance of B7:CTLA4 signaling in inhibiting T cell activation or inducing tolerance has been confirmed in numerous transplant models. However, studies using $B7^{-/-}$ mice as recipients of cardiac

allograft demonstrated that CTLA4 signaling is not absolutely required to induce long-term graft survival.[15]

B7 and CD28 Homologues

In recent years, several new molecules have been described which are homologous to CD28 and to B7 respectively, and are also important for T cell activation and differentiation.[56] These molecules include the CD28 homologue ICOS (inducible co-stimulator), which binds the B7 homologue B7h (also called B7RP-1,GL-50, B7H2, or LICOS). B7h-ICOS signaling appears to interact with B7:CD28 signaling,[57]but neither B7-1 nor B7-2 bind directly to ICOS, and B7h does not bind to either CD28 or CTLA4.[58] In a mouse cardiac transplantation model, elimination of ICOS signaling by anti-ICOS antibody, an ICOS-Ig fusion protein, or the use of ICOS$^{-/-}$ recipients prolonged graft survival to some extent, although much less dramatically than B7 or CD40L blockade.[59] However, the combination of anti-ICOS and cyclosporin led to long-term survival, and the combination of anti-ICOS and anti-CD40L prevented the development of chronic vascular rejection. Other B7 and CD28 homologous such as the PD-1 (programmed death -1):PDL-1/ 2 pathway are described elsewhere in this book, but their role in transplantation has not yet been reported.

In addition to the contribution by ICOS and PD-1 pathways, it is likely that other B7:CD28 related pathways also play an important role in transplantation. For example, mouse heart transplants placed into CD28$^{-/-}$ recipients showed minimally prolonged survival,[52] while grafts placed into B7-1/B7-2$^{-/-}$ recipients survived long term.[15] This suggested the possibility that B7 molecules might mediate rejection by interacting with a non-CD28 receptor. Studies in CTLA4/CD28$^{-/-}$ mice suggest that in fact B7 molecules interact with a stimulatory receptor that is neither CTLA4 nor CD28.[39] In vitro, CTLA4/CD28$^{-/-}$ T cells show decreased proliferation in the presence of B7$^{-/-}$ APCs or CTLA4-Ig blockade, and increased proliferation with APCs transfected with B7. In addition, CTLA4-Ig prolongs mouse cardiac allograft survival in CTLA4/CD28$^{-/-}$ recipients. Thus, both in vitro and in vivo data suggest that B7 binds a still unidentified receptor that is neither CTLA4 nor CD28.

Overview of ex Vivo Blockade of the CD28/CTLA-4:B7 Pathway for Graft-versus-Host Disease (GVHD) Prevention

GVHD is a major complication of allogeneic BMT. GVHD is caused by donor alloreactive T cells that recognize MHC or minor histocompatibility antigenic differences between the donor and the recipient.[60,61] Donor T cells with alloreactive potential encounter recipient disparate antigens, activate, expand, and infiltrate into GVHD target tissues (skin, liver, gastrointestinal tract, and lung). Tissue destruction ensues. Approaches to prevent GVHD have included the in vivo administration of immunosuppressive agents and the ex vivo depletion of donor T cells from the bone marrow inocula.[60,61] However, such approaches render the recipient prone to the complications associated with an immune deficiency state. Preclinical strategies, discussed below, have included attempts to induce antigen-specific tolerance by blocking donor T cells from receiving signals delivered via costimulatory pathways. Most approaches have involved the in vivo administration of antibodies or fusion proteins that block either CD28/CTLA-4:B7 or CD40 ligand (CD154)/CD40 interactions. As an alternative to regulating donor anti-host T cell responses in vivo by costimulatory pathway blockade, approaches to tolerize donor T cells to recipient alloantigens by ex vivo CD28:B7 blockade prior to infusion would be advantageous for several reasons: 1) The recipient is not exposed to materials that may have adverse side-effects in vivo; 2) As compared to in vivo therapy, ex vivo costimulatory receptor blockade can be ensured, less material is needed since donor T cells to be targeted are

concentrated in a smaller volume, and the efficiency of tolerization can be accurately monitored; 3) Tolerance induction is more likely to be specifically directed against host alloantigens than leukemia or viral antigens which may reach critical antigenic threshold levels during the course of in vivo tolerance induction.

Ex Vivo Blockade of B7:CD28/CTLA-4 Interactions in Human Mixed Lymphocyte Reaction (MLR) Cultures

Costimulatory blockade in human MLR cultures was first investigated by Tan et al in 1993.[62] In their initial and comprehensive study, they showed that peripheral blood mononuclear cells (PBMC) could be rendered specifically hypo-responsive to alloantigen if CTLA4-Ig was used to block B7-mediated costimulation. Their model was to use PBMC-PBMC allo MLR with HLA mismatched cells. MLR were cultured for 7 days and then the cells washed and rested for 3 days. Secondary MLR were stimulated with PBMC from either the same or third party sources. CTLA4-Ig treatment inhibited primary MLR proliferation by 50-85%. The treated cells were specifically hypo-responsive to restimulation, with average 70% reduction in secondary MLR proliferation. Blockade with CD28 fab fragments resulted in similar results. Cytotoxic lymphocyte (CTL) development was inhibited four-fold. Interestingly, when cells were primed in a primary MLR and then restimulated under CTLA4-Ig blockade, they too developed restimulation hyporesponsiveness. IL-2 when added to the primary MLR (10U/ml) prevented the induction of hyporesponsiveness.

Using a similar PBMC model system, Gribben et al in 1996 further characterized ex vivo the costimulatory blockade system.[63] They proposed such an approach as a method for the prevention of GVHD in a BMT setting. Using ostensibly the same culture conditions, PBMC-PBMC in flasks at a 1:1 ratio at 10^6 cells/ml concentration, with CTLA4-Ig, they reported a greater degree of inhibition of primary MLR proliferation, approximately 90%. With combined monoclonal antibodies to B7-1 and B7-2 they noted a slightly more profound inhibition than with CTLA4-Ig. Cytokine analysis in the supernatant revealed complete prevention of detectable IL-2 accumulation, and by a quantitative PCR technique, a marked inhibition of IL-2 mRNA accumulation with CTLA4-Ig (100-fold) and anti-B7-1 plus anti-B7-2 antibodies (1000-fold). T cells in costimulatory pathway blocked cultures did not mount significant alloantigen restimulation responses. In contrast, MLR cultures treated with high concentrations (1 mM ~1000 ng/ml) of cyclosporin (CsA), anti-MHC Class II mAb, or anti-ICAM-1 mAb blocked cultures had suppressed primary responses but mounted significant responses to alloantigen restimulation and third party PBMC, presumably because such treatment resulted in a state of immunological ignorance by precluding TCR signals. Helper T lymphocyte precursor (HTLp) assays were also performed to quantitate effects of costimulatory blockade. A 100-fold reduction was noted in HTLp frequency for random donor full HLA mismatched combinations. These data suggest near complete blockade of proliferative responses and IL-2 production, a hallmark of anergy induction.

Human Clinical Trial of ex Vivo B7:CD28/CTLA4 Blockade for GVHD Prevention

On the basis of the initial in vitro studies, a clinical trial was initiated to test whether anergy induction would prevent GVHD by Guinan et al.[64] A marrow harvest (containing approximately 10-30% T cells from the marrow and peripheral blood contaminating the harvest) was cultured with irradiated patient/recipient PBMC for 36 hours in a culture bag to which CTLA4-Ig has been added. Notably, in the aforementioned preclinical studies, these same investigators demonstrated that costimulatory blockade treatment had no untoward ef-

fects on marrow stem/progenitor cells assays. Results from the reported trial involved 12 patients, 5 of whom survived long term. In haploidentical marrow transplant trials without post graft immunosuppression, $3x10^4$ T cells/kg is considered to be the maximum safe number of transplantable mature T cells. Because haploidentical marrow grafts were infused without T cell depletion and since patients received approximately $3x10^7$ T cells/kg, 70-90% of patients would be expected to develop accelerated GVHD if alloantigen hypo-responsiveness was ineffective. However, only (3 of 12) total and (2 of 5) long term survivors, experienced GVHD, which was mild-moderate in severity and responded to corticosteroid treatment. Thus, these preliminary results seem impressive.

In addition to anergy, a number a mechanisms may be at work with this method. The mechanics of T cell activation may be different in the marrow bag. The high density cultures may allow for alloreactive T cells to get activated, yet they may not traffic properly to lymph nodes as chemokine and homing receptors expression patterns are altered. The patient PBMC may contain circulating leukemic HLA-DR positive blasts which possibly could facilitate anergy induction. As stem cells were not purified, high doses of allogeneic NK cells may be transferred which may help prevent GVHD.

Adjuvants to B7 Blockade

Several groups have attempted to augment anti-B7 mediated anergy induction by the addition of other agents, either CSA or inhibition of CD40L (CD154):CD40 interactions. Using lymphoblastoid cell lines (LCLs) as potent APCs for anergy induction, Van Gool et al in 1994[65] first showed anti-B7-1 mAb and CSA formed a potent combination for anergy induction as assessed using a more stringent assay, inhibition of CTL development. Comoli et al in 2001[66] have extended this work by studying the combination of CTLA4-Ig and low dose CSA (200ng/ml), reporting profound inhibition of primary MLR, with subsequent profound inhibition of secondary MLR. Using flow cytometry analysis, these investigators observed that minimal activation occurred during the primary MLR culture. Third party reactivity as assessed by proliferative T lymphocyte precursor assays (PTLp) and HTLp was observed. However, the lack of a concurrent control to assess naïve responses at the time of the secondary MLR makes it difficult to determine whether there was functional inactivation of alloreactive cells (anergy), prevention of priming (ignorance) to alloantigen, or both processes operative. It is possible that low concentrations of CsA do not prevent anergy induction as did the high CsA concentration used in the Gribben study.[63] Low dose CSA may also block other accessory costimulatory signals for IL-2 production.

Van Gool et al[67] also studied combining anti-B7 mAbs with a blocking anti-CD40 mAb. A more profound inhibition of IL-2 secretion and prevention of CTL induction was observed with the combination approach. Naïve cells generated by culture without stimulators for one week will quickly form CTL on "restimulation" while cells exposed to costimulatory blockade would not, indicating that the combined anti-B7/anti-CD40L mAb approach is not just preventing the MLR response. Costimulatory blocked cultured cells were noted to have a deviated cytokine profile on secondary restimulation, with slightly increased IL-10 production, and reduced Th1 (IL-2, IFN-gamma) and Th2 (IL-5, IL-13) cytokines. Combined blockade did not result in anergy when LCL were used as stimulators. Koenen et al also have studied combined blockade of the CD28/CTLA-4:B7 and CD40L:CD40 pathways.[68] Again using ostensibly the same system, PBMC-PBMC allogeneic MLR cultures at 1:1 ratio, a more modest blockade of primary MLR proliferation was observed. Flow cytometry revealed blast transformation during blocked primary MLR, and secondary MLR hyporesponsiveness (4-fold) similar to the Tan study. They also noted a profound hyporesponsiveness on tertiary stimulation, even if IL-2 is present during secondary MLR. Lastly, they detected modest downregulatory properties of costimulatory pathway blocked T cell blasts.

Modifications of ex Vivo B7:CD28/CTLA4 Costimulatory Pathway Blockade of Human MLR Cultures Designed to Improve the Efficacy of Tolerization

The systems described to date all have utilized unpurified PBMC populations as responders and stimulators with similar conditions: 1:1 ratio of responders to stimulators and plating in flasks/flat well culture plates at a density of 10^6 cells/ml. However, the results of these studies differ slightly. In the studies by Tan and Koenen, costimulatory blockade does not completely block the MLR. While proliferative responses are inhibited, responders can proliferate to some extent and form blasts. These cells are hypo-responsive upon secondary alloantigen restimulation, but proliferation is reduced only by 3-4 fold. Therefore, responders are not completely unresponsive, although these cells may exert immunoregulatory functions. On the other hand, the studies by Gribben (CTLA4-Ig), Comoli (CTLA4-Ig/low dose CSA) and Van Gool (combined CD28/B7 and CD40L:CD40 pathway blockade) seem to generate cultures which are completely (or nearly completely) blocked. The resulting cells are not blasts and mount weak, delayed, or non demonstrable secondary responses with weak stimulation. Cells in these cultures appear to be a mixture of anergic and ignorant cells.

Krampf et al[69] have noted that the response to costimulatory blockade is critically dependent on culture conditions and T cell/APC ratios which can be manipulated in a defined manner. By titrating APC numbers, systems were generated where there is near complete blockade (low APC) or incomplete blockade with some blast formation despite costimulatory blockade (high APC). These cultures generate anergic/ignorant T cells, or altered blasts with immunoregulatory activity. Recently, Boussiotis et al have reported that blockade of B7/CD28 in anergizing MLR cultures results in the generation of alternatively activated macrophages that had a decreased ability for antigen presentation and when added to fresh T cells could suppress T cell responses. Therefore, besides direct inhibition of T-cell costimulation, blockade of B7/CD28 may facilitate induction of T cell unresponsiveness by generating alternatively activated macrophages. Thus there appears to be a spectrum of effects, and it is unclear where the clinical trial fell on this spectrum, or where the optimum might be for tolerance induction. The human studies by Guinan et al provided evidence that CD28/CTLA-4:B7 blockade was sufficient to induce alloantigen hyporesponsiveness as assessed in vitro and would result in a low incidence of GVHD even when haploidentical donor T cells were infused in large numbers in vivo. Due to the GVHD risk, post-transplantation immune suppression was administered. Therefore, the true efficacy of GVHD prevention was difficult to accurately quantify.

Ex Vivo Tolerization of Murine MLR Cultures by Costimulatory Pathway Blockade As a Means of Achieving GVHD Prevention

Murine studies designed to induce alloantigen hyporesponsiveness by adding saturating concentrations of CTLA4-Ig to a 3- or 4-day MLR culture consisting of donor splenocytes and T cell depleted MHC class I + II disparate stimulator cells, showed that hyporesponsiveness could be achieved as assessed by in vitro assays.[71] However, such tolerization attempts did not reproducibly prolong recipient survival rates. To further improve this ex vivo approach, several modifications were made to the culture system: 1) Anti-B7-1 plus anti-B7-2 antibodies were substituted for hCTLA4-Ig, based upon the more effective results obtained by Gribben et al in a human MLR system;[63] 2) The duration of the MLR culture system was extended to 10 days, based upon anergy induction experiments performed by Roncarolo and colleagues,[72] who showed that the induction of alloantigen- specific nonresponsiveness in human $CD4^+$ T cells in an MLR culture was optimum after 10 days of incubation; 3) The responding population was restricted to $CD4^+$ T cells and the stimulator to an isolated MHC class II disparity, since the best evidence for anergy induction in mice and humans involves that T cell subset.[73-5]

With the implementation of these modifications, a profound degree of alloantigen hyporesponsiveness was achieved as measured in primary and secondary MLR cultures.[73-75] Importantly, the in vivo infusion of cultured cells that had been exposed to alloantigenic stimulators in the presence of anti-B7 antibodies resulted in complete GVHD lethality protection against an otherwise uniformly lethal dose of T cells. Whether these tolerized cells have retained beneficial biological functions (e.g., anti-viral, anti-leukemia, or bone marrow engraftment promoting capacities) has yet to be studied. Finally, an interesting aspect of these ex vivo tolerization studies was the demonstration that $CD4^{+}25^{+}$ regulatory cells were required for tolerance induced by anti-B7 mAbs.[75] Specifically, MLR cultures containing anti-B7 mAbs but initiated with $CD4^{+}25^{-}$ cells were not hyporesponsive to alloantigen-bearing stimulator cells as measured either in primary or secondary proliferation assays. Moreover, no protection against GVHD lethality was observed. Thus, the presence of $CD4^{+}25^{+}$ cells was a pre-requisite for ex vivo tolerization by anti-B7 antibodes. If confirmed in human MLR cultures, strategies to optimize the number of $CD4^{+}25^{+}$ responder cells could improve upon the degree of tolerization achieved ex vivo, and presumably in vivo, by CD28/CTLA-4:B7 blockade.

In Vivo Blockade of B7:CD28/CTLA4 for GVHD Prevention

Although ex vivo tolerization clearly has certain advantages as compared with in vivo tolerization, bulk MLR cultures performed on a large scale for clinical infusion purposes can be cumbersome from both a technical and regulatory affairs point-of-view at academic institutions. Therefore, the exportability and widespread implementation of such approaches would be more difficult than the injection of monoclonal antibodies or fusion proteins, provided by the pharmaceutical industry, to block the CD28/CTLA-4:B7 pathway. Initial proof-of-principle experiments in that CD28/CTLA-4:B7 blockade could inhibit GVHD-induced lethality was derived from studies utilizing CTLA4-Ig infused into heavily irradiated recipients of a fully allogeneic or semi-allogeneic donor graft.[76,77] In both experimental settings, CTLA4-Ig reduced GVHD-side effects and/or lethality, albeit to a variable extent. The findings of partial GVHD protection conferred by CTLA4-Ig infusion in heavily irradiated allogeneic recipients were confirmed in subsequent studies and in other studies focused upon recipients of minor histocompatibility only disparate donor grafts.[78,79] In no instance did CTLA4-Ig completely eliminate lethality or prevent GVHD mediated tissue damage in heavily irradiated recipients, although data by the groups of Hakim and Via both showed the inhibition of acute GVHD responses in non-irradiated F1 recipients of parental donor grafts.[80,81] The lack of complete GVHD protection in heavily irradiated recipients could be due to the contribution of pro-inflammatory cytokines to GVHD pathology and lethality, participation of other pathways in providing T cell costimulation, and/or relatively more rapid clearance of the fusion protein in the setting of GVHD or irradiation-induced tissue injury. With respect to the latter mechanism, sera obtained from heavily irradiated recipients of allogeneic grafts that had received repetitive CTLA4-Ig were sub-therapeutic when measured 9 days after the last injection[76]. These data suggested that the incomplete anti-GVHD effect of CTLA4-Ig may be related to its size, affinity, avidity and clearance rate accentuated by an enhanced intestinal and capillary protein loss state of GVHD and/or conditioning regimen injury.

Rather than infuse CTLA4-Ig, high affinity and avidity mAbs directed toward B7-1 and B7-2 were used to extend the duration of effective CD28/CTLA-4:B7 blockade. We observed that the infusion of anti-B7 antibodies was highly effective in eliminating GVHD lethality induced in sublethally irradiated recipients of purified MHC class I or II disparate $CD8^{+}$ or $CD4^{+}$ T cells, respectively.[82] Consistent with our findings, Saito et. al have shown that anti-B7 mAbs were highly effective in preventing GVHD lethality, but not the hematological or histological features of GVHD in lethally irradiated F1 recipients of parental grafts.[83,84] In comparison to studies using CTLA4-Ig to block GVHD lethality in heavily irradiated recipients of

allogeneic donor grafts, the survival achieved with anti-B7 antibody treatment appeared superior. Moreover, using the same model system utilized for CTLA4-Ig studies, anti-B7 mAb infusion reproducibly led to survival rates ranging from approximately 40-60%, in contrast to the more variable and generally lower survival rates of 0-63% following CTLA4-Ig infusion (B. R. Blazar, P. A.Taylor: unpublished data).

Mechanism(s) of GVHD Inhibition by B7:CD28/CTLA-4 Blockade

In heavily irradiated recipients of MHC disparate splenocytes, anti-B7 mAbs were shown to inhibit donor $CD4^+$ and $CD8^+$ T cell expansion by almost 100-fold as measured by enumerating thoracic duct lymphocytes (TDLs) obtained early post-transplant[82]. Similarly, the groups of Hakim and Via both observed that CTLA4-Ig was effective in preventing the engraftment and/or inhibiting the expansion of donor $CD4^+$ and $CD8^+$ T cells, as well as donor B cells, in non-irradiated F1 recipients of parental donor grafts.[80,81] TDLs retained anti-host responsiveness indicating that not all T cells were anergic.[82] Anasetti's group has shown that targeting the CD28 receptor with a specific monoclonal antibody modulates the receptor in vivo, selectively inhibits donor anti-host alloreactive T cell expansion in vivo, and prevents GVHD.[78] CTLA-4 signaling was necessary for this effect because treatment with CTLA4-Ig did not prevent GVHD as effectively as anti-CD28 antibody. Collectively, these data indicate that CD28/B7 blockade reduces the number of GVHD-causing donor T cells capable of responding to host alloantigenic disparities.

CTLA4-Ig and anti-B7 monoclonal antibodies block CD28/B7 and CTLA-4/B7 interactions. CTLA-4/B7 inhibits cell cycle entry, reduces IL-2 secretion, and is necessary for tolerance induction.[85-7] Blockade with B7 antagonists interferes with CD28:B7 and CTLA-4:B7 interactions, which may have opposing effects. To maintain the down-regulatory capacity of CTLA-4:B7 on alloreactive T cells responses, several groups have used $CD28^{-/-}$ donor mice as a source of GVHD-causing T cells.[86,88-90] A critical role of B7:CD28 was demonstrated by markedly compromised expansion of $CD28^{-/-}$ T cells and diminished GVHD lethality when limited numbers of purified $CD4^+$ or $CD8^+$ $CD28^{-/-}$ T cells were infused.[90] In vitro, alloresponses of CD28 $^{-/-}$ T cells are markedly lower than controls. In sublethally irradiated recipients of purified $CD28^{-/-}$ $CD4^+$ or $CD8^+$ T cells, GVHD lethality capacity was reduced by > 10-fold and > 3-fold, respectively, as compared to $CD28^{+/+}$ cells and as assessed by titration of donor T cell numbers.[90] With the notable exception of studies by Saito et al , lethality was observed in a high proportion of recipients when high numbers of T cells were infused or mice were heavily irradiated, indicating that the requirement for CD28:B7 interaction was lessened. A complicating feature in the interpretation of studies in which $CD28^{-/-}$ T cells are used to prevent CD28/B7 signaling was the recent discovery that $CD4^+25^+$ regulatory cells are present in low numbers in $CD28^{-/-}$ mice.[91] The importance of $CD4^+25^+$ cells in the ex vivo tolerance inducing capacity of anti-B7 mAbs has been discussed above. In addition, recent data indicates that the purposeful depletion of $CD4^+25^+$ cells from fresh splenocytes prior to infusion into lethally irradiated, sublethally irradiated, or non-irradiated recipients markedly augments GVHD lethality capacity.[92] In contrast, the addback of fresh or ex vivo cultured and expanded $CD4^+25^+$ cells inhibits GVHD lethality in non-conditioned recipients of allogeneic donor T cells.[92]

Interestingly, in the aforementioned Saito study, the administration of anti-B7 mAbs into heavily irradiated recipients of $CD28^{-/-}$ donor splenocytes augmented the hematological and histological manifestations of GVHD, suggesting that CTLA-4:B7 interaction served to downregulate GVHD responses.[86] Direct proof for this hypothesis was derived from studies using anti-CTLA-4 mAb infusion in our lethally irradiated model in which MHC class I + II disparate donor grafts are given.[90] Under these conditions, anti-CTLA-4 monoclonal antibody enhanced in vivo donor T cell expansion. GVHD lethality was accelerated by anti-CTLA-4

monoclonal antibody infusion given early post-BMT. This accelerated GVHD lethality was most readily apparent when wild-type rather than $CD28^{-/-}$ T cells were administered.

In addition to the dominant donor anti-host T cell response that supports the generation of GVHD-induced lethality, host T cells present at the time of allogeneic T cell administration serve to temper the GVHD response. For example, non-irradiated severe combined immune deficient (SCID) mice are especially susceptible to a rapidly lethal GVHD response after the infusion of allogeneic T cells.[82] $CD28^{-/-}$ recipients are more susceptible than wild-type recipients to GVHD lethality induced by the infusion of splenocytes later post-BMT.[93] Mice that are thymectomized prior to BMT are more susceptible than euthymic mice due to a paucity of host T cells.[94] Pre-BMT depletion of host $CD25^{+}$ cells markedly increases GVHD lethality.[92] In each of these instances, there would exist a paucity of thymic-derived, host $CD4^{+}25^{+}$ regulatory cells that would be available and capable of down-regulating alloreactive donor T cells.

In aggregate, these data provide compelling evidence that blockade of CD28/B7 mediated costimulation can diminish GVHD responses especially when limited numbers of T cells are infused or recipients are not heavily irradiated. GVHD inhibition can be further optimized by preserving CTLA-4:B7 interactions and ensuring that sufficient numbers of donor and host $CD4^{+}25^{+}$ cells are available to down-regulate the GVHD response. These data illustrate the complex dynamics of a GVHD response as influenced by CD28/CTLA-4:B7 signaling processes.

Role of CD28/CTLA-4:B7 in Graft-versus-Leukemia (GVL)

Allogeneic donor T cells also have been shown to eliminate host leukemia cells in vivo. This process, known as a graft-versus-leukemia (GVL) effect, can be mediated either as a consequence of donor T cell recognition of MHC or minor histocompatibility antigenic disparities present on leukemia cells of host origin or via the generation of donor T cells that recognize leukemia antigens. As for GVHD responses, in vivo immunosuppressive agents or ex vivo donor T cell depletion can increase the likelihood of relapse post-BMT, as is most evident in patients with myeloid leukemia. Thus, although blockade of CD28/CTLA-4:B7 interactions can be advantageous for inhibiting GVHD dependent upon the experimental settings tested, donor anti-host alloresponses can have beneficial effects in preventing leuekmia relapse post-BMT. Potential mechanisms for this response include the expression of MHC and minor histocompatibility antigens on host leukemia cells, cross-presentation of leukemia antigens in the context of host APCs, or non-specific amplification of anti-leukemia T cell responses via the elaboration of soluble factors from bona fide alloreactive donor anti-host T cells. Therefore, it was not surprising that we observed a higher survival rate with B7-1 and B7-2 transduced leukemia cells as compared to control transduced leukemia cells in heavily irradiated, BM reconstituted BMT recipients.[95] Similarly, we found that anti-B7 mAbs completely eliminated the GVHD-side effects and the GVL response of delayed lymphocyte infusions (DLI) given later post-BMT to recipients of non-transduced leukemia cells.[95] Conversely, anti-CTLA-4 mAb-treated was strikingly advantageous in facilitating the GVL effects of donor lymphocyte infusions given later post-BMT, albeit GVHD also was augmented.[90] These data, along with the previously discussed observations that $CD28^{-/-}$ recipients are more susceptible to GVHD-induced lethality by DLI than wild-type mice,[93] interject a note of caution in the purposeful blockade of CD28/B7 interactions, especially during the time period of donor lymphocyte infusions later post-BMT. These data are important since DLI has become a mainstay for the treatment of certain types of leukemia relapse post-BMT.

Role of CD28/CTLA-4:B7 in Alloengraftment

The importance of the CD28/CTLA-4:B7 pathway on inducing optimal GVHD and GVL responses suggested that blockade of this pathway could be exploited to dampen host anti-donor alloreactive T cell responses which can result in graft rejection. Host T cells can

recognize donor MHC and minor histocompatibility antigens present on donor bone marrow cells. When this occurs, the bone marrow cells are eliminated by the host and the recipient is left either with no bone marrow cells or with a return of their own cells. Therefore, investigators have developed preclinical rodent models to study approaches that would facilitate alloengraftment. Most of these rodent models involve the use of low doses of total body irradiation (TBI). Recently, investigators have focused upon very low doses of TBI which will not ablate either the immune or hematopoietic system and result in a high propensity toward host cell recovery. In this section, we will concentrate upon the consequences of regulating the CD28/CTLA-4:B7 interactions on donor bone marrow engraftment.

Sykes and colleagues have used low dose TBI to condition recipients that then received conventional doses of allogeneic bone marrow. This platform results in host cell recovery without evidence of donor cell engraftment. These investigators showed that the inclusion of two peri-BMT doses of CTLA4-Ig given along with anti-CD40L mAb will induce stable chimerism in the vast majority of mice averaging > 40% donor cells in all lineages.[96] However, the same protocol without anti-CD40L mAb did not induce chimerism in any animal at any time post-BMT. Low dose TBI could be eliminated if a high doses of bone marrow cells was given at high doses.[97] Only transient chimerism was detectable if CTLA4-Ig was not given. In other studies using low dose TBI and conventional bone marrow cell doses, we have shown that anti-CD40L mAb is highly effective in promoting alloengraftment, in contrast to anti-B7 mAbs used at doses and schedules that are inhibitory to GVHD generation.[98] Low dose TBI and anti-B7 mAbs alone was inadequate to promote alloengraftment. Conversely, $CD28^{-/-}$ recipients treated with low dose TBI did not engraft better than wild-type recipients. Moreover, anti-CD40L mAb was ineffective in promoting alloengraftment in $CD28^{-/-}$ mice. These latter data could be consistent either with a lack of host $CD4^{+}25^{+}$ cells to downregulate the graft rejection process, greater dependency upon other costimulatory pathways (e.g., CD40L:CD40) for graft rejection, or the fact that $CD28^{-/-}$ T cells do not receive sufficient signals to be actively induced to become tolerigenic. Nonetheless, taken together, these data indicate that although CD28/CTLA-4:B7 blockade alone is ineffective in promoting alloengraftment, blockade of this pathway can act in concert with anti-CD40L mAb to facilitate alloengraftment. The reasons for the relatively low efficacy of CD28/CTLA-4:B7 blockade on impairing the host anti-donor graft rejection process are unknown.

Because CTLA4-Ig and anti-B7 mAbs block both CD28/B7 and CTLA-4/B7 pathways, we analyzed the engraftment effects of anti-CTLA-4 mAb in the context of sublethal but higher dose TBI.[90] In two different systems, anti-CTLA-4 mAb enhanced the rejection of allogeneic T cell-depleted marrow. Moreover, anti-CTLA-4 mAb abrogated the engraftment promoting effects of anti-CD40L mAb, consistent with reports indicating that CTLA-4/B7 interactions are critical for tolerance induction. Thus, the selective and transient blockade of CD28/B7 interactions early post-BMT, which preserve CTLA-4:B7 interactions, would be preferable to blocking both pathways. Alternatively, the infusion of host $CD4^{+}25^{+}$ cells in the peri-BMT would represent a new approach that may serve to down-regulate host anti-donor T cell responses in vivo, leading to improved allograft acceptance.

Future Directions

Much work still is required to determine treatment protocols that will permit antigen-specific tolerance in humans. As additional cell surface, intracellular signaling and molecular targets related to the B7 family are described, strategies to block costimulatory pathway responses will become increasingly sophisticated. In addition, the field is only starting to understand how currently used immunosuppressive drugs might best be combined with agents targeting the B7 family. However, as this section demonstrates, a great deal of progress has been made in prolonging graft survival using agents that target the B7 family and its receptors.

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Chapter 10

B7 Family of Costimulatory Molecules in the Induction and Regulation of Autoimmunity

Claudia Jabs, Arlene H. Sharpe and Vijay K. Kuchroo

Introduction

Normally, most autoreactive T cells are deleted during thymic development. The autoreactive T cells, which escape thymic deletion are kept in check by the mechanisms of peripheral tolerance, which include clonal anergy and active suppression. Autoimmune diseases do not occur until there is appropriate activation, expansion and differentiation of autoreactive T cells in the peripheral immune compartment. In the organ specific autoimmune diseases like multiple sclerosis (MS) and type 1 diabetes, the T cells have to migrate across the blood brain barrier/blood tissue barrier to access the target organ. In the target tissues the autoreactive T cells have to be further activated by the local antigen presenting cells (APCs) to initiate inflammation and mediate tissue injury.[1]

Consistent with the two-signal model, autoreactive T cells in the periphery do not become activated until they encounter antigen (either self or mimicry non-self peptide) in the context of relevant MHC molecules, together with appropriate costimulatory signals (as discussed below). Activation of T cells is important at several stages of induction of an autoimmune disease, including stimulation and clonal expansion within peripheral lymphoid tissues, entry and reactivation of autoreactive T cells into the target tissue to initiate tissue destruction within tissue parenchyma of the target tissue (for a review, see ref. 2). Therefore, expression of appropriate costimulatory molecules on the antigen presenting cells in the peripheral immune compartment and also at the target tissue site is essential for the induction and effector functions of the autoreactive T cells and induction of autoimmune disease.

The interactions between a number of ligands expressed on APCs and their receptors expressed on T cells appear capable of providing a costimulatory signal[3] for T cell activation and expansion. However, the critical importance of the B7-CD28 pathway is underscored by its unique capacity to induce IL-2 production, expand and differentiate T cells and prevent the induction of anergy.[4-6] Signaling through the B7-1/B7-2:CD28/CTLA4 costimulatory pathway is complex due to dual specificity of the two B7 family members, B7-1[7-11] and B7-2 [12-14] and two CD28 family members, CD28[15] and CTLA4,[16] providing the potential for redundancy in the interactions between B7 and CD28 family members. An additional level of complexity is provided by the opposing outcomes of the CD28 and CTLA4 mediated signaling.[17,18]

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Whereas CD28 provides a positive signal for T cell activation and expansion, CTLA4 (cytotoxic T lymphocyte antigen-4) induces a negative signal into T cells. In addition to the B7-CD28/CTLA4 pathway, in the last two years a number of molecules with homology to B7 or CD28 have been cloned and shown to either induce or inhibit T cell activation or differentiation. Two new pathways have been delineated recently, ICOS-ICOS-L and PD-1- PD ligand pathways.

This chapter focuses on recent advances in our understanding of the role of B7 family molecules in the development of autoimmune diseases. The published reports suggest that costimulation of autoreactive T cells is important not only in the experimentally antigen-induced models of autoimmunity (like autoimmune encephalomyelitis) but also spontaneous models of autoimmunity like type 1 diabetes in the NOD mice. This is further substantiated by genetic analysis of susceptibility/resistance to a number of experimental autoimmune diseases in animals and human autoimmune diseases showing that susceptibility to autoimmune diseases is linked to genetic intervals that encompass B7-costimulatory receptors and ligands.[19,20]

Interestingly, the role of costimulation in the induction or inhibition of the two autoimmune disease models (spontaneous and induced) in not always the same, thus providing an important basis for understanding the role of costimulatory molecules in the induction and regulation in induced vs. spontaneous autoimmune diseases. For this reason, the chapter will summarize and compare the role of costimulation in experimental autoimmune encephalomyelitis (EAE) an antigen-induced autoimmune disease and insulin-dependent diabetes mellitus (IDDM) a spontaneous autoimmune disease in NOD mice.

EAE and IDDM

EAE is a chronic inflammatory demyelinating disease of the Central Nervous System (CNS) and has been studied as an animal model for human Multiple Sclerosis (MS). EAE can be induced in several susceptible mouse strains by immunization with myelin antigens in a strong adjuvant like CFA or by the transfer of myelin-specific $CD4^+$ Th1 cells. EAE can be induced in animals from different genetic backgrounds including SJL, PLSJLF1 and C57BL/6 by immunization with different myelin-derived components like Myelin Proteolipid protein (PLP), Myelin Basic Protein (MBP) or Myelin Oligodendrocyte Glycoprotein (MOG) peptides resulting in different clinical disease phenotypes. While PLP peptide induces a relapsing-remitting disease in the SJL mice, immunization with MOG peptide induces a chronic or chronic- progressive disease in the C57Bl/6 mice, providing clinical equivalent of various forms of human MS. Several different T cell receptor (TcR) transgenic mice have been generated that express TcRs for myelin antigens like MBP and PLP and these mice do develop a spontaneous autoimmunity of the CNS. The role of various costimulatory pathways in the induction of spontaneous forms of EAE in the transgenic mice has not been studied.

IDDM in the nonobese diabetic (NOD) mouse is a mouse model for human type 1 diabetes. IDDM/Autoimmune diabetes is a highly regulated autoimmune disease targeting the pancreatic islet β cells and leading to hyperglycemia. In contrast to EAE, IDDM develops spontaneously in NOD mice and is mediated by both $CD4^+$ and $CD8^+$ T cells specific for pancreatic autoantigens. NOD mice develop diabetes by 10-20 weeks of age, preceded by a period of peri-insulitis without clinical disease, which begins at 2-4 weeks of age. The relative importance of costimulation seems to differ in the two models. Blocking the B7-CD28 pathway, for example, can protect from EAE[21,22] whereas IDDM is exacerbated.[23] Manipulating costimulatory pathways may therefore have very different effects depending on the model of autoimmune disease. Understanding the roles of different costimulatory pathways in different models of autoimmune disease therefore may provide a more comprehensive understanding of the requirement for costimulation in the development of autoimmune disease.

Manipulation of B7 Costimulatory Pathway

The first evidence for an important role of the B7-CD28/CTLA4 pathway in autoimmunity came from studies with CTLA4Ig, a fusion protein that binds to both B7-1 and B7-2 and blocks B7-1 and B7-2 interactions with its counter-receptors CD28 and CTLA4.[24,25] In EAE, when CTLA4Ig is given at the time of immunization,[22,26,27] shortly after immunization[28] or when MBP-specific T cells are restimulated in vitro in the presence of CTLA4Ig before adoptive transfer,[22] the development of EAE can be prevented or ameliorated. However, when activated MBP-reactive T cells are adoptively transferred and the recipient is treated with CTLA4Ig, EAE is not inhibited.[22] In addition, treating mice with CTLA4Ig at onset of clinical signs[29] results in exacerbation of EAE. Therefore, blocking the B7-CD28/CTLA4 pathway by administration of CTLA4Ig at different stages of EAE results in different outcomes, which may indicate different roles of the costimulatory molecules at different stages of disease. Like in EAE, the effect of CTLA4Ig in treatment of diabetes in NOD mice varied depending upon the timing of administration of the reagent. When NOD mice were treated early in their life (2-3 weeks of age) with CTLA4Ig, they were protected from the development of diabetes. In contrast, CTLA4Ig treatment had no effect on the development or progression of diabetes when CTLA4Ig was administered at the onset of diabetes (10 weeks of age).[30] These studies suggest that the timing of B7 blockade by CTLA4Ig administration have different effects on the clinical outcome of the disease. Whereas blocking B7-costimulation at the induction of disease is effective in inhibiting disease, perhaps by blocking induction of autoreactive T cells, B7-blockade by CTLA4Ig administration however is not effective in inhibiting disease induced by previously activated autoreactive T cells.

Since CTLA4Ig can effectively bind to both B7-1 and B7-2, these results raised the question whether differential blockade of either ligand B7-1 or B7-2 independently might affect the development of autoimmune diseases differently. In vivo studies with anti-B7-1 and anti-B7-2 mAbs suggest that B7-1 and B7-2 can differentially regulate autoimmune responses. Anti-B7-1 mAbs administered at the time of immunization have been shown to reduce the incidence and severity of acute EAE, whereas anti-B7-2 mAb therapy either increased or had no effect on disease severity.[27,31,32] In vivo administration of both B7-1 and B7-2 antibodies together had no effect on amelioration of EAE.[31] In EAE and MS, B7-1 has been shown to become a dominant costimulatory molecule and it is possible that the blocking effects of anti-B7-1 antibody in EAE might be due to this reason.[32,33] In searching for the mechanism by which B7-1 and B7-2 could have such contrasting effects, several studies suggested that B7-1 and B7-2 molecules differentially affect Th cell differentiation. It was clear from our studies that anti-B7 antibodies did not significantly inhibit the induction of T cells to the immunizing antigen but altered the cytokine profile of the responding T cells.[31] These studies suggested that administration of anti-B7-1 antibody in vivo at the time of immunization resulted in predominant generation of Th2 clones whose transfer both prevented induction of EAE and abrogated established disease. Since EAE is thought to be mediated by Th1-type cytokines,[34] these results suggest that anti-B7-1 mAbs block the development of Th1 T cells and consequently the progression of disease, while anti-B7-2 mAbs block the development of protective Th2 cells[31] resulting in more severe disease. More recent studies suggest that the expression of B7-1 on the T cells actually regulates IL-4 production from the T cells in that the loss of B7-1 from the T cells (in the B7-1 deficient mice) leads to a preferential development of IL-4 producing Th2 cells.[35] We and others have previously shown that activated T cells express B7-1 on their surface[36,37] and the blocking effect of anti-B7-1 antibody might be due to its effects on blocking B7-1 on the surface of T cells rather than APCs.

In contrast to the EAE studies, in the NOD mouse model, anti-B7-2 antibody treatment at the time of insulitis blocked the development of disease (which is also a Th1 dependent autoimmune disease), whereas anti-B7-1 treatment exacerbated diabetes in the female NOD mice and even induced disease in the male NOD mice that are normally resistant to diabetes.[30] EAE in SJL mice and diabetes data in the NOD mice are paradoxical. This may relate to the spontaneous vs. induced models of autoimmunity or the difference in the effector cells used in the two models. It is also possible that some of the differences might be, instead of blocking interactions with their receptors, anti-B7 mAbs could have activating properties and lead to signaling into the T cell or the APC. Another caveat is that the antibodies or CTLA4Ig might not be able to penetrate the blood-brain barrier and that might be responsible for the differential effects observed in the two disease models.

To directly address these issues, we and others have bred B7-1 and B7-2 deficient mice on several different autoimmune susceptible backgrounds including B6, SJL and NOD mice. Using B7-1, B7-2 and B7-1/B7-2 double deficient mice to further study the relative importance of B7-1 vs. B7-2 costimulation in the induction of EAE, we first tested B7 deficient mice on the B6 background.[21] The B7-1 or B7-2 deficient mice were equally susceptible to active EAE on the B6 background following immunization with MOG^{35-55} peptide. In contrast, we found that the mice lacking both B7-1 and B7-2 or CD28 are highly resistant to EAE induction by active immunization or by adoptive transfer of encephalitogenic T cells. Whereas the wild-type mice developed a significant inflammation and demyelination in the CNS, the B7-1/B7-2 double deficient mice had the inflammatory cells restricted to meninges consistent with a picture observed with the $CD28^{-/-}$ mice.[21,38,39] These studies suggest that in B6 background, B7-costimulation is not only important in the induction phase of the disease but also in the effector phase as it appears to be crucial for reactivation, survival and or/expansion of effector cells in the target organ. However, $CD28^{-/-}$ mice have been shown to be susceptible to EAE induction by either adoptive transfer of primed T cells or by double-immunization, demonstrating that the lack of CD28 can be overcome by increasing the dose of immunizing encephalitogenic antigen.[39-41] It should be noted though that effects seen in the B7-1/$B7\text{-}2^{-/-}$ mice are not always equivalent to $CD28^{-/-}$ mice as has been highlighted by transplant studies.[42]

We have also tested EAE susceptibility of B7-1/B7-2 double deficient mice on the SJL background following immunization with the PLP 139-151 peptide. In contrast to the B6 B7-1/B7-2 double deficient mice, the B7-1/B7-2 double deficient on the SJL background remained susceptible to EAE.[43] This difference in EAE-susceptibility in the B7-1/B7-2 double deficient mice on the two backgrounds could not be attributed to changes in expansion of Th cells or the cytokine profile. The difference in the susceptibility in the B7-1/B7-2 double deficient mice on the two backgrounds was not due to different immunogens used for elicitation of EAE but appears to be due to the genetic differences. This data is further substantiated by testing B7-deficient mice on NOD background for the development of EAE. The blockade of the B7-CD28 pathway on the NOD background by using either $CD28^{-/-}$ mice or treating wild-type mice with anti-B7-1/B7-2 Abs results in significantly reduced EAE severity.[39] The myelin peptide-specific T cells were shown to be effectively primed, however the peptide-specific delayed-type hypersensitivity responses were significantly decreased, suggesting a critical role for CD28 costimulation in in vivo trafficking and systemic immunity.[39] Thus difference in susceptibility of B7-1/B7-2 deficient mice to EAE on the three different genetic backgrounds suggests that requirement for B7 costimulation to induce autoimmunity will vary depending upon the genetic background of the individual. This conclusion is particularly important from the point of view of B7-based therapies that are currently being tested in humans.

CD28$^{-/-}$ mice and CTLA4Ig transgenic mice on the NOD background demonstrate that the loss of B7-CD28 costimulation promotes the development and progression of spontaneous autoimmune diabetes,[23] a result very different from the effect of CD28 deficiency in the induction of EAE. Functional analyses of T cells isolated from CD28$^{-/-}$ mice have demonstrated that the B7-CD28 pathway affects Th1 versus Th2 differentiation and cytokine secretion, thereby affecting the outcome of disease. The CD28$^{-/-}$ and CTLA4Ig transgenic mice on the NOD background showed a significant skewing towards a Th1 phenotype and thus promoted induction of a more severe spontaneous diabetes. In addition, B7-1/B7-2$^{-/-}$ NOD mice were more susceptible to IDDM than wild-type controls, although in this case a skewing towards a Th1 phenotype does not seem to be responsible for a more severe spontaneous diabetes.[44] However, B7-1/B7-2$^{-/-}$ NOD mice were shown be more susceptible to IDDM because they are severely deficient in CD4$^+$CD25$^+$ regulatory T cells[44] and adoptive transfer of wild-type regulatory cells into the B7-1/B7-2$^{-/-}$ NOD mice ameliorated the development of spontaneous disease. It should be noted that CD4$^+$CD25$^+$ cells in B7-1/B7-2$^{-/-}$ mice on the B6 background are reduced yet we do not see any enhanced EAE in these mice (unpublished data). The increased susceptibility of CD28$^{-/-}$, B7-1/B7-2$^{-/-}$ and CTLA4tg NOD mice to IDDM suggests that B7-CD28 signaling is not required for the development of autopathogenic diabetogenic T cells. Instead, B7-CD28 interactions in the NOD mice may modulate IDDM susceptibility, possibly by regulating Th1/Th2 differentiation and/or by affecting the regulatory T cell population. The effect of loss of B7-1 or B7-2 on the NOD background in the development of diabetes has not been analyzed in detail, but recent studies suggest that B7-2$^{-/-}$ mice on the NOD background were protected from diabetes, but developed a spontaneous autoimmune peripheral polyneuropathy,[45] very similar to the human disease chronic inflammatory demyelinating polyneuropathy (CIDP). Affected mice exhibited limb paralysis and severe demyelination in the peripheral nerves, but no lesions in the central nervous system. Polyneuropathy could be transferred to NOD.SCID mice by transferring CD4$^+$ T cells isolated from affected animals.[45] Thus, blocking the CD28-B7 pathway had a severe impact on the susceptibility/resistance of autoimmune diseases in NOD mice.

Blocking Negative Costimulatory Signals in Autoimmune Diseases

CTLA4 has now been shown to induce a negative signal into T cells. CTLA4 deficient mice develop a severe lymphoproliferative disorder and die at a young age due to multi-organ autoimmune disease.[46,47] Inhibition of the negative costimulatory molecule CTLA4 by antibody blockade led to exacerbation of both EAE and diabetes. This contrasts with the opposing effects of blocking CD28/B7-1/B7-2 costimulation in EAE and diabetes. Whereas the absence of CD28 and B7-1/B7-2 molecules leads to amelioration of EAE, the loss of these molecules led to the exacerbation of spontaneous diabetes in the NOD mice. Several laboratories reported that in vivo administration of anti-CTLA4 antibody exacerbated EAE induced either by active immunization or by adoptive transfer of encephalitogenic T cells.[29,48-50] Administration of anti-CTLA4 at the onset of clinical EAE increased mortality and enhanced subsequent relapses by increasing epitope spreading, clearly supporting the role of CTLA4 in downregulating autoreactive T cells and inhibiting autoimmune reactions. Similarly CTLA4 has been shown to have a downregulatory role in IDDM. When NOD BDC2.5 TcR transgenic mice, in which T cells recognize an unidentified pancreatic autoantigen, were treated with anti-CTLA4 antibody, the transgenic mice developed rapid diabetes with a more aggressive pancreatic infiltrate.[51] However, the timing of anti-CTLA4 administration was crucial for this outcome. If antibody was administered at the initiation of insulitis, the anti-CTLA4 antibody had no effect on the disease progression. However, administration of anti-CTLA-4 accelerated diabetes when given before the onset of insulitis. This suggests that there might be a time window during an

autoimmune reaction when CTLA4 must exert its effect in inhibiting an autoimmune disease in vivo.

Besides its role in inhibiting T cell activation, CTLA4 has also been shown to play a role in tolerance induction by inducing T cell anergy or induction of regulatory T cells that produce TGF-β. Furthermore, more recent studies suggest that preferential ligation of CTLA4 by B7s may induce T cell unresponsiveness.[52] Studies with CTLA4$^{-/-}$ T cells have shown that CTLA4 has an essential role in determining the outcome of T cell encounter with a tolerogenic stimulus.[53] Following exposure to a tolerogenic stimulus, CTLA4$^{-/-}$ T cells but not wild-type T cells were able to proliferate and produce IL-2. In contrast to wild-type T cells which become tolerant and were blocked in the late G1 to S restriction point of the cell cycle, CTLA4$^{-/-}$ T cells entered the S phase of the cell cycle, proliferated and produced effector cytokines. Thus, tolerance resistance in the CTLA4$^{-/-}$ T cells was shown to be related to a role for CTLA4 in regulating cell cycle progression.[53]

Recent studies suggest that CTLA4 might also be critical for the function of CD4$^+$CD25$^+$ regulatory T cells. The result that NOD B7-1/B7-2 deficient mice have increased incidence and severity of autoimmune diabetes, which can be corrected by adoptive transfer of CD4$^+$CD25$^+$ cells,[44] further supports this notion that CTLA4 may play a crucial role in the generation or regulating function of CD4$^+$CD25$^+$ regulatory T cells. In addition, CD4$^+$CD25$^+$ regulatory cells express CTLA4 on their surface.[54,55] Therefore, CTLA4 may exert its downregulatory effects on autoimmunity by multiple mechanisms, including inhibition of T cell activation, induction of anergy, generation of CD4$^+$CD25$^+$ regulatory T cells and production of immuno-suppressive cytokines that may inhibit induction or progression of an autoimmune disease.

ICOS-B7h Costimulation in Autoimmune Diseases

Like CD28, ICOS has positive costimulatory activity enhancing cytokine production, upregulation of CD40L expression and help for Ig production by B cells. However, it has several properties that are distinct from CD28 that make it a particularly interesting molecule. Whereas CD28 is constitutively expressed, ICOS expression is inducible. ICOS expression is stimulated by TcR and CD28. Although it was initially thought that ICOS costimulation was important for Th2 cytokine production, recent studies indicate that ICOS also regulates Th1 cytokine production. In contrast to CD28, ICOS can stimulate IL-10 production, a key immunoregulatory cytokine. Thus ICOS costimulation could impact autoimmunity by regulating effector cytokine production and/or production of IL-10.

Several studies suggest a crucial role for this pathway in autoimmunity. ICOS deficient mice on the 129xB6 mixed background show exacerbated MOG 35-55-induced EAE as compared to wild-type littermate controls.[56] Exacerbation of EAE can be demonstrated by higher severity of clinical disease, more inflammatory foci in the brain, and more CD4$^+$ T cells in the CNS producing IFNγ. The increased severity of EAE in the ICOS$^{-/-}$ mice was attributed to a decrease in IL-13 and increase in IFNγ production by the responding T cells. In vivo blockade of ICOS-B7h pathway by administration of anti-ICOS antibody[57] or ICOS-Ig[58] further supported these studies.

However, more recent studies indicate that blockade of the ICOS-B7h pathway at different stages of disease might have different outcomes. Treating mice during the induction phase of EAE seems to result in exacerbation of disease whereas treatment during the effector phase seems to result in amelioration of disease.[57] Although the mechanism for this diverse effect was not explored, the blockade of B7h-ICOS pathway at the induction of EAE affects T cell differentiation and promotes generation of autopathogenic, IFNγ producing Th1 cells. However, administration of anti-ICOS antibody in the effector phase of the disease might delete or inhibit effector functions of the previously activated autopathogenic T cells.

Blocking the ICOS-B7h pathway with ICOS-Ig[58] during the in vitro activation of MBP-reactive transgenic $CD4^+$ T cells inhibited the ability of these T cells to transfer EAE. Similarly, ICOS-Ig treatment of mice after the onset of EAE ameliorated clinical disease. ICOS-Ig was found to increase apoptosis in the transgenic T cells, particularly effecting the memory population. ICOS-Ig did not prevent IL-2 production, but suppressed IFNγ and IL-10 production. Therefore signaling through the ICOS-B7h pathway seems to promote activation and viability of previously activated encephalitogenic T cells, supporting the anti-ICOS antibody data.[57]

These observations provide a mean by which ligation of ICOS on effector T cells could be used to treat an autoimmune disease.

PD-1:PD-L1/PD-L2 Pathway and Autoimmunity

The newest pathway in the B7-CD28 family consists of PD-L1 (B7-H1) and PD-L2 (B7-DC) that bind to PD-1. In contrast to other CD28 family members, PD-1 is not only expressed on T cells but also on myeloid and B cells. Its ligands, PD-L1 and PD-L2 are also not only expressed on professional antigen presenting cells but also on non-lymphoid tissue. PD-L1 and PD-L2 have distinct expression patterns. PD-L1 is expressed on T cells, B cells, myeloid DCs, heart, fetal liver and placenta and is IFNγ -induced.[59,60] PD-L2 is expressed on DCs, heart, lung, placenta, liver and pancreas and is induced by IL-4 (reviewed in ref 61).

A role for PD-1 in regulating tolerance is indicated by the phenotype of PD-1 deficient mice. Aged $PD\text{-}1^{-/-}$ mice develop a spontaneous lupus like autoimmune disease on C57Bl/6 background and autoimmune dilated cardiomyopathy on the Balb/c background resulting in heart failure and sudden death.[63] The $PD\text{-}1^{-/-}$ mice show significantly augmented serum levels of IgG2b, IgGA and IgG3 and a reduction of CD5 on B-1 cells, a molecule known to negatively regulate B cell responses, suggesting that the PD-1/PD-L pathway may not only affect T cell activation but may also regulate certain aspects of B cell activation and differentiation. Since PD-1 deficient mice develop autoimmune disease of peripheral organs and since PD-L1 and PD-L2 are expressed on the parenchymal cells of various organs, this suggests that the PD-1/PD-L pathway might provide a negative signal to T cells and suggests a mechanism by which tissue tolerance is maintained. This provides a unique mechanism for the induction of T cell tolerance in peripheral tissues and prevention of organ specific autoimmune diseases. Since PD-1 is expressed on B cells and T cells, PD-1 may regulate both B and T cell tolerance. It is not clear whether the B cell defects are secondary to T cell defects or whether both B and T cells are primarily affected because of loss of PD-1. No studies have yet been reported on the consequence of manipulating this pathway in various models of autoimmunity.

Since the first description of the B7 family members and the identification of their role in T cell costimulation, the field has become complex with the identification of newer members of the family and potential for both positive and negative signaling within and outside the immune system. Thus the members of the B7-family not only are important in enhancement and inhibition of T and B cell responses, but the expression of their ligands in the parenchyma of the peripheral tissues provide a way of regulating autoimmune diseases and inducing and/or maintaining tissue tolerance. The new pathways may provide avenues for the inhibition of inappropriate activation or effector functions of autoreactive T cells and provide new drug targets for the regulation and treatment of autoimmune diseases.

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Chapter 11

Operation Enduring Costimulation:

Modulation of B7 Receptors to Elicit Anti-Tumor Immunity

Eugene D. Kwon, Qingyong Ji and Arthur A. Hurwitz

In the war against the evil-doer tumor, the battle lines are clearly drawn. Marshalling the T cell infantry can be a powerful way to neutralize the enemy. The weaponry used by the assaulting immunoforces has been the focus of strategic planning for the past decade. The identification of the triggers that launch T cell-mediated immunity to a variety of tumors has led to considerable enthusiasm about the possibility of a successful attack on the evil neoplasm. Our understanding of the role of the critical costimulatory interactions during the activation of T cells has contributed to the development of many promising strategies for recruiting T cells to the on-going war on cancer.

As described elsewhere, T cell activation requires at least two distinct signals resulting from interactions with antigen presenting cells (APCs). The first signal is antigen-specific, mediated by T cell receptor (TcR) occupancy by antigen/major histocompatibiliy complex (MHC) molecules. In the context of tumor immunity, this refers to recognition of a 'tumor antigen' at both the priming and effector stages. Also, to be destroyed by effector T cells, tumors must express detectable MHC. The second signal is antigen-independent and is referred to as a costimulatory signal. Initially, this signal was identified as CD28 ligation by B7-1 (CD80). Subsequently, B7-2 (CD86) was identified as an additional CD28 ligand and more recently, additional members of the CD80 and CD86 family have been reported. In general, expression of CD80 and CD86 (hereafter collectively referred to as B7) is largely restricted to professional APC's (dendritic cells, macrophages, and B cells), and rarely expressed by tumor cells.

Turning Up the Offensive

Most tumor cells do not express costimulatory ligands and as such, are incapable of priming T cells and thus may evade the immune system.[1] Based on this, it was hypothesized that conferral of B7 expression by gene transfer may be a useful way to enhance tumor immunity. In support of this, the cavalry of Chen and Allison successfully demonstrated that transduction of a weakly immunogenic melanoma (K1735) with B7-1 was sufficient to promote regression in immunocompetent mice.[2,3] In these studies, it was also demonstrated that tumor regression was dependent on $CD8^+$ cells and further, that mice rejecting the $B7^+$ tumor were immune to rechallenge with the unmodified or 'parental' tumors. Taken together, these findings suggested that the B7-expressing tumor may function as an APC and directly prime the T cells, by-passing the need for host-derived professional APCs. However, a more complex story was later revealed (see below). These original results were followed by many other studies that demonstrated that tumors of different tissue origins could be modified to express B7-1 (or later, B7-2) and this modification was sufficient to promote their regression (reviewed in ref. 4). Paradoxically, it

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was suggested that B7-2 may antagonize the effects of B7-1 in tumors expressing both costimulatory ligands.[5,6] However, to date, this has not been confirmed in tumors of other tissue origin. Although some studies have suggested that differences exist in the effectiveness between B7-1 and B7-2 to promote an anti-tumor response,[7,8] at present, no consensus has been reached.

Initial reports demonstrating that T cell immunity to tumors can be enhanced by B7 gene transfer were met with excitement. However, it is now apparent that the success of this approach is limited to tumors exhibiting some degree of inherent immunogenicity.[9] For instance, tumors such as the poorly immunogenic B16 melanoma and its sublines were refractory to the effects of B7 transduction whereas more immunogenic murine tumors such as K1735, the EL4 thymoma, and the P815 mastocytoma lines were susceptible to B7-mediated regression. Even more discouraging was the observation that the B7-expressing tumors typically did not elicit a robust therapeutic response. Many studies have since demonstrated that B7-expressing tumor cell vaccines were useful to treat recently established, immunogenic tumors, but that $B7^+$ tumor cell vaccination was insufficient against more established tumors (i.e., those with a diameter greater than 5 mm).[10] Only tumors that co-expressed B7 and another immunostimulatory antigen (e.g., class II MHC[11]) could elicit a more potent, curative anti-tumor response.

Subsequent to the observation that $B7^+$ tumors potentiate anti-tumor immunity, a greater understanding of the mechanism by which this occurs has evolved. The infantry of Levitsky and Pardoll demonstrated that the mechanism by which B7-1-transduced tumors enhanced the immunogenicity of tumors was not as simple as just converting the tumor into an APC. In one report, Wu et al used the poorly immunogenic $B16/F_{10}$ melanoma tumor to demonstrate that immunity elicited by the B7-transduced B16 line was dependent on both $CD8^+$ T cells and NK cells.[12] Unlike the previous studies using more immunogenic tumors, sensitization to the B7-1-expressing B16 cells, which express low levels of MHC I, did not confer immunity to rechallenge with the parental tumors. The idea that B7-expressing tumors could recruit an NK response is consistent with the findings that murine NK cells express CD28 and that triggering CD28 via B7 can enhance NK cell responsiveness.[13,14] However, the clinical relevance of this is not clear as expression of CD28 by human NK remains controversial.[15-17] Interestingly, potentiation of antitumoral immunity by 4-1BB ligand—another costimulatory molecule from the TNF-receptor family that costimulates $CD8^+$ T cell activation or anti-4-1BB FV—have similarly been reported to be NK cell-dependent.[18,19]

In a second report, Levitsky and colleagues demonstrated that vaccination with a $B7^+$ tumor elicited immunity through a mechanism that was dependent on antigen transfer to host-derived APC's (a.k.a., cross-presentation or cross-priming).[20] This finding was in contrast to previous suggestions that conferring B7 expression to a tumor converted it into an APC. Using the 'moderately' immunogenic CT26 colorectal carcinoma transduced to express B7-1 and a strongly immunogenic viral antigen (influenza NP), Huang et al demonstrated that priming of NP-specific (and presumably tumor-specific) T cells was principally mediated by host-derived bone marrow cells and not by the B7-1-expressing CT26 cells. It was only after repeated immunization that T cells were shown to be primed against the tumor-derived antigen, suggesting that antigen transfer, and thus cross-priming, is the more efficient method of priming tumor-specific T cells.

Thus, depending on the inherent immunogenicity of the tumor, two possible mechanisms for priming by $B7^+$ tumors may exist. For poorly immunogenic tumors that express limited class I MHC, B7 may trigger NK cells to elicit tumor cytolysis. NK-mediated lysis may initiate the cross-presentation pathway which may be followed by recruitment of a $CD8^+$ T cell response. These two responses may not be sufficient to confer long-lasting immunity to a poorly immunogenic tumor. In contrast, B7-expression by more immunogenic tumors may further enhance the immunogenicity of the tumor and facilitate cross-presentation of

tumor-associated antigens. This may, in turn, elicit a stronger and more persistent memory T cell response. In either case, it is clear from these and other studies that the effects of endowing tumors with B7 expression extend beyond simply converting the tumor into a more potent APC that can directly trigger T cells.

An Alliance of Mediators

In many tumor models, conferral of B7 expression was not sufficient to induce a potent anti-tumor immune response. In general, success of B7-mediated regression was correlated with the inherent immunogenicity of the tumor. To bolster induction of tumor immunity, several groups have explored co-expression of B7 with immunostimulatory cytokines. In general, most of these studies have employed the use of T_H1-associated cytokines, reflecting the belief that eliciting T_H1 responses provides a more favorable anti-tumor immune response. In support of this, one of the first reports demonstrated that B7-1 and IL-12 synergized to promote regression of poorly immunogenic tumors. In this study, IL-12 was either co-expressed by the tumor[21] or administered systemically.[22] Since a downstream effect of IL-12 secretion is interferon-γ (IFN-γ) production, it is not surprising that a subsequent report demonstrated synergy between B7-1 and IFN- γ as well.[23] Other cytokines that have been demonstrated to enhance the immunogenicity of $B7^+$ tumors include IL-2,[24] IL-7,[25] IL-10,[26] and GM-CSF.[27] In each of these studies, co-expression of B7 with the cytokine was sufficient to potentiate the anti-tumor immune response and induce immunity to the parental tumor.

Another approach to enhance the effects of B7-mediated gene transfer is based on the observation that a potent and curative anti-tumor response may be facilitated by provision of a tumor-specific $CD4^+$ T cell response.[28] Ostrand-Rosenberg and co-workers have waged the battle on tumors using vaccines that co-express class II MHC and B7 (reviewed in ref 29). Their studies demonstrated that co-expression of class II MHC and B7 (either B7-1 or B7-2) is not only sufficient to promote regression of primary tumors,[30,31] but also recently established tumors[11] and pulmonary metastases.[32] Such findings suggest that endowing a tumor with a more complete repertoire of ligands expressed by professional APCs (i.e., class II MHC and B7) renders it more immunogenic and thus more suitable as a vaccine.

The above studies support the idea that anti-tumor immune responses can frequently be enhanced by transfer of B7 expression to tumor cells. Most of the approaches reported thus far, however, rely on ex-vivo manipulations of tumor cells (often cell lines) as recipients of gene transfer. Clearly, such ex-vivo approaches are highly cumbersome, as individual tumors would need to be processed, cultured, and genetically modified. Thus, in vivo gene transfer would be a more favorable alternative. Consequently, great attention has been directed towaûås in situ methods to directly transfer both traditional as well as newly-discovered costimulatory molecules into tumors. Schlom, Hodge and their troops at the NCI have generated a pox vector (designated 'TRICOM') that encodes B7 and two other alternative costimulatory ligands, ICAM-1 and LFA-3. This vector was shown to elicit potent T cell responses both in vivo and in vitro.[33,34] Moreover, it was shown that this vector can be used alone or in combination with a tumor-associated antigen to provide potent T cell-mediated anti-tumor responses.[35] Given that in vivo gene transfer of B7-1 using this system did not elicit autoimmune responses,[36] this approach may have significant utility for in situ viral-mediated gene transfer of costimulatory ligands, representing a more practical and desirable approach for anti-tumor immunotherapy.

Another approach that eliminates the need for ex vivo gene transfer utilizes a soluble form of B7.[37] This construct ('B7-Ig') was capable of costimulating T cells in vitro and when administered to tumor-bearing mice, could facilitate regression of recently established tumors (7 day-old tumors) with as few as two doses. B7-Ig synergized with a cell-based vaccine, enhancing survival of mice challenged systemically with the poorly immunogenic B16/F10

Table 1. B7 family and its receptors in tumor immunity

Costimulatory Receptor	Ligand	Functional Outcome	Effect of Ligand on Tumorigenicity
CD28	B7-1/B7-2	STIM	+
CTLA-4	B7-1/B7-2	INHIB	BLOCKADE
ICOS	B7h	STIM	+
PD-1	B7-H1/PD-L2	INHIB	(BLOCKADE)
PD-1	B7-DC	STIM	(+)
?	B7-H3	STIM	(+)

?=unidentified role or receptor
STIM=stimulates T cell activation
INHIB=inhibits T cell activation
BLOCKADE=blocking inhibitory receptor to enhance tumor immunity
+=stimulates tumor immunity ( )=projected effect

variant. Interestingly, both B7-1 and B7-2 fusion proteins were capable of eliciting immunity and the anti-tumor response was only dependent on $CD8^+$ T cells. The mechanism by which these fusion proteins preferentially signal through CD28 and not CTLA-4, an inhibitory receptor for B7-1 and B7-2 (see below), is not clear. Nevertheless, they represent an attractive approach to overcome the inability of most tumors to provide costimulation for priming T cells.

Shutting Down the Defense

As described above, the use of B7-expressing tumor cells to elicit T cell-mediated immunity is based on the observation that ligation of the constitutively expressed CD28 is required for efficient T cell priming. Once activated, though, T cells express another B7 receptor termed cytotoxic T lymphocyte antigen-4 (CTLA-4). Although CTLA-4 is highly homologous to CD28 and was originally thought to deliver a redundant costimulatory signal, it is now well-appreciated that CTLA-4 delivers an inhibitory signal to T cells.[38] CTLA-4 binds B7-1 and B7-2 with 20 to100-fold greater affinity than CD28.[39] Studies by Allison and Bluestone showed that in vitro antibody crosslinking of CTLA-4 inhibits anti-CD3-induced T cell proliferation and IL-2 production, whereas blockade of CTLA-4 with soluble intact antibody or F_{ab} fragments enhances T cell proliferative responses.[40] Similarly, it was shown that in vivo administration of soluble anti-CTLA-4 antibody or Fab fragments (which block CTLA-4/B7 interactions) greatly augments T cell responses to nominal peptide antigen or Staphylococcal superantigens.[41,42] Finally, CTLA-4 knockout mice exhibit a severe lymphoproliferative disorder.[43,44] Together, these observations have implicated CTLA-4 as a critical inhibitory receptor that downregulates T cell responses.

In 1996, it was first reported that in vivo blockade of the inhibitory CTLA-4 receptor can be exploited to augment T cell-mediated anti-tumor responses.[45] Leach and colleagues showed that systemic administration of a monoclonal anti-CTLA-4 antibody accelerated the complete rejection of $B7^+$ colorectal carcinoma cells (51BLim10). Remarkably, CTLA-4 blockade was further shown to induce rapid rejection of unmodified 51BLim10 colorectal carcinoma tumors as well as SAI/N fibrosarcoma cells. Since, it has been shown that blocking inhibitory signaling caused by interaction of B7 with CTLA-4 can be used to trigger T cell-mediated rejection of a number of unmodified murine tumors.[46-49]

While the mechanism(s) whereby CTLA-4 blockade enhances anti-tumor immunity have not been fully elucidated, several points are clear. CTLA-4 blockade-mediated tumor regression typically occurs in a $CD8^+$ and sometimes $CD4^+$ T cell-dependent manner. Additionally, manipulations that boost host tumor antigen presentation can markedly enhance antitumoral responses raised during CTLA-4 blockade. Thus, CTLA-4 blockade has been shown to synergize with GM-CSF-transduced tumor cell vaccination to reduce tumor burden in several transplantable as well as primary tumor models.[47,50,51] Even more striking is the observation that in mice that reject B16 melanoma tumors following CTLA-4 blockade, a depigmentation of the hairs often ensues.[51] This finding is reminiscent of the vitiligo-like syndrome observed in melanoma patients that undergo immunotherapy.[52] Thus, reactivity following this sensitization scheme is not limited to anti-tumor immunity, but extends to autoimmunity against normal melanocytes. Similarly, CTLA-4 blockade combined with GM-CSF prostate tumor cell vaccination manifests in the induction of prostatitis in mice bearing primary prostatic tumors as well as wild-type male mice.[47] These findings are consistent with the observations that CTLA-4 blockade may permit modulation of tolerance to nominal antigen[53,54] and autoantigen.[55]

Given the observed synergy between CTLA-4 blockade and GM-CSF-expressing vaccines (that presumably enhance APC activation and antigen presentation), it is not surprising that activation of host APCs via the CD40-CD40 ligand pathway can also synergize with CTLA-4 blockade to induce tumor cell-specific CTL activity. In one study, the combination of anti-CD40 (agonstic) and anti-CTLA-4 (antagonsitic) administration resulted in marked increases in antigen-specific CTL activity following liposomal delivery of antigenic peptides. Moreover, responses elicited by vaccination with liposomal pRL1a, a tumor antigen, and co-administration of anti-CD40 and anti-CTLA-4 were sufficient to extend the survival of mice challenged with a pRL1a-expressing leukemia.[56]

Alternate strategies to extend the effectiveness of CTLA-4 blockade as an antitumoral therapy have included its use in combination with chemotherapy or surgery. Low dose melphalan administration followed immediately by CTLA-4 blockade has recently been shown to cause complete tumor regression, and improve survival of mice initially bearing small, recently established subcutaneous plasmacytoma tumors (MOPC-315).[57] The putative mechanism whereby low-dose melphalan enhanced the effectiveness of CTLA-4 blockade in these studies was initially attributed to the ability of melphalan to shift cytokine profiles within MOPC-tumors towards a pro-inflammatory, T_H1 environment favoring the generation of tumor-specific $CD8^+$ T cells. However, more recent studies have suggested that chemotherapeutics, including melphalan, and radiation can upregulate the expression of B7 by a variety of tumors.[58,59] The mechanism by which these treatments unexpectedly upregulate B7 expression by many non-hematopoietic tumors is not clear, although it was suggested that NFKB is involved.[60] However, these findings do suggest that the combination of chemotherapy and immunotherapy may yield some interesting synergistic effects.

Finally, it was also reported that CTLA-4 blockade can be effective as an adjunctive immunotherapy to eliminate established residual metastases following primary (transplantable) prostate tumor (TRAMPC2) resection.[48] Elimination of residual metastases following CTLA-4 blockade in this study resulted in increased disease-free survival, presumably consequent to an increase in the number of cytotoxic effector cells relative to tumor cell targets or relative reductions in tumor-induced impairments in host immune function. Collectively, the above studies underscore the potential of combining CTLA-4 blockade with other immunotherapeutic and/or standard treatments to improve the overall effectiveness of cancer therapy. Most importantly, given the difficulties associated with ex vivo manipulation of patient-derived tumor cells and that CTLA-4 blockade may obviate such requirements, CTLA-4 blockade constitutes a particularly practical approach suitable for clinical cancer treatment.

The New Breed…

More recently, new members of the B7 family have been identified. The first cloned B7 homologue was B7h,[61] later identified by the alternative names B7RP-1, B7H2, GL50 and LICOS.[62-65] B7h is constitutively expressed on professional antigen presentation cells such as B cells, macrophages, and dendritic cells. Low levels of B7h are also expressed on non-lymphoid tissues such as kidney, peritoneum, and testes but can be up-regulated by TNF-α or LPS. The receptor for B7h, the inducible costimulatory molecule (ICOS), is structurally similar to CD28 and CTLA-4. Unlike CD28, which is constitutively expressed on naïve T cells, ICOS is only expressed on activated T cells and resting memory T cells.[65] In normal mice, ICOS expression is mainly detected in the germinal centers and T cell zones of lymph nodes and Peyer's patches. Through ligation by B7h, ICOS co-stimulates the proliferation of $CD4^+$ cells and preferentially induces IL-4, IFN-γ, and IL-10 expression in vitro.[66] It can also costimulate $CD8^+$ T cells as well.[67,68] The importance of ICOS/B7h interactions is supported by studies demonstrating a critical role for ICOS in a variety of murine models of human autoimmune and inflammatory diseases.[69-71]

Two studies have examined the role of B7h in enhancing anti-tumor immunity. Liu et al found that in vivo expansion of tumor antigen (P1A)-specific T cells was enhanced when tumors expressed B7h.[67] B7h expression increased the immunogenicity of the tumor (J558 plasmacytoma) as demonstrated by a 40% reduction in tumor incidence. Interestingly, challenge with $B7h^+$ and $B7h^-$ tumor cells resulted in outgrowth of only $B7h^-$ tumors. In addition, it was proposed that B7h expression by the J558 tumor enhanced susceptibility of the tumor to CTLs and expanded tumor reactive T cells in a mechanism dependent on expression of B7-1 or B7-2 by host APC. These latter findings suggest a potentially important role for cross-priming in the initiation of the anti-tumor response in this model system.

In a separate report, Wallin et al extended these studies by demonstrating that B7h-expressing tumors could be rejected in a CD4-independent mechanism and that immunity to the parental (SAI/N) tumor was long-lasting.[68] Interestingly, using an in vitro assay, these authors showed that B7h was more effective at stimulating a secondary, recall response than a primary T cell response. They further showed that the secondary response was comparable to that stimulated by B7-2-expressing tumors, despite the suggestion that ICOS expression was greater on naïve cells undergoing primary stimulation. Taken together, these reports suggest that B7h may have an important role in both eliciting and potentiating anti-tumor responses.

B7-H1 (also reported as PD-L1) is another molecule exhibiting homology to B7-1 and B7-2.[72] B7-H1 binds to Programmed Death-1 (PD-1), an inhibitory receptor with homology to CTLA-4.[73] Despite the inhibitory role proposed for PD-1, B7-H1 has been suggested to have a costimulatory role in T cell activation. Chen and colleagues demonstrated that B7-H1-transfected COS cells costimulate T cell proliferation in an IL-2-dependent manner, but that the outcome is most notably detected by IL-10 production. Another report defines B7-H1 (therein referred to as PD-L1) as an inhibitory ligand.[73] A second PD-1 ligand, PD-L2, was recently identified by Freeman and Sharpe and also appears to deliver inhibitory signals to T cell PD-1.[74] B7-DC was also reported to be a PD-1 ligand in mice, yet it appears to deliver a strong, positive costimulatory signal.[75] B7-DC is the murine homolog of PD-L2. More recently, it was suggested that two different receptors for PD-L1 and PD-L2 may exist and these receptors may deliver opposing signals.

Although B7-DC may bind additional receptors on T cells besides PD-1, it is clear from these studies that the role of PD-1 in regulating T cell responses is quite complex. To date, no studies have reported using PD-L1, PD-L2, or B7-DC to modulate tumor immunity. However, the observation that several tumor lines express both PD-L1and PD-L2 suggests that

inhibitory signals delivered by these costimulatory ligands may contribute to escape from immune surveillance. Thus, it will be of considerable interest to study how this novel, yet complex costimulatory axis can be exploited to enhance T cell-mediated anti-tumor responses.

B7-H3 is another member of B7 family of costimulatory ligands that was cloned from a dendritic cell cDNA library.[76] B7-H3 mRNA is expressed in spleen, lymph nodes, bone marrow and thymus, as well as in several tumor cell lines. B7-H3 is not expressed on peripheral blood mononuclear cells, but can be induced on the surface of dendritic cells, monocytes and T cells by GM-CSF, IFN-γ, or the combination of phorbol myristate acetate (PMA) + ionomycin. The receptor for B7-H3 has not been identified, but CD28, CTLA-4, and PD-1 were excluded as potential cognate ligands.[76] Similar to ICOS, CTLA-4, and PD-1, the unidentified B7-H3 receptor is apparently expressed on activated T cells but not resting T cells. Costimulation by B7-H3 promoted the proliferation of $CD4^+$ and $CD8^+$ T cells, stimulated IFN- γ production and induced primary CTL generation. Tumor cells (624mel melanoma) transfected with B7-H3 induced CTL activity of purified primary human T cells. This suggests that B7-H3 may convert tumor cells into more potent APCs. To date, no studies have tested the effect of B7-H3 on tumorigenicity in vivo. However, given its ability to elicit CTL in vitro, it would be reasonable to hypothesize that conferral of B7-H3 expression to tumors would enhance tumor immunity.

Future Battles

It has become clear that the process of T cell activation involves a complex interaction of multiple signaling pathways. In addition to antigen-specific signals through the TcR, T cells must integrate both stimulatory and inhibitory signals. Adding complexity to this is the observation that single ligands (e.g., B7-1 or –2) can deliver both types of signals (CD28 and CTLA-4) and the possibility that a single receptor (e.g., PD-1) may receive both inhibitory (PD-L1 and PD-L2) and stimulatory (B7-DC) signals, depending on the ligand interaction. Thus, it is not surprising that a variety of approaches have been applied to enhance anti-tumor immunity. These include gene transfer into tumor cell vaccines, blockade of inhibitory signals, and in situ gene transfer into primary tumors.

Combinatorial approaches that combine multiple costimulatory and cytokine pathways have also proven to be effective against poorly immunogenic tumors. The recent identification of novel B7-family members will allow even more sophisticated and elaborate approaches to stimulating tumor immunity. It should be noted that members of the TNF- and TNFR-family ascribed to costimulatory activity have been shown to enhance tumor immunity. Thus, 4-1BB,[77,78] OX-40,[79,80] and CD40/CD40L[81-83] pathways are potential candidates for combinatorial therapy with B7-family members. By combining modulation of multiple pathways, both stimulatory and inhibitory, one can envision that even more potent approaches will be developed.

The official, government-sanctioned War on Cancer began during the Nixon Presidency.[84,85] However, drafting of Immunologists became fashionable in the late 1980's. As the war raged on, the mechanisms by which T cells become activated substantially contributed to the weaponry of tumor immunologists. Now armed with a plethora of costimulatory receptors upon which to base future battles, the task becomes clear: identify the appropriate combination of costimulatory pathways, cytokines and/or traditional therapies that permits the elimination of established tumors and metastases. Although simple in words, design of this strategy will require significant time and effort to accomplish. However, given that a victory of this battle has such great stakes, it is clear that the continued efforts of all researchers are contributing to an important war that must be won.

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Index

A

Adjuvants 108, 119
Affinity 3, 9, 24, 101, 110, 131
Alignment 3-7, 9, 91-95
Allogeneic 97, 106, 108, 110-113, 117
Allograft 86, 87, 101-106, 113
Anergy 17, 33, 38, 39, 51, 67, 100, 105, 107-109, 118, 123
Antigen-presenting cells 2, 12, 23, 38, 39, 50, 63, 67, 71, 100, 105, 118, 124, 128
Anti-tumor immunity 40, 128, 129, 132-134
Apoptosis 14, 20, 30, 49, 51, 53-55, 73, 124
Autoantibody 55, 60
Autoimmune diseases 40, 42, 48, 55, 56, 67, 82, 84, 86, 87, 118-124
Autoimmunity 38, 40, 43, 72, 78, 119-121, 123, 124, 132
Avidity 3, 24, 41, 96, 110

B

B7-1 *see* CD80
B7-2 *see* CD86
B7-DC (PD-L2) 2, 55, 59-65, 91, 92, 96, 124, 131, 133, 134
B7h (B7RP-1, B7-H2, LICOS) 2, 13, 40, 67-78, 84, 86, 87, 91, 92, 96, 106, 123, 124, 131, 133
B7-H1 (PD-L1) 2, 48-56, 59, 61-65, 68, 91, 92, 96, 124, 131, 133, 134
B7-H3 12, 91-94, 96-98, 131, 134
BCR (B cell receptor) 61, 62
Beta-sheets 2, 3, 5
Beta-strands 2-5, 7, 9
Binding site 1, 4, 5, 7-9, 19, 20, 22, 26, 29

C

CD25 54, 60, 64, 82, 84, 112, 122, 123
CD28 1-9, 12-30, 39, 41-43, 48, 50, 51, 59-62, 64, 65, 67, 68, 70-76, 82-88, 100, 101, 104-113, 118-124, 128, 129, 131, 133, 134
CD80 (B7-1) 1-3, 6-10, 12, 13, 23, 24, 39-43, 48-50, 52, 54, 55, 62-65, 84, 87, 91, 92, 94, 96, 97, 100, 101, 103, 104, 106-110, 112, 118, 120-123, 128-131, 133, 134
CD86 (B7-2) 1-3, 6-10, 12, 13, 23, 24, 40-43, 48-50, 52, 54, 62-65, 84, 86, 87, 91, 92, 96, 100, 101, 103, 104, 106, 107, 109, 110, 112, 118, 120-123, 128-131, 133
CD152 *see* CTLA-4
Comparative modeling 4, 5
Conformation 4, 5, 7-9, 16
Consensus residues 3, 5, 6, 7
Costimulation 1, 3, 13-17, 19, 21, 22, 39-43, 50-52, 54, 55, 67, 70-73, 75, 82, 84, 86, 87, 91, 100-104, 107, 109, 110, 112, 119-124, 131, 134
Costimulatory molecule 13, 38-42, 50, 70, 91, 100, 104, 118-120, 122, 129, 130, 133
Counter-receptor 48, 50, 120
Crosslinking 16, 17, 21, 60, 87, 131
CTLA-4 (CD152) 1-4, 6-9, 12-14, 21, 23-30, 41-43, 48, 50, 59, 62, 65, 68, 70, 72, 74-77, 82-84, 87, 96, 100-102, 104-113, 118-120, 122, 123, 131-134
CTLA-4Ig 24, 50, 84, 85, 87, 96, 101, 102, 104-111, 113, 120-122
Cytokine 12, 13, 16, 39, 40, 48, 50-54, 61-64, 70-72, 76, 77, 84, 91, 97, 107, 108, 110, 120-123, 130, 132, 134
Cytolytic T cell (CTL) 40, 52, 53, 64, 87, 96, 97, 107, 108, 132-134

D

Database 91-94, 96
Development 13, 14, 22, 38, 40-42, 72, 78, 84, 86, 87, 102, 104, 106-108, 118-122, 128

E

Ex vivo 76, 106, 107, 109-112, 130, 132
Experimental autoimmune encephalomyelitis (EAE) 40, 71, 76, 77, 84, 86, 119-124

F

Family 1-4, 7, 12, 13, 16-21, 26, 27, 39, 40, 42, 59-62, 65, 68, 82-84, 91-94, 96, 100, 113, 118, 119, 124, 128, 129, 131, 133, 134
Flow cytometry 85, 108
Fusion protein 24, 71, 73-78, 84, 86, 96, 101, 106, 110, 120, 131

G

Gene knock-out 23
Genome 39, 91, 93-95
GM-CSF 60, 64, 70, 82, 84, 96, 130, 132, 134
Graft versus host disease (GVHD) 77, 106-113
Graft-versus-leukemia (GVL) 112

H

Homologue 62, 64, 67, 68, 91, 100, 106, 133
Hypersensitivity 67, 76, 86, 121

I

ICOS 2, 3, 4, 12, 13, 14, 18, 40, 50, 59, 62, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 82, 83, 84, 85, 86, 87, 88, 96, 106, 119, 123, 124, 131, 133, 134
IFN-γ (Interferon-gamma) 40, 49, 50, 52, 54, 63, 64, 70-72, 76, 77, 82, 84, 86, 87, 91, 96-98, 123, 124, 130, 133, 134
IgV-like domain 92
IL-2 38, 39, 48, 54, 64, 70, 72, 82, 84, 86, 87, 107, 108, 111, 118, 123, 124, 130, 131, 133
IL-4 13, 64, 82, 84, 86, 70-72, 97, 98, 120, 124, 133
IL-5 13, 54, 64, 70-72, 82, 86, 108
IL-10 13, 50-55, 64, 70-72, 82, 84, 86, 97, 98, 108, 123, 124, 130, 133
IL-13 64, 70, 72, 76, 77, 84, 108, 123
Immunoglobulin superfamily (IgSF) 2-7, 9, 61, 83, 91
In silico cloning 91
Inflammation 41, 71, 73, 74, 76, 118, 121
Insulin-dependent diabetes mellitus (IDDM) 119, 122
Intracellular domain 48
Isoform 17, 19, 20, 92

L

LICOS *see* B7h
Ligand 1-5, 12, 13, 16, 17, 21, 23-25, 28-30, 39, 41, 48, 50, 51, 54, 55, 59-68, 70, 72, 74, 82-85, 87, 96, 100, 101, 104, 106, 118-120, 124, 128-134
Lipid kinase 27, 29
Lipid raft 14, 15, 18, 20, 22, 29

M

MHC (major histocompatibility complex) 1, 2, 7, 38-40, 43, 52, 53, 64, 100-103, 105-107, 109-113, 118, 128-130
Mixed lymphocyte reaction (MLR) 107-110
Monoclonal antibody (mAb) 5, 24, 39, 41-43, 49, 51-55, 60, 64, 65, 70, 73, 74, 76, 77, 82, 84, 86, 96, 97, 101, 107, 108, 110-113, 120, 121
Motif 2, 3, 7-9, 13, 14, 16-18, 21, 22, 25-30, 50, 59, 62, 68
Multiple sclerosis (MS) 71, 76-78, 118-120
Mutagenesis 1, 4, 5, 7-9, 51
Myelin basic protein (MBP) 77, 119, 120, 124

N

Negative costimulation 42
NK (natural killer cells) 60, 69, 108, 129
NOD (nonobese diabetic mouse) 40, 42, 119-123

P

PD-1 2, 12-14, 48-52, 55, 59-65, 68, 83, 96, 106, 119, 124, 131, 133, 134
PD-L1 *see* B7-H1
PD-L2 *see* B7-DC
Phosphatase 18, 21, 25, 27, 28, 42, 61, 62

S

Second signal 38, 128
SHP-2 27-29, 42, 61, 62
Signal transduction 1
SMAC 14, 15, 19
Structure 1-10, 21, 48, 59, 62, 68, 82, 83, 94, 104
Superfamily 2, 48, 59, 61, 65, 83, 91
Synapse 14, 15, 19, 22, 28

T

T cell activation 12-16, 23, 24, 26, 38, 39, 41, 42, 55, 60, 63-65, 76, 82, 84, 86, 87, 91, 100, 104-106, 108, 118, 119, 123, 124, 128, 129, 131, 133, 134
T cell receptor (TCR) 1, 2, 8, 9, 12-30, 38, 39, 41-43, 51, 53, 54, 59-64, 67, 70, 73, 74, 82, 83, 86, 91, 97, 100, 104, 105, 107, 119, 128
T lymphocytes 2, 12-16, 18-22, 96, 97, 107, 108, 119, 131
Tolerization 107, 109, 110
Trafficking proteins 26
Transplantation 67, 71, 75, 78, 100-102, 104-106, 109
Tumor necrosis factor (TNF) 12, 40, 54, 129, 133, 134
Tumor-associated B7-H1 52, 53
Tyrosine kinase (PTKs) 15, 16, 25-28

X

Xenotransplantation 102

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