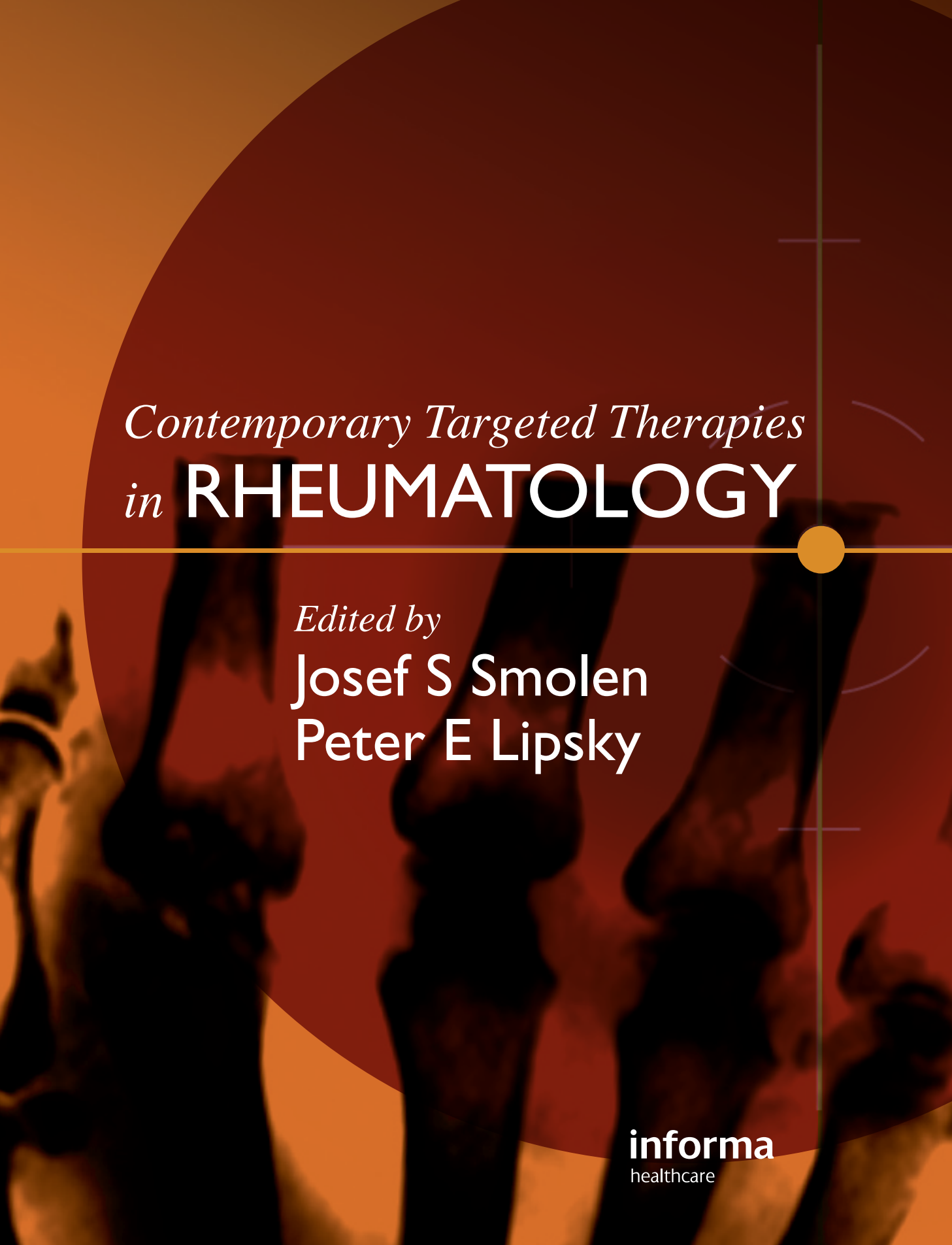
Contemporary Targeted Therapies in RHEUMATOLOGY

Edited by
Josef S Smolen
Peter E Lipsky



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1

T cells – overview – update

Hendrik Schulze-Koops and Joachim R Kalden

Introduction • Lymphopenia and autoimmunity • T-cell-directed therapy by immunosuppressive drugs • T-cell-directed therapy with biologicals • T-cell-directed therapy by blocking T-cell costimulation • T-cell-directed therapy by blocking T-cell migration • T-cell-directed therapy with statins • T-Cell-directed therapy in non-rheumatic diseases • Conclusion • Acknowledgment • References

INTRODUCTION

Because of the central role that $CD4^+$ T cells play in the pathogenesis of autoimmune diseases, different T-cell-directed therapies were introduced for the treatment of autoimmune rheumatic diseases. The initial approaches that aimed to ameliorate inflammatory activity by reducing T-cell numbers, however, provided only modest and inconsistent clinical benefit. Compounds that specifically interfere with T-cell activation – such as some of the disease-modifying anti-rheumatic drugs currently used as standard therapy in rheumatic inflammation – are clinically effective in a majority of patients, but are still associated with a number of side effects related to toxicity and general immunosuppression. Owing to the substantially increased knowledge of cellular and molecular mechanisms of the pathogenesis of rheumatic diseases and the increased understanding of molecular and cellular biology, molecules (biologicals) can now be specifically designed to exclusively target only those cells perpetuating the chronic inflammation, with minimal effects on other aspects of the immune or inflammatory systems. Various T-cell-directed biologicals have been employed in rheumatic diseases with different clinical successes. This chapter updates the currently available clinical data on T-cell-directed interventions in rheumatic diseases.

T cells are central for both the induction and the effector phases of specific immune responses in autoimmune diseases. Of particular importance for initiating, controlling, and driving inflammatory autoimmune responses are $CD4^+$ T cells that, once activated, determine to a large extent the outcome of immune reactions by activating different effector functions of the immune system. Thus, T cells and in particular $CD4^+$ T cells represent an ideal target for immunotherapy in diseases driven by specific immunity to autologous antigens.

LYMPHOPENIA AND AUTOIMMUNITY

However, initial T-cell-directed therapies that were designed to control disease progression by means of reducing the number of T cells, for example, by total lymphoid irradiation or thoracic duct drainage,[1–3] have provided only modest and inconsistent clinical benefit and have been associated with a number of side effects. It became obvious from these approaches that the generation of T-cell lymphopenia is insufficient to combat established autoimmune responses. Moreover, numerous studies have subsequently shown that manipulations that generate functional T-cell lymphopenia in animals result in the development of a variety of organ-specific autoimmune disease in these models.[4] Impressive examples of such manipulations

include the interleukin (IL)-2 knockout (KO) mouse, that develops prominent autoimmune colitis,[5] the T-cell receptor (TCR) α-chain deficient mice which develop inflammatory bowel disease associated with an array of autoantibodies ,[6,7] TCR-α chain transgenic mice,[8] neonatal application of cytotoxic intervention protocols, such as cyclosporin A,[9] total lymphoid irradiation[10] or thymectomy,[11] and lymphotoxic treatment of adult animals.[12] Further studies revealed that the development of autoimmunity was critically dependent on α/β $CD4^+$ T cells, indicating that lymphopenia promotes the induction of autoimmune inflammation by self-reactive peripheral blood CD4 T cells in these animals. In fact, it could be demonstrated that the peripheral T-cell population that emerged in mice in which lymphopenia was induced by cytotoxic treatment with cyclophosphamide or streptozotocin, preferentially consisted of interferon (IFN)-γ secreting pro-inflammatory Th1-like cells.[13] Although lymphopenia is not sufficient for the development of autoimmune diseases in humans,[14] it is conceivable that lymphopenia in patients with existing autoimmune diseases permits the homeostatic expansion of autoreactive T cells, thereby resulting in the reappearance of autoimmune inflammation and, thus, the reoccurrence of clinically overt autoimmune phenomena.

T-CELL-DIRECTED THERAPY BY IMMUNOSUPPRESSIVE DRUGS

Owing to the significant advances in the understanding of T-cell biology, compounds were designed in recent years that specifically interfere with T-cell activation without reducing T-cell numbers. Cyclosporin A and FK506 (tacrolimus), for example, inhibit T-cell activation by interfering with calcineurin-mediated transcriptional activation of a number of cytokine genes, such as IL-2, IL-3, IL-4, IL-8, and IFN-γ. Leflunomide, a potent non-cytotoxic inhibitor of the key enzyme of the *de novo* synthesis of uridine monophosphate,[15] dihydro-orotate dehydrogenase, blocks clonal expansion and terminal differentiation of T cells as activated T cells critically depend on the *de novo* pyrimidine synthesis to fulfill their metabolic needs. These compounds are clinically effective in ameliorating autoimmune inflammation and are important components of the current therapeutic repertoire in autoimmune diseases. It is of interest to note that besides the established ability of some of these so-called disease-modifying anti-rheumatic drugs (DMARDs), such as cyclosporine, FK506, or leflunomide, to directly inhibit T-cell activation, many DMARDs have been associated with a shift in the balance of proinflammatory Th1 cells to immunomodulatory Th2 cells.[1,16] This immunomodulatory effect might contribute to the beneficial therapeutic potential of DMARDs in inflammatory autoimmune diseases that reflect ongoing inflammation largely mediated by activated proinflammatory Th1 cells without the sufficient differentiation of immunoregulatory Th2 cells to down-modulate inflammation, such as rheumatoid arthritis (RA).[17–20]

T-CELL-DIRECTED THERAPY WITH BIOLOGICALS

Despite the progress that has been made in the treatment of rheumatic diseases, standard immunosuppressive therapy (even if T-cell-directed) is still clinically ineffective in many patients and is associated with a number of side effects related to toxicity and general immunosuppression. Moreover, as yet standard therapy with DMARDs and corticosteroids has failed to interrupt and permanently halt autoimmune inflammation. The substantial progress in our understanding of molecular and cellular biology in recent years has permitted the design of therapeutic tools with defined targets and effector functions ('biologicals') that might fulfill these hopes of an optimal therapy. Based on the increased knowledge of molecular mechanisms involved in the pathogenesis of rheumatic diseases, biologicals have been developed to selectively target only those cells and/or pathways driving the disease, while maintaining the integrity of the remainder of the immune system. Based on the concept that activated T cells are the key mediators of chronic autoimmune inflammation, a number of approaches have been designed in autoimmune diseases to specifically target mature circulating T cells. However, although the concept of T-cell-directed immunotherapy with biologicals is evidence-based and

has been successfully employed in animal models of autoimmune diseases, T-cell-directed biologicals have generally failed to induce sustained clinical improvement in patients with RA.[1,21]

A number of reasons, such as the selection of the targeted molecules, the design of the biologicals, and the selection of patients at advanced stages of their disease, might have contributed to the unfavorable results of some T-cell-directed therapies with biologicals in man. A further problem in targeting specifically the disease-promoting T cells in human autoimmune rheumatic diseases is the fact that neither the eliciting (auto)antigens nor the specific disease initiating or perpetuating T cells are known. Therefore, the most rational approach to treat human autoimmune diseases has been interference with the activation of $CD4^+$ T cells in a rather non-antigen-specific manner.

T-cell-directed therapies have been performed with biologicals that target T-cell surface receptors or disrupt the cell/cell interactions that are important for the recruitment of T cells to sites of inflammation and/or for T-cell costimulation. The T-cell surface receptors that have been targeted in clinical trials include CD2, CD3, CD4, CD5, CD7, CD25, and CD52. These molecules are more or less specific for T cells or T-cell subsets and were thus considered promising targets in attempts to down-modulate sustained inflammation by virtue of interfering with T-cell activation. A detailed review of experiences with the *in vivo* use of monoclonal antibodies (mAbs) to these individual surface receptors and the outcome of clinical trials with such mAbs was presented in our earlier review.[1] Although some of the mAbs employed were clearly associated with convincing and prolonged clinical benefit, the conception arose from these trials that targeting surface receptors of CD4 T cells by mAbs was generally not sufficient to ameliorate established autoimmune inflammation.[1,21] Of importance, the induction of permanent unresponsiveness of autoreactive T cells that would have resulted in sustained clinical improvement without the need for continuous immunosuppressive therapy was never achieved in any of the studies. With the exception of a limited number of trials with biologicals blocking CD2,[22] CD3,[23] or CD4,[24] clinical studies with mAbs to T-cell surface receptors in rheumatic diseases have largely been discontinued for the past few years.

T-CELL-DIRECTED THERAPY BY BLOCKING T-CELL COSTIMULATION

An alternative approach to inhibit T-cell activation in inflammatory diseases is to interrupt the interaction between T-cells and neighboring cells by blocking the ligand for a T-cell surface molecule on the surface of the cells interacting with T cells, thereby preventing receptor/counter receptor interaction. This approach has been successfully employed in an attempt to block CD28-mediated costimulation in T cells.[25–28] Costimulation is an absolute requirement for the activation of naive T cells. Therefore, costimulation controls the initiation of specific immunity. In fact, activation of a naive T-cell through its TCR without providing appropriate costimulation renders the T cell anergic, which essentially restricts the initiation of specific immune responses to professional antigen-presenting cells (APCs), such as dendritic cells, that are able to engage costimulatory molecules on naive T cells. CD28-mediated costimulation can be blocked by coating the binding partners of CD28 on APCs, CD80, and CD86, with a soluble immunoglobulin fusion protein of the extracellular domain of CD152 (cytotoxic T-lymphocyte antigen 4, CTLA-4). CTLA-4 is a homolog to CD28 and is expressed by activated T cells. It can bind both CD80 and CD86 with higher affinity than CD28. Because CD152 has a high affinity for CD80 and CD86, soluble forms of CTLA-4 inhibit the interaction of CD28 with its ligands. The various clinical trials in which signaling through CD28 was inhibited will be discussed in detail elsewhere in this book.

An alternative costimulatory pathway involved in T-cell activation is the CD2/CD58 pathway. Following the promising results from an open-label study with alefacept, a soluble fully human recombinant fusion protein comprising the first extracellular domain of CD58 and the hinge, CH2 and CH3 sequences of human IgG1, in patients with psoriatic arthritis,[29] a phase II study of alefacept in combination with methotrexate for psoriatic arthritis has recently

been presented.[22] Three months after a 12-week period of weekly intramuscular application of 15 mg alefacept, 54% of the verum-treated patients (compared with 23% of the placebo-treated control) achieved an ACR20 response. The data suggest that prevention of T-cell activation by targeting CD2/CD58 interactions is feasible and might result in reduction of autoimmune joint inflammation. Further studies are required to substantiate these observations.

Together, the successful therapy of clinically active rheumatic diseases with biologicals interrupting T-cell costimulatory pathways clearly emphasize the important role of T cells in the pathogenesis even at advanced stages of these diseases. Importantly, as in contrast to naive T cells, memory and effector T cells are independent of costimulation, the data also strongly suggest that inflammatory joint activity in RA and psoriasis depends on the continuous activation and recruitment of naive T cells.

T-CELL-DIRECTED THERAPY BY BLOCKING T-CELL MIGRATION

T-cell recruitment to sites of inflammation was successfully prevented with a murine mAb to CD54 (ICAM-1), which is critical for transendothelial migration of T cells and their subsequent activation.[30] Because of the immunogenicity of this mAb, however, retreatment with this agent was associated with immune complex-mediated side effects, including urticaria, angioedema, and serum complement protein consumption[31] and therefore further studies were not conducted. The concept of modulating autoimmune inflammation by selectively interfering with T-cell migration, however, was tested again in a more recent randomized placebo-controlled trial of an antisense oligodeoxynucleotide to ICAM-1 in patients with severe RA.[32] In this study, clinical efficacy was not noted, presumably because of insufficient dosage, as suggested by a subsequent study in Crohn's disease, in which the dose required for therapeutic efficacy was higher than the dose employed in the RA trial.[33] Thus the clinical value of an antisense oligodeoxynucloetide approach to CD54 in RA remains to be shown.

T-CELL-DIRECTED THERAPY WITH STATINS

Apart from the treatment principles described herein in more detail, other innovative T-cell-directed therapeutic strategies have been defined, some of which have already entered preliminary clinical trials. For example, the anti-inflammatory role of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) has been documented in a murine model of inflammatory arthritis. Simvastatin not only markedly inhibited developing but also clinically established collagen-induced arthritis in doses that were unable to significantly alter cholesterol concentrations *in vivo*.[34] Importantly, simvastatin reduced anti-CD3/anti-CD28-induced T-cell proliferation and IFN-γ production and, moreover, demonstrated a significant suppression of collagen-specific Th1 humoral and cellular immune responses. Studies in humans, though, have not been reported to date.

T-CELL-DIRECTED THERAPY IN NON-RHEUMATIC DISEASES

In non-rheumatic autoimmune diseases, several interesting T-cell-directed approaches have been performed. For example, altered peptide ligands (APLs) of myasthenogenic peptides that are single amino acid-substituted analogons of the pathogenic peptides were able to inhibit the proliferative responses of the pathogenic peptide-specific T-cell lines *in vitro* and to prevent *in vivo* priming to the myastenogenic peptides.[35] A dual APL composed of two tandemly arranged single altered peptide analogs was also able to inhibit those responses *in vitro* and *in vivo*. Interestingly, the dual APL activated $CD4^+CD25^+$-expressing regulatory T cells in the lymph nodes of injected mice, suggesting that the active suppression exerted by the dual APL is mediated by the recently identified $CD4^+CD25^+$ regulatory T-cell population. The potency of these cells in ameliorating autoimmune inflammation has been documented in non-obese diabetic mice, in which small numbers of antigen-specific $CD25^+$ regulatory T cells were able to reverse diabetes after disease onset.[36] As it was possible to expand these antigen-specific regulatory T cells *in vitro*, the vaccination with $CD4^+CD25^+$ regulatory

T cells might open novel avenues for T-cell-mediated cellular immunotherapy in autoimmune diseases. Whether the obstacle of unknown antigens in most human autoimmune diseases can be overcome and whether the numbers of regulatory T cells required for down-modulating systemic autoimmune inflammation in humans can be generated *in vitro* remain to be shown.

CONCLUSION

Based on the concept that activated T cells are the key mediators of chronic autoimmune inflammation, different T-cell-directed approaches have been introduced for the treatment of inflammatory rheumatic disease. Whereas attempts to down-modulate rheumatic inflammation by reducing T cell numbers have largely failed, novel treatment approaches with biologicals that specifically inhibit T-cell activation by preventing costimulation are associated with considerable clinical efficiency. These compounds have clearly established the feasibility of targeted T-cell-directed interventions and the clinical benefit induced by inhibiting T-cell activation supports the dominant role of T cells in rheumatic inflammation even at advanced stages of the diseases. Some interesting novel treatment approaches have been tested in animal models of autoimmune disease, but their value for clinical use in humans needs to be established.

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2

Pathways of T-cell costimulation

Brendan J Classon, Steven G Nadler and Suzanne J Suchard

Introduction • Costimulatory pathways • Coinhibitory pathways • Conclusion and summary • References

INTRODUCTION

It has been recognized since the 1970s that T cells require at least two signals for full activation leading to maximum proliferation and cytokine production.[1–3] The first signal is provided by the clonotypic cell surface T-cell receptor (TCR) when it engages a specific major histocompatibility complex (MHC) molecule–peptide complex on an antigen-presenting cell (APC). The second activating signal(s) is provided by costimulatory ligands expressed on the T-cell surface that engage cognate receptors on the surface of APCs. The initiation and progression of the immune response is controlled by spatial and temporal regulation of the expression of costimulatory and coinhibitory ligands and their receptors. In general, T cells that receive only the first signal through the TCR in the absence of a second costimulatory signal become anergic and non-responsive. However, in certain circumstances, T cells may become activated after receiving a potent agonist signal via the TCR. In addition to receiving costimulatory signals, T cells may also receive coinhibitory signals, which results in the attenuation of costimulatory signals and interruption of T-cell activation and cytokine secretion. The expression pattern of costimulatory and coinhibitory ligands and their receptors is regulated over the course of the immune response, ensuring an optimal balance of stimulatory and inhibitory signals to enable effective clearance of antigen or pathogen and a diminution of the response once the antigen or pathogen is cleared. Thus, T-cell costimulation and coinhibitory pathways have evolved to facilitate initiation of appropriate immune responses, which are subsequently regulated to avoid uncontrolled T-cell activation and the attendant potential risk of autoimmunity.

There are two major families of cell surface costimulatory molecules that can be classified according to their structural characteristics (Figure 2.1). Firstly, there is the CD28:B7 family, whose ligands and receptors comprise immunoglobulin (Ig)-like domains. The second family of costimulatory molecules is the CD40/CD40L family, whose ligands are homologous to tumor necrosis factor (TNF), and whose receptors are homologous to the TNF-receptor. In contrast to costimulatory molecules, the cell surface coinhibitory ligands and their receptors are predominantly composed of Ig-like domains. This chapter reviews the important costimulatory and coinhibitory ligands and receptors, with an emphasis on their function in normal immune responses, and how these functions may contribute to the pathogenesis of autoimmune disease, particularly, rheumatoid arthritis.

COSTIMULATORY PATHWAYS

CD28 AND CD80/CD86

The most well characterized T-cell costimulatory ligand is CD28, which interacts with the costimulatory receptors CD80 and CD86. CD28 is a transmembrane protein comprising a single

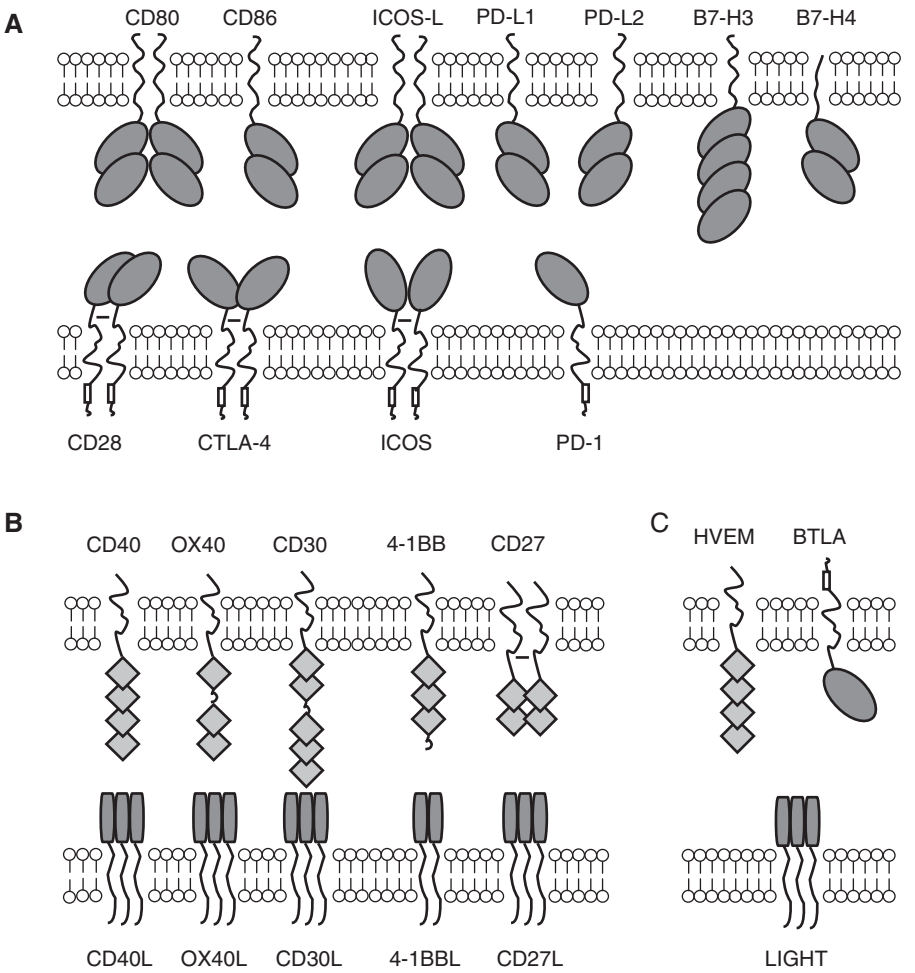

Figure 2.1 (A) Molecular interactions between costimulatory and coinhibitory receptor/ligand pairs of the immunoglobulin superfamily. Putative cognate ligands for the B7-H3 and B7-H4 receptors remain unidentified. (B) Molecular interactions between costimulatory receptor/ligand pairs of the TNF/TNFR superfamily. (C) Receptor/ligand interactions that mediate costimulatory (LIGHT/HVEM) and coinhibitory response (BTLA/LIGHT) that involve members of both the Ig and TNF/TNFR superfamilies.

extracellular Ig variable-like (IgV) domain.[4] In humans and in mice, CD28 is constitutively expressed on the majority of $CD4^+$ T cells and a subset of $CD8^+$ T cells.[5,6] Costimulation through CD28 in T cells leads to initiation of the cell cycle, enhanced metabolic activity, up-regulation of anti-apoptotic genes, and enhanced cytokine production, particularly IL-2.[7–9] CD28 plays a key role in activation of naive T cells; however, recent data suggest that CD28 also plays a role in the activation of memory T cells.[10]

The first receptor to be identified for CD28 was CD80 (also termed B7-1).[11] The second receptor, CD86 (also termed B7-2), was identified subsequently.[12,13] CTLA-4 is a second ligand that binds CD80 and CD86, but CTLA-4 function differs from CD28 because it plays an important role in coinhibitory signaling (see below). The CD80 receptor is expressed constitutively at very low levels on APCs including dendritic cells (DCs), B cells, and Langerhans cells, but its expression is markedly up-regulated on APCs and T cells following activation, which may occur during infection and exposure to proinflammatory cytokines.[14] In contrast, CD86 is expressed constitutively and increased only

moderately after APC stimulation.[12] CD80 and CD86 are structurally similar and they have overlapping but distinct functions.[15] Considering their tissue distribution and timing of expression, it is generally believed that CD86 is critical for the initiation of immune responses, whereas CD80 plays a more prominent role in maintenance and subsequent attenuation of the immune response.[16]

Extensive *in vitro* analysis of CD28 and CD80/CD86 function has been performed using monoclonal antibodies (mAbs) and fusion proteins, including CTLA-4-Ig, in blocking experiments. Antibody blockade of CD28 was shown to inhibit T-cell proliferation in a mixed lymphocyte reaction (MLR).[17,18] Similarly, antibody blockade of either CD80 or CD86 also inhibited T-cell proliferation, although dual blockade of both CD80 and CD86 was required for maximum inhibition.[19] A soluble derivative of the alternative CD80/CD86 ligand, CTLA-4, expressed as an Ig-fusion protein (CTLA-4-Ig) was also shown to inhibit T-cell proliferation.[19] In this study, the degree of inhibition observed with CTLA-4-Ig was similar to that observed with CD80 plus CD86 mAbs, a result that is consistent with the notion that CTLA-4-Ig is an effective dual antagonist of CD80 and CD86. Several studies have demonstrated that CTLA-4-Ig is also an effective inhibitor of the CD80/CD86 interaction with CD28 *in vivo*,[20,21] and the functional effect of this reagent has been studied in a significant number of experimental animal models of autoimmune disease.[22] CTLA-4-Ig inhibits T-cell-dependent antibody responses, ameliorates autoimmune disease progression and severity, and prolongs allograft survival.[23,24] In the collagen-induced arthritis model, CTLA-4-Ig inhibits anti-collagen antibody production, paw swelling, serum cytokine production, and bone erosion.[25]

Analysis of CD28-deficient mice has confirmed a key role for CD28 in the activation of naive T cells, differentiation of T-helper cells, Ig isotype class switching, and T-cell survival.[26] Furthermore, the impaired T-cell responses observed in these mice have established that CD28 is the single major CD80/CD86 binding costimulatory ligand on T cells.[27] Similarly, it has been shown that mice deficient for either CD80 or CD86 also exhibit impaired T-cell responses. CD80-deficient mice exhibited reduced T-cell proliferative responses in MLRs[28] and CD86-deficient mice are defective in Ig isotype switching and have impaired splenic germinal center formation.[29] CD80/CD86-double deficient mice exhibited a similar phenotype to CD86-deficient mice, although the deficiency in Ig isotype switching was more pronounced.[29]

To date, CTLA-4-Ig (abatacept) is the only clinically approved drug that selectively targets CD28-mediated costimulation. A phase I clinical trial was conducted in patients with psoriasis, where approximately half the abatacept-treated patients exhibited a sustained improvement in disease symptoms.[30] In this study, it was established that abatacept effectively inhibited Ig production in response to a neoantigen as well as measures of inflammation in the psoriatic lesion. Abatacept was also shown to be effective in reducing the signs and symptoms of rheumatoid arthritis in phase III clinical trials.[31–33] In December 2005, abatacept was approved for the treatment of moderate to severe rheumatoid arthritis in the United States and Canada.[22] Other biologics that target the CD28 pathway and have been in development for autoimmune disease but are now discontinued include anti-CD80 (Galiximab) and anti-CD86. A small molecule inhibitor of CD80[34] is currently entering a phase II clinical trial in rheumatoid arthritis patients.[35]

ICOS and ICOS-L

Inducible costimulatory molecule (ICOS) is a CD28-related molecule whose expression is induced on differentiated T cells following activation.[36] Costimulation of T cells through ICOS initiates secretion of cytokines other than IL-2.[37] The lack of IL-2 production following ICOS costimulation limits the long-term expansion potential of ICOS-costimulated T cells.[38] Because ICOS seems to play a more prominent role in driving T-cell effector function, rather than expansion, it has been suggested that ICOS facilitates rapid activation of T-cell memory responses.[9] Thus, while CD28 costimulation is required for initiation of the response, ICOS appears to play a more prominent role in the

ongoing response through maintenance of T-cell effector function.[39]

ICOS binds B7h (also called B7RP-1, B7H2, LICOS, and GL50), which is expressed on activated myeloid cells. ICOS does not bind CD80 or CD86, and B7h does not bind CD28 or CTLA-4. Both ICOS- and B7h-deficient mice display similar phenotypes, suggesting that they function as a monogamous receptor/ligand pair. Analysis of mice deficient for ICOS or B7h suggests that the ICOS/B7h interaction is not an obligate requirement for T-cell expansion, but rather ICOS acts co-operatively with CD28 in T-cell costimulation.[40–42] The major functional role of ICOS/ICOS-L appears to be in the induction of T-cell effector function during T-cell differentiation. The phenotype of ICOS-deficient mice and analysis of mice treated with soluble ICOS-Ig fusion protein to block ICOS/B7h interactions reveal that ICOS is required for Ig isotype switching and germinal center formation.[40,41,43] ICOS seems to play little if any role in the generation of $CD8^+$ T-cell effector function, since viral CTL and antibody responses in mice treated with soluble ICOS-Ig fusion protein were relatively unaffected.[44] The importance of ICOS in late B-cell differentiation and Ig class switching was confirmed following the identification of a homozygous loss of ICOS in a subset of patients suffering from adult-onset common variable immunodeficiency.[45,46]

CD40 and CD40L

CD40 is a member of the TNFR superfamily, which was first identified as a B-cell surface receptor capable of inducing polyclonal activation and differentiation into antibody-producing cells.[47] CD40 is constitutively expressed on B cells, monocytes, macrophages, DCs, epithelial cells, endothelial cells, fibroblasts, and platelets.[48] The ligand for CD40 is CD40L (CD154), which was reported to induce contact-dependent differentiation of B cells.[49,50] CD40L is a type II transmembrane protein expressed predominantly by activated $CD4^+$ T cells and activated platelets.[51] CD40L expression on T cells is induced shortly after T-cell activation, and thus represents an early activation marker of T lymphocytes. The expression of CD40L on activated platelets is thought to mediate recruitment of inflammatory cells to the damaged endothelium. In a manner similar to membrane-bound TNF-α, CD40L is cleaved from the cell surface of activated T cells by a matrix metalloproteinase, releasing a homotrimeric form of sCD40L into the circulation.[52] Like membrane-bound CD40L, sCD40L can also promote B-cell activation and differentiation.

Engagement of CD40 by CD40L induces up-regulation of CD80 and CD86 on B cells, and up-regulation of CD54 and CD86 on DCs.[53,54] Ligation of CD40 on DCs induces the secretion of cytokines such as IL-8, TNF-α, MIP-1α, and IL-12. Functional interactions between CD40L and CD40 are bidirectional, and engagement of CD40L on T cells by $CD40^+$ APCs can induce apoptosis in $CD4^+$ T cells and leads to the generation of $CD8^+$ memory T cells.[55,56] Ligation of CD40 on endothelial cells triggers production of chemokines and cytokines such as IL-8, MCP-1, MIP-1α, RANTES, IL-1, IL-6, IL-12, and TNF-α, and leads to the up-regulation of adhesion molecules and matrix metalloproteinases.[57–60]

Cognate interactions between CD40 and CD40L are crucial for the switch in recombination and synthesis of immunoglobulins by B cells.[61] In addition to its role in Ig isotype switching, binding of CD40L to CD40 is crucial for activation, proliferation, and maturation of B cells. A critical role for CD40L in B-cell function was confirmed when genetic mutations in CD40L were reported in patients suffering from hyper-IgM syndrome.[62] These individuals exhibit defective antibody production manifest by a lack of circulating IgG and IgA due to the inability of the B cell to switch the IgM isotype. Similar defects were recapitulated in mice following genetic disruption of the CD40/CD40L pathway.[63,64]

Antibody blockade of CD40L has been a relatively successful immunosuppressive strategy in animal transplantation models. In combination with CTLA-4-Ig, CD40L blockade has both additive and synergistic effects in the context of prolonging kidney allograft survival in primates and skin graft survival in mice.[65,66] Preclinical animal models also demonstrate the potential for antagonizing the CD40L/CD40 pathway for the treatment of autoimmune diseases. Treatment with an anti-CD40L mAb suppresses

the development of collagen-induced arthritis, ameliorating disease symptoms including joint inflammation, cartilage erosion, and infiltration by inflammatory cells of the subsynovial tissue.[67] In a transgenic mouse model of Ig-mediated arthritis, anti-CD40L mAb significantly diminished the development of arthritis in a prophylactic treatment regimen.[68] In a mouse model of lupus, animals treated with continuous anti-CD40L mAb infusion exhibited a delay in disease onset, with increased efficacy in combination with CTLA-4-Ig. In both cases, there was a decrease in anti-dsDNA autoantibodies and the spleens from these animals had reduced numbers of B cells.

Monotherapeutic applications of anti-CD40L mAbs in human lupus have been published. In a phase II trial, a humanized anti-CD40L antibody (IDEC-131) was shown to be safe and well tolerated but failed to demonstrate significant efficacy over placebo.[69] In another study, another humanized anti-CD40L antibody (BG9588) appeared to have a beneficial impact on the course of disease[70] but the study was terminated early due to adverse thromboembolytic complications.[71]

OX40 and OX40L

OX40 (CD134) is a member of the TNFR superfamily originally identified by an antibody generated against activated rat T cells.[72,73] Subsequently, the human OX40 homolog was identified.[74] OX40 is absent from resting T cells but is expressed on $CD4^+$ and some $CD8^+$ T cells following activation.[75] Costimulation through OX40 has been implicated in the generation of T-helper responses as well as in the maintenance of memory T-cell populations.[76] OX40L (gp34) was first identified as a type II transmembrane protein induced by HTLV-1 infection of T cells.[77] OX40L is expressed on activated T cells, B cells, DCs, macrophages, epithelial cells, and endothelial cells, and similar to OX40, OX40L expression is prolonged for several days following cell activation.[78]

Studies using OX40L or anti-OX40 antibody to mediate T-cell activation demonstrated that OX40 signaling on T cells enhances cytokine production and proliferation of $CD4^+$ T cells, an effect that can occur in the absence of CD28 signaling.[79,80] OX40/OX40L interactions appear to be important in sustaining T-cell function at later stages of the primary immune response and during the memory response.[75,81] T cells from OX40-deficient mice produce IL-2 and proliferate normally, but as the response proceeds, T-cell expansion and cytokine production are not sustained.

Agonistic anti-OX40 antibody can elicit $CD4^+$ and $CD8^+$ T-cell expansion *in vivo*, with transient splenomegaly and lymphadenopathy observed in non-human primates.[82] CD40L transgenic mice demonstrate an accumulation of activated $CD4^+$ $OX40^+$ T cells in the B-cell follicles of secondary lymphoid organs following antigenic stimulation, suggesting that OX40 regulates T-cell homing within secondary lymphoid organs.[83] $OX40^+$ T cells have been demonstrated at the site of inflammation in a number of animal models of autoimmunity, including experimental allergic encephalomyelitis (EAE), rheumatoid arthritis (RA), and graft versus host disease (GVHD).[84–86] Consistent with these observations, transgenic expression of OX40L by DCs increases the number of antigen-responding $CD4^+$ and autoimmune events in rodents.[83] Administration of soluble OX40-Ig fusion protein to colitic mice ameliorates disease, with a concomitant reduction in T-cell infiltrates and TNF-α, IL-1, IL-12, and IFN-γ production.[87] In mice, a neutralizing anti-OX40L antibody administered before, but not after a second immunization with a model autoantigen (type II collagen), inhibits the development of collagen-induced arthritis,[88] suggesting that the OX40/OX40L interaction is involved in the early stages of disease induction. Treatment with anti-OX40L antibody or depletion of $OX40^+$ T cells has been shown to ameliorate EAE symptoms in both an induced disease model and an adoptive transfer model.[85,89] OX40L antibody treatment does not inhibit the development of pathogenic T cells but rather their accumulation in the spinal cord.[90] The opposite effect is observed with an activating OX40 antibody which can exacerbate disease.[91] Similarly, agonistic anti-OX40 can break peripheral T-cell tolerance induced in mice by administering antigen-specific peptides. In general, these data support a role for

OX40/OX40L in maintaining the ongoing immune response following antigen-specific T-cell activation, and as such, manipulation of the OX40/OX40L pathway has significant clinical potential for treatment of autoimmune disease.

4-1BB and 4-1BBL pathway

4-1BB (CD137) was first discovered as a TNFR-related cDNA whose expression was induced in activated mouse T-cell clones.[92] 4-1BB is absent from resting T cells, but is expressed on activated $CD4^+$ and $CD8^+$ T cells, some DCs, and on activated natural killer (NK) cells, with expression peaking at 42–72 hours after activation.[93,94] The ligand for 4-1BB (4-1BB-L) was first identified in a human B-cell line, using a 4-1BB-Ig fusion protein as a probe for the counter structure.[95] Although a member of the TNF superfamily, 4-1BBL is unusual in that it exists at the cell surface as a disulfide-linked homodimer, rather than the more typical homotrimer. 4-1BB-L is expressed on mature DCs, activated T cells and B cells and macrophages.[96,97]

Antibody-induced cross-linking of 4-1BB on anti-CD3 activated mouse T cells was shown to enhance T-cell proliferation.[93] Likewise, engagement of 4-1BB by 4-1BBL was shown to elicit a similar response.[95] Engagement of 4-1BB on activated T cells by its ligand leads to a preferential expansion of $CD8^+$ T cells, rather than $CD4^+$ T cells.[98] Thus, signaling through 4-1BB appears to be important for $CD8^+$ T-cell survival, enhancing cytokine production and differentiation of CTL effector function.[98,99] In addition to 4-1BB signaling on T cells, 4-1BBL is capable of inducing a 'reverse' signal to APCs. For example, engagement of 4-1BBL induces B-cell proliferation and inflammatory cytokine production by monocytes.[97,100,101] In T cells, proliferation induced by anti-CD3 antibody is inhibited by cross-linking of 4-1BBL, which ultimately leads to apoptosis.[102]

Although 4-1BB mAbs have been shown to effectively costimulate $CD4^+$ and $CD8^+$ T cells, they can also block the development of humoral immunity when administered early during immunization.[103] Consistent with this finding, an anti-CD137 mAb can effectively block the onset of SLE in young mice and block its progression in animals with advanced disease.[104] Administration of the 4-1BB agonist antibody at the time of collagen immunization blocks development of disease in a model of collagen-induced arthritis,[105,106] but it has only a modest effect on progression of established disease. [106]

HVEM and LIGHT

LIGHT is a TNF-related cell surface ligand that was originally identified from a human activated T-cell library. It is expressed on the surface of activated T cells, NK cells, and immature DCs.[107–109] LIGHT is a homotrimeric cell surface protein, which exists in three distinct forms. Full-length LIGHT is expressed on the cell surface, an alternatively spliced isoform lacking the transmembrane domain is retained in the cytoplasm, and there is a soluble form which is released from the cell surface by a metalloprotease activity.[110,111]

There are two cell surface receptors that interact with LIGHT. The first, HVEM (herpesvirus-entry mediator), is expressed on T cells, B cells, monocytes, and immature DCs[112,113] and the second, lymphotoxin-β receptor (LTβR), is expressed on epithelial cells and stromal cells but not on lymphocytes.[107] HVEM expression decreases following T-cell activation and it has been suggested that LIGHT may be responsible for this phenomenon.[111] This reciprocal regulation of LIGHT and HVEM expression may be important for limiting the duration of LIGHT-HVEM-mediated T-cell activation. HVEM also interacts with the BTLA (B- and T-lymphocyte attenuator) ligand. BTLA is a coinhibitory ligand which down-regulates B- and T-cell responses and will be discussed further below. LIGHT can also costimulate T-cell proliferation in a manner that is CD28-independent.[108,114,115] This response can be inhibited with either an anti-HVEM antibody or an HVEM/Fc fusion protein, indicating that LIGHT-mediated T-cell immune responses are mediated through its interaction with HVEM. Splenocytes from HVEM-deficient mice fail to proliferate in response to triggering with an anti-TCR antibody plus recombinant soluble LIGHT, demonstrating that HVEM signaling is essential for LIGHT-mediated costimulation.[116] $CD8^+$ T cells from LIGHT-deficient mice exhibit

reduced *in vitro* proliferative responses,[115,117] although LIGHT deficiency does not appear to impact their cytolytic effector function.

Transgenic mice with enhanced LIGHT expression on T cells exhibit a lymphoproliferative phenotype, with expanded populations of both $CD4^+$ and $CD8^+$ T cells.[118] In transplantation models, antibody blockade of LIGHT or targeted disruption of the LIGHT gene has been shown to ameliorate graft rejection and GVHD, further supporting a role for LIGHT in regulation of T-cell effector function.[114,119] Blockade of the LTβR/LIGHT interaction by a soluble LTβR-Ig fusion protein has been shown to ameliorate disease severity in a colitis model.[118,120] Consistent with these findings, transfer of transgenic T cells overexpressing LIGHT into $RAG^{-/-}$ recipient mice induces a rapid disease onset with a pathology similar to Crohn's disease.[116] Up-regulation of LIGHT is also associated with active disease in Crohn's patients, suggesting that LIGHT may contribute to pathogenesis of Crohn's disease.[116]

CD27 and CD70

The CD27 receptor is a member of the TNFR superfamily originally identified as a novel T-cell differentiation antigen.[121] CD27 is a disulfide-linked homodimer expressed on $CD4^+$ and $CD8^+$ T, NK cells, and antigen-primed B cells. The CD27 counter structure, CD70, is expressed on activated T and B cells, activated DCs, NK cells, and Hodgkin's lymphoma cells.[121,122] CD70 expression on T cells is up-regulated following antigen activation and it is further modulated by cytokines.[123] On DCs, CD70 expression is induced by CD40 ligation.[124] Interestingly, anomalous expression of CD27 in B cells has proved a useful marker for assessing disease activity in lupus patients.[125]

Costimulation mediated by CD27/CD70 induces expansion and differentiation of effector T-cell and memory T-cell populations.[126] Engagement of CD27 on B cells promotes cell expansion, germinal center formation, plasma cell differentiation, and Ig production.[126,127] CD27-deficient mice have reduced numbers of antigen-specific T cells in lymphoid organs and recruitment of $CD4^+$ and $CD8^+$ effector T cells to sites of viral challenge in these animals is also impaired.[128] CD27 signaling in T cells is thought to enhance cell survival rather than directly affecting proliferation. CD70 transgenic mice exhibit an accumulation of $CD4^+$ and $CD8^+$ effector T cells, which leads to progressive depletion of naïve T cells in secondary lymphoid tissue.[129] In a vascularized cardiac transplant model, CD70 blockade has little effect on $CD4^+$ T-cell function but prevents $CD8^+$ T-cell-mediated graft rejection.[130]

CD30 and CD30L

CD30 (Ki-1) is a TNFR-related cell surface receptor originally discovered as a marker of Reed-Sternberg cells in Hodgkin's lymphoma, where it was discovered that CD30 overexpression led to malignancy.[131] CD30 is expressed on activated T cells and B cells, and some NK cells, and is inducible on T cells by signaling through the TCR in combination with CD28 or IL-4 signaling. $CD30^+$ cells are also present at inflammatory sites in several human diseases, including atopic dermatitis, RA, chronic GVHD, and systemic sclerosis.[132] CD30L (CD153) is primarily expressed by $CD4^+$ T cells, B cells, and some tumors. [133]

Signaling through CD30 can induce proliferation, differentiation or apoptosis depending upon the cell type, stage of development, and other stimuli.[133–136] CD30-deficient mice have an impaired capacity to sustain follicular germinal center responses and have reduced recall responses to T-dependent antigens.[137] Memory T-cell responses are reduced in these mice because the T cells fail to receive adequate survival signals from $CD30^+$ $OX40L^+$ accessory cells in B-cell follicles. Consistent with this finding, a non-depleting anti-CD30L mAb inhibits class switching in antibody responses to T-dependent antigens, but it does not affect primary antibody responses.[132] As expected, the phenotype of CD30L-transgenic mice is generally the opposite of that observed with the CD30-deficient mice. It has been suggested that CD30 is a candidate for a diabetes-susceptible gene (*Idd 9*) in NOD mice,[138] and an anti-CD30L antibody has been used to implicate the CD30/CD30L pathway in autoimmune diabetes.[139] The CD30/CD30L pathway has also been implicated

in $CD4^+$ T-cell-mediated GVHD disease.[139,140] Currently, there are anti-CD30 antibodies in the clinic for the treatment of hematopoietic malignancies but none for autoimmune disease.

COINHIBITORY PATHWAYS

CTLA-4 AND CD80/CD86

The coinhibitory ligand counterpart to CD28 is CTLA-4, which was first identified as an activation-induced gene in mouse T cells.[141,142] In contrast to CD28, CTLA-4 delivers a negative signal to T cells, and even low levels of constitutively expressed cell surface CTLA-4 are capable of inhibiting early events in T-cell activation and IL-2 secretion.[143] CTLA-4 binds both CD80 and CD86, although the apparent affinity of CTLA-4 interaction with CD80 is significantly higher than the corresponding interaction with CD86, as a consequence of multivalent avidity enhancement.[144] The mechanism of CTLA-4 function involves inhibition of TCR signal transduction through binding of CTLA-4 to the zeta chain of the TCR, with concomitant inhibition of tyrosine phosphorylation via phosphatases associated with the cytoplasmic tail of CTLA-4.[145] More recently, CTLA-4 was shown to effect the immune synapse and length of time of interaction between T cells and APCs.[146,147] An alternatively sliced variant of CTLA-4 which lacks the CD80/CD86 binding domain has been identified and shown to induce potent inhibition of T-cell proliferation and cytokine secretion,[148] suggesting that CTLA-4 can function as a negative regulator of T cell responses in a CD80/CD86-independent fashion. Thus, the mechanism of CTLA-4-mediated down-regulation of T-cell activation is complex, because it involves ligand competition, perturbation of the immune synapse, and recruitment of intracellular phosphatase activity, which may occur in a ligand-dependent or a ligand-independent fashion.[149]

The crucial role of CTLA-4 in down-regulating T-cell responses was most clearly evident in the phenotype of CTLA-4-deficient mice. These animals develop a pronounced lymphoproliferative disorder and die at around 3 weeks of age as a consequence of multiorgan lymphocytic infiltration and tissue destruction.[150] In humans, susceptibility to the cluster of autoimmune disorders including Graves' disease, autoimmune hypothyroidism, and type I diabetes is correlated with lower levels of an alternatively spliced transcript which encodes a soluble form of CTLA-4.[151]

PD-1 and PDL-1/PD-L2

Programmed death-1 (PD-1) was initially described as an abundant transcript in a mouse T-cell hybridoma undergoing programmed cell death. Subsequently, it was shown that PD-1 is also expressed on activated T cells, B cells, and myeloid cells in humans and mice. PD-1 is a single IgV-like domain, but unlike CD28 and CTLA-4, it exists as a monomer at the cell surface. Like CTLA-4, even low levels of PD-1 at the cell surface are sufficient to mediate inhibition of T-cell activation. PD-1 has two counter-receptors, PD-L1 (B7h1) and PD-L2 (B7-DC), which share 38% sequence identity. PD-L1 mRNA is widely expressed in parenchymal tissue, including heart, placenta, skeletal muscle, and lung, but PD-L1 protein appears to be restricted to cancer cells, activated myeloid cells, and a subset of activated T cells. PD-L2 expression is restricted to activated macrophages and DCs.

Genetically modified mice deleted for expression of PD-1, PD-L1, or PD-L2 exhibit immune phenotypes consistent with an inhibitory role for this receptor ligand/pair in T-cell activation. PD-1-deficient mice spontaneously develop a lupus-like disease or autoimmune dilated cardiomyopathy, depending on the genetic background. PD-1-deficient mice crossed with $H\text{-}2L^d$-specific TCR transgenic mice on an $H\text{-}2^{b/d}$ background develop splenomegaly and a lethal GVHD. In NOD mice, PD-1 deficiency or blockade accelerates progression of autoimmune diabetogenic disease. In humans, PD-1 has been identified as a candidate gene within a disease-susceptibility locus for systemic lupus erythematosus (SLE). In PD-L1-deficient mice, $CD8^+$ T cells exhibited enhanced clonal expansion and were capable of secreting higher levels of IFN-γ. Likewise, PD-L2-deficient mice exhibit enhanced *in vivo* T-cell activation and augmented APC function. A second study of PD-L2-deficient mice reported contradictory findings of

diminished $CD4^+$ T-cell-dependent humoral responses and impaired $CD8^+$ T-cell anti-tumor responses, suggesting a costimulatory function for PD-L2. These studies suggest that mouse PD-L2 possesses dual stimulatory and inhibitory functions, invoking the presence of an additional costimulatory ligand analogous to PD-1. Nevertheless, it appears that the primary role of PD-L2 in humans is inhibitory.

Consistent with its function as a coinhibitory ligand, it has been shown that PD-1 attenuates immune responses to both viruses and tumors. In humans and mice, it has been shown that during chronic viral infection, T cells that have become non-responsive as a consequence of PD-1 overexpression, can be functionally reactivated following PD-1 blockade. It has been demonstrated that overexpression of PD-L1 in human cancer cells confers resistance to cytolysis by T cells, thought to be mediated by PD-L1-induced apoptosis in the T cell. Similarly, enhanced anti-tumor responses were observed in mice treated with T cells activated by APCs under conditions where PD-L1 function was blocked. Taken together, these results show that the primary function of the PD-1-PD-L1/PD-L2 pathway is to mediate down-regulation of the immune response.

B7-H3 (B7RP-2)

B7 homolog 3 (B7-H3) was first identified in humans as a truncated sequence derived from a more abundant, full-length sequence ubiquitously expressed in many tissues including, heart, liver, lung, kidney, pancreas, and colon. Human B7-H3 protein is expressed on immature and mature DCs, activated monocytes, subsets of activated T cells, B cells, and NK cells, and also neuroblastomas and many other cancer cell lines. Mouse B7-H3 is similarly expressed in a wide variety of tissues, including osteoblasts. In mice, the abundant form of B7-H3 corresponds to the truncated form of human B7-H3, a consequence of selective exon loss that occurred in rodents, but not in humans. The putative counter structure for B7-H3 has not been identified.

Blocking experiments conducted with soluble B7-H3-Ig fusion proteins and antibodies have yielded conflicting functional results, although in mice, B7-H3 appears to function predominantly as an attenuator of immune responses. For example, it has been shown that immobilized soluble B7-H3-Ig fusion protein induces a dose-dependent inhibition of mouse T-cell proliferation and reduced IL-2 and IFN-γ production. Furthermore, blockade of B7-H3 with an antagonist antibody was shown to enhance T-cell proliferation and enhance EAE. However, it has been shown that B7-H3 was capable of delivering a positive costimulation signal leading to expansion of antigen-specific $CD8^+$ cytolytic cells.

Genetically modified mice lacking B7-H3 developed more severe disease symptoms in both an airway inflammation model and an EAE model. An inhibitory role for B7-H3 was further supported by the finding that mouse APCs lacking B7-H3 expression exhibit enhanced stimulatory capacity. B7-H3-deficient mice developed a spontaneous autoimmune phenotype and develop anti-DNA autoantibodies with age. The relatively broad tissue distribution of B7-H3 suggests pleiotropic effects outside the immune system and a recent report describing reduced bone mineral density in B7-H3-deficient mice suggests a role for B7-H3 in osteoblast differentiation and bone mineralization. In humans, there is less evidence to support the role of B7-H3 as an inhibitory receptor, although it has been reported that B7-H3 expressed ectopically on APCs can attenuate T-cell proliferative responses. Interestingly, a recent report has shown that B7-H3 expression affords tumor cells protection from NK cell-mediated lysis. Taken together, it appears that B7-H3 functions predominantly as a coinhibitory receptor. The lack of structural conservation between human and mouse B7-H3 may further reflect species-specific differences in B7-H3 function in mice and humans.

B7-H4 (B7x, B7s1)

B7-H4 was identified by sequence database mining based on similarities to other known members of the gene family. Like B7-H3, B7-H4 mRNA is also expressed in many tissues, including lung, kidney, stomach, and small intestine, but the relatively broad distribution of the mRNA contrasts with the more restricted expression pattern of B7-H4 protein. In mice,

B7-H4 protein is expressed on splenic B cells and peritoneal macrophages, but not on T cells. In humans, B7-H4 is absent from resting T cells, B cells, monocytes, and DCs, but its expression is up-regulated on activated T cells and B cells. Aside from its expression on lymphoid cells, B7-H4 is highly expressed in human cancers, including breast, ovarian, renal, and kidney cancers. Moreover, B7-H4 is abundantly expressed on tumor-infiltrating macrophages in ovarian cancer. Initially, B7-H4 was thought to interact with the coinhibitory ligand BTLA (see below), but this has since been disproved. Binding studies with soluble B7-H4 have demonstrated a putative ligand on activated human T cells, although it remains unidentified.

There are relatively few data on the functional outcome of B7-H4 blockade on T-cell responses. However, one study reported that antisense oligo-mediated down-regulation of B7-H4 expression on tumor-infiltrating macrophages enhanced their ability to prime T cells when coinjected into ovarian tumors in a xenogeneic model. Recently, analysis of B7-H4-deficient mice revealed a mild enhancement in the magnitude of Th1-type responses, but the overall impact of B7-H4 deficiency on immune responses is subtle, possibly indicative of overlapping function between the ubiquitously expressed coinhibitory receptors such as B7-H3 and B7-H4.

BTLA

B- and T-lymphocyte attenuator (BTLA) was initially discovered in a mouse Th2 T-cell clone[183] and was subsequently shown to be also expressed at high levels on mouse B cells. In humans, BTLA is constitutively expressed on B and T cells and expression is diminished following activation.[184] The receptor for BTLA has been identified as HVEM,[185,186] a TNFR-related molecule which is relatively widely expressed on T cells, B cells, NK cells, DCs, and myeloid cells (see above). BTLA and HVEM are structurally unrelated and, as such, this interaction represents an unusual example of cross-talk between the two major structural classes of costimulatory and coinhibitory ligands and their receptors.[187]

In humans, cross-linking BTLA with an agonistic mAb inhibited T-cell proliferation and cytokine production in primary $CD4^+$ T-cell responses and secondary $CD4^+$ and $CD8^+$ responses.[184,188] At present, the function of BTLA on other cells is less clear. Unlike other costimulatory and coinhibitory ligands, BTLA is polymorphic and several alleles have been identified in various inbred mouse strains.[158] In humans, a BTLA gene polymorphism has been associated with an increased risk of RA.[190]

CONCLUSION AND SUMMARY

A regulated balance of activating and inhibitory signals is required to ensure an effective immune response while preserving self-tolerance. After its initiation, the magnitude and progression of the immune response are governed by an array of costimulatory and coinhibitory receptor ligand pairs whose expression is modulated both spatially and temporally. At the outset of the response, T-cell activation is directed largely by a combination of an antigen-specific signal via the TCR and a costimulatory signal delivered by CD28 ligation. As the response progresses, costimulation through other costimulatory ligands, such as ICOS, regulates the differentiation of T-cell effector function. At the height of the response, expression of coinhibitory molecules such as CTLA-4, BTLA, and PD-1 is up-regulated, which serves to dampen the response as the pathogen or antigen is cleared. While CTLA-4 is the predominant coinhibitory ligand, additional inhibitory signals are provided by BTLA, B7-H3, and B7-H4. The relatively broad distribution of some of the more recently discovered coinhibitory receptors suggests a key role for these molecules in the maintenance of tolerance and the regulation of immune responses in peripheral tissues. The molecules that direct costimulatory and coinhibitory signaling pathways represent tractable targets for therapeutic intervention in inflammatory autoimmune disease.

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3

Regulatory T cells

Harald von Boehmer

Introduction • Characteristics of regulatory T cells • Intra- and extra-thymic generation of Tregs • Lifestyle of Tregs • Function of Tregs • Concluding remarks • References

INTRODUCTION

Cellular therapy employing Foxp3-expressing regulatory T cells (Tregs) holds the promise to replace and/or supplement indiscriminatory immunosuppression by drugs. In order to achieve this goal in the clinic we need to learn more about the generation, lifestyle, and function of Tregs. One way to generate Tregs of any desired antigen specificity is the retroviral introduction of the Foxp3 gene into activated CD4 T cells. Foxp3 is mostly but not exclusively a transcriptional repressor that interferes with T-cell receptor (TCR)-dependent activation of genes and may exert its effect, at least in part, by compromising NF-AT-dependent gene activation. Another way of generating Tregs extrathymically *in vivo* is the introduction of low amounts of peptides under subimmunogenic conditions. Such artificially induced Tregs have a long lifespan in the absence of the inducing antigen and can thus mediate antigen-specific tolerance. Antigen specificity of Tregs-mediated immunosuppression is due to effective co-recruitment and expression of Tregs and T effector cells to antigen-draining lymph nodes and sites of inflammation such that Tregs effectively suppress neighboring effector T cells at early or late stages of their differentiation. The latter allows for interference with already established unwanted immunity and may thus be employed to treat rather than prevent unwanted immune reactions.

The notion that the immune system employs different mechanisms to prevent autoimmune disease or maintain self-tolerance has been around for decades, but definitive evidence emphasizing the essential role of negative selection as well as that of suppressor or regulatory T cells is of more recent origin. Today we distinguish negative selection in the form of deletion[1] of certain antigen-specific cells as well as in the form of 'anergy'[2] by cell-autonomous mechanisms, also referred to as 'recessive' tolerance, from tolerance that relies on the silencing of immune cells by regulatory or suppressor T cells by non-cell-autonomous mechanisms,[3] also referred to as 'dominant' tolerance. Both forms of tolerance can achieve antigen-specific non-responsiveness of the immune system in contrast to pharmacological interventions that usually result in undesirable general immunosuppression with potentially deadly side effects. In many clinical situations antigen-specific non-responsiveness represents the desired goal but in general present day treatment does not achieve that goal. For that reason it remains a great challenge for immunologists to design strategies and protocols that achieve antigen-specific non-responsiveness, since there is little hope that the pharmaceutical industry will come up with suitable procedures to effectively and specifically interfere with unwanted immunity in the near future. Given this goal, it appears a reasonable strategy to exploit evolutionarily selected mechanisms effective in self-tolerance

for clinical purposes. This requires a thorough understanding of how the immune system manages to avoid self-aggression. It is now appreciated that so-called negative selection of potentially self-reactive T cells by antigens inside and probably also outside the thymus essentially contributes to self-tolerance.[4] Likewise it has become clear that the generation of Foxp3-expressing regulatory T cells is mandatory to achieve self-tolerance.[5] The progress in understanding the contribution of such reasonably well-defined mechanisms to tolerance has thus established the somewhat limited usefulness of models that solely consider the absence of 'danger' signals as an essential feature of self-tolerance.

While we have some basic ideas about mechanisms that can be exploited to induce antigen-specific non-responsiveness, much needs to be learned in detail before this will become clinically applicable. Experiments have shown that overexpression of certain crucial self-antigens (such as insulin) that results in more profound tolerance by negative selection,[6] can be helpful in preventing autoimmune disease, perhaps because certain autoimmune diseases, such as type 1 diabetes, begin with a rather limited autoimmune response to antigens such as insulin,[6,7] while later on a variety of other antigens in pancreatic β cells are recognized. However, clinically, such maneuvers would be limited to introducing such antigens prior to disease outbreak or when the immune system is 'reset' after elimination of mature lymphocytes by x-irradiation and/or cytotoxic drugs.

In contrast, the manipulation of regulatory T cells appears to represent a more widely applicable approach not only to prevent but potentially also to interfere with already ongoing unwanted immunity. With such a clinical goal in mind it is clear that we need to have a much better understanding of how antigen-specific regulatory T cells are and can be generated and/or amplified and how they can achieve antigen-specific non-responsiveness. It is the purpose of this chapter to review recent progress in the understanding of several aspects of regulatory T cells with the hope that some of this information may find its way into the clinic, with the challenge that ensuing procedures will eventually replace or at least supplement the present day practice of indiscriminate immunosuppression.

CHARACTERISTICS OF REGULATORY T CELLS

Recent years have seen rapid progress in the characterization of regulatory T cells (Tregs). There is not one particular cell surface marker that defines Tregs but the CD25 surface molecule is at least expressed on the vast majority of cells that express the Foxp3 transcription factor, which has become a signature gene expressed in Tregs. The recognition that $CD25^+$ cells are enriched in Tregs has thus contributed considerably to establishing their role in suppressing the activation and function of other lymphocytes.[8] In the meantime other molecules such as neuropilin 1,[9] CD103,[10] GPR83,[11] GITR,[12] and CTLA-4[13] have been shown to have a characteristic expression profile in Tregs and thus can be helpful in achieving optimal purification in combination with the CD25 marker. Recent evidence shows that $CD4^+25^+$ Tregs are IL-7R-negative, in contrast to $CD4^+25^+$ cells that just represent activated T cells without obvious regulatory function.[14] Intracellular staining by Foxp3 antibodies represents a useful means to identify Tregs in various tissues[15] and in the meantime various Foxp3 reporter mice[16,17] have become available, which allow purification of functional Foxp3-expressing cells. While Foxp3 expression represents a good signature for Tregs it can have its drawbacks, because Foxp3 can be transiently expressed in activated T cells that do not qualify as stable Tregs.[15]

A variety of studies indicate that stable Foxp3 expression is sufficient to confer a regulatory T-cell phenotype to CD4 T cells.[18–20] Thus retroviral Foxp3 transduction is a valuable means to endow antigen-specific T cells with a regulatory phenotype. This represents an important tool because, unlike the *in vitro* expansion[21,22] of Tregs that have been preformed *in vivo*, it allows production of Tregs of any desired specificity.

Recent data suggest that Foxp3 can interact with NF-AT in a DNA binding complex to regulate gene expression such as down-regulation of the IL-2 gene and up-regulation of CTLA-4 and CD25 molecules.[23] It is presently not clear

whether all Foxp3-dependent gene regulation involves NF-AT and whether NF-AT plays a crucial role in the generation of Tregs. It has become clear from the combined analysis of Foxp3 binding and genome-wide gene expression, however, that Foxp3 is predominantly but not exclusively a repressor that silences genes that are normally activated after T-cell stimulation, especially genes associated with T-cell receptor (TCR) signaling.[43] This fact may contribute to the relatively poor response of Tregs in response to antigenic stimulation *in vitro*, while exogenous growth factors may permit effective clonal expansion *in vivo*. The latter feature is likely essential for effective *in vivo* suppression.

Among the genes that fail to be up-regulated in Foxp3-expressing cells is the PTPN22 phosphatase that has a role in dephosphorylating $p56^{lck}$ and Zap-70. Interestingly, a gain of function mutation of this gene is associated with several autoimmune diseases and it is presently not clear whether this mutant affects Tregs that control autoimmune disease or effector T cells that cause autoimmune disease.[24]

Another important characteristic of Tregs is that they do express an αβ,TCR that confers antigen specificity. This is worthwhile pointing out, since many studies on Tregs ignore this fact. It is our belief that antigen specificity of Tregs is absolutely crucial for antigen-specific suppression of immune responses and hence considerable attention has to be paid to the role of TCR specificity in the generation, homing, and effector function of Tregs.[25] As all T cells with αβ, TCRs, Tregs also undergo stringent TCR-dependent selection in primary and secondary lymphoid organs,[26] which eventually may be exploited to generate Tregs of any desired specificity and to interfere specifically with unwanted immune responses in the clinic.

INTRA- AND EXTRA-THYMIC GENERATION OF TREGS

Experiments in TCR transgenic mice, in which the transgenic TCR was the only TCR expressed, by developing T cells have clearly shown that ligation of the αβ,TCR by strong agonist ligands plays an essential role in the intrathymic generation of Tregs.[27,28] These results are compatible with analysis of the Tregs TCR repertoire in normal mice, suggesting a focus on self-antigens.[29] It became especially obvious that expression of TCR ligands by thymic epithelial cells represented a powerful means to commit developing $CD4^+$ T cells to the Treg lineage.[28] In this context it is of considerable interest to note that thymic epithelial cells, and especially thymic medullary epithelial cells, can express 'ectopically' a variety of proteins that otherwise would be considered 'organ-specific' such as preproinsulin2 that is expressed in pancreatic β cells but also in thymic medullary epithelial cells.[30,31] Such ectopic expression can be regulated, at least in part, by the AIRE (autoimmune immune regulator) transcription factor[32] and it is thus conceivable that the ectopic expression of 'organ-specific' antigen by thymic epithelium plays a decisive role in the generation of Tregs specific for such antigens, even though experiments addressing that question have so far yielded negative results.[33,34] However, negative results by no means rule out the possibility that AIRE-regulated antigens contribute to the generation of Tregs under more favorable experimental conditions.

The intrathymic generation of Tregs by strong agonist ligands appears to require costimulation of developing cells by B7-1 (CD80)[35] ligands that are expressed on thymic epithelial cells as well as on antigen-presenting cells (APCs) of hemopoietic origin, at least under certain experimental conditions. This is a somewhat astonishing observation in the light of findings that Treg generation in peripheral lymphoid tissue is most effective under conditions that avoid costimulation (see below). Conceivably this could be due to the different stages of development of thymic and extrathymic T cells, which may require different signaling inputs for Treg commitment. From thymus transplantation experiments it is clear that Tregs generated by ligands expressed on thymic epithelium only can migrate into peripheral lymphoid tissue and patrol the body for long periods of time without being confronted with the same ligand that was involved in their generation.[28,36] This does not exclude the possibility that lower affinity ligands in peripheral lymphoid tissue may contribute to survival, much as they can contribute to survival of CD4 and CD8 conventional T cells.[37]

Considering the intrathymic generation of Tregs it is of interest to note that generation of Tregs from cells with one particular αβ,TCR is not mutually exclusive to deletion of some of these cells.[28] Thus both processes depend on recognition of agonist ligands by developing $CD4^+$ T cells but under some conditions such recognition results in deletion and under other conditions in Treg generation, even within the same thymus, perhaps because some of these cells encounter their TCR ligands on different cells, i.e. either on cross-presenting dendritic cells (DCs) or directly on thymic epithelial cells.[38]

Whereas the intrathymic generation of Tregs would mostly depend on instruction of lineage commitment by self-antigens, the peripheral generation of Tregs may also include instruction by foreign antigens. It is therefore of considerable interest to define conditions permissible for extrathymic Treg generation. To this end we have exploited protocols of subimmunogenic antigen presentation, because circumstantial and historic evidence suggested that one might be able to induce 'dominant' tolerance in this way. Indeed it was found that either constant delivery of peptides by osmotic mini-pumps[39] or by targeting DCs with peptide-containing fusion antibodies directed against the DEC205 endocytic receptor on DCs allowed the conversion of naïve T cells into Foxp3 regulatory T cells.[15] The conversion process depended on an intact TGF-βRII receptor on naïve T cells a similiar and conditions that avoided activation of DCs as well as IL-2 production by naïve T cells. It was clear that Tregs were generated by conversion rather than expansion of already committed Tregs, since the experiments were performed in mice expressing only one particular transgenic TCR in the absence of coexpression of a TCR agonist ligand, resulting in the unique constellation that none of the generated $CD4^+$ T cells initially exhibited a Treg phenotype and only a certain percentage (15–20%) assumed it after the artificial introduction of the respective TCR agonist ligand.[15] Importantly, the Tregs generated in this way exhibited i.e. was TGF-β-dependent the same global gene expression pattern as intrathymically generated Tregs[38] and much like intrathymically generated Tregs exhibited a long lifespan that was independent of further supply of the TCR agonist ligand. Thus by these maneuvers a Treg 'memory' to external TCR ligands could be induced, resulting in the subsequent suppression of immune responses elicited by the same agonist ligand, i.e. this protocol succeeded in generating specific immunological tolerance to one particular antigen ("by stander" supression, see below). Hopefully this protocol can be extended to many other antigens and thus help the prevention of unwanted immune responses. Of note, this particular protocol only works with naïve T cells and not with T cells that have already been activated *in vivo* and thus can presumably not be used to suppress already established autoimmunity in which most antigen-specific T cells are already activated. In such cases the *in vitro* generation of Tregs by Foxp3 transduction would likely be more appropriate (see below).[38]

LIFESTYLE OF TREGS

As pointed out above, Tregs can survive for relatively long periods of time as resting cells at an intermitotic stage but as soon as they encounter their TCR agonist ligand they will express activation markers and begin to home to antigen-draining lymph nodes and undergo considerable expansion.[21,22,36] This is usually accompanied by loss of CD62L and acquisition of CD44 expression and followed by expression of the α_E integrin (CD103) receptor (at least in the mouse). Such activated cells to extravasate and accumulate together with other T effector cells in inflamed tissue.[10] It is in fact the co-recruitment of CD4 and/or CD8 effector cells with activated Tregs in draining lymph nodes and/or inflamed tissue that determines the specificity of immunosuppression:[36] since Tregs suppress neighboring T cells in a 'bystander' fashion it can only be effective when most antigen-specific effector cells are co-recruited to the same anatomical location, which depends on presentation of TCR ligands in these places, such as antigen-draining lymph nodes.[20] Thus while Tregs may suppress 'innocent' bystanders that happen to be in their vicinity, this will not result in general immunosuppression, because the majority of such 'innocent' cells will be distributed throughout the body and not recruited by antigen such that they will not be subject to suppression. It is for

this reason that injection of Tregs specific for a pancreas-derived antigen are far more effective in suppressing diabetes than polyclonal Tregs that will not all accumulate and be activated in pancreatic lymph nodes.[20]

'Bystander suppression' is well documented by the fact that, for instance, $CD4^+$ Tregs recognizing a class II major histocompatibility complex (MHC)-presented epitope from one particular protein can suppress CD8 T cells recognizing a different class I MHC-presented epitope from the same protein.[40] Thus the antigen specificity of Tregs and effector T cells does not need to match for effective immunosuppression to occur: it is sufficient that the different T cells are co-recruited to the same tissue. This of course is good news since this will permit a Treg of one particular specificity to suppress a variety of effector cells with different specificity, as long as all these different epitopes are present within the same draining lymph node or anatomical site.

Since many intrathymically generated Tregs are specific for self-antigen it is perhaps not surprising that normally there are always 'activated' Tregs present in the organism[41] and some of these Tregs may be engaged in locally preventing autoimmunity. In fact neonatal removal of Tregs will result in the 'scurfy' phenotype associated with multiorgan-specific autoimmunity.[42] Other Tregs are apparently not 'in action' and patrol the body by exhibiting a phenotype of naïve T cells that do not divide.[30,41]

FUNCTION OF TREGS

One of the questions that has remained rather elusive concerns the molecular mechanisms by which Tregs control other T cells. There are probably several not mutually exclusive mechanisms that may dominate in certain situations.[25] *In vitro* data have emphasized the role of close cell-to-cell contact and a nonessential role of cytokines such as IL-10 or TGF-β. All *in vivo* data published so far have emphasized the crucial role of the TGF-βRII on suppressed cells, since a dominant negative form of that receptor is usually associated with ineffective Treg suppression and with generalized autoimmunity. It is still not clear whether this results from the fact that Tregs produce TGF-β (which they do but only in moderate amounts) or whether in general TGF-β-induced signaling 'conditions' effector cells for more stringent suppression by a mechanism that does not involve increased TGF-β production but depends on specific Treg activation.[25] A good example for such a scenario is the suppression of tumor-specific CD8 T cells by CD4 Tregs that crucially depends on an intact TGF-βRII receptor on the CD8 T cells. In this particular model the suppression affects the function of fully differentiated cytotoxic T lymphocytes (CTLs), notably the secretion of cytolytic granules. However, *in vitro* experiments with fully differentiated CTLs have shown that TGF-β does not have any negative impact on cytolysis when added during the effector phase. This is consistent with the hypothesis that TGF-β-dependent signaling 'conditions' the CD8 T cells for Treg suppression rather than representing the sole suppressor mechanism.[40]

These experiments also make another important point, namely that it is apparently never too late to interfere with an immune response by Treg suppression, since the experiments show that suppression can affect fully differentiated effector cells. This is good news in the sense that the obviously effective suppression late during an immune response can revert rather than prevent unwanted immunity, a concept that may become extremely useful in the clinic.

Different experiments attempting to reverse rather than prevent diabetes are fully consistent with that view: CD4 T cells specific for an islet-derived antigen of unknown nature could be activated *in vitro* and retrovirally transduced with Foxp3 such that within 24 hours they assumed a phenotype of Tregs. When 10^5 of such converted cells were injected into NOD mice that had become just diabetic because of beginning destruction of their islet cells, these islet-specific Tregs cured the mice of diabetes and they remained diabetes-free for at least 3 months when the experiment was terminated. Again this experiment suggests that Tregs can silence already fully developed effector cells.[20]

Additional controls make important points with regard to the role of Treg antigen receptors in this process and hence the specificity of immunosuppression: while the injection of 10^5 cells with islet-antigen specificity was sufficient to abolish disease, the injection of even 10^6 Tregs

with specificity for a large variety of different antigens or the injection of Tregs with specificity for an antigen not present in the pancreatic lymph node did not have any effect and the animals died several days later from complete destruction of β cells and resulting diabetes that obviously at this point could no longer be reversed by Tregs.[20] These results and similar results by others employing *in vitro* expanded Tregs[21,22] are very encouraging, since they suggest that by adoptive Treg therapy early-diagnosed diabetes may be cured, in spite of the fact that the generation of sufficient numbers of islet-antigen-specific Tregs still represents a staggering logistic problem.

Thus in spite of our ignorance concerning molecular mechanisms of Treg-mediated suppression (even though a variety has been proposed)[25] we have promising evidence from murine models of disease that Tregs have the capacity to interfere with unwanted immunity early and/or late during the immune response in an antigen-specific way, since they interfere with such immunity in a local milieu only while leaving the rest of the immune system intact.

There is also no compelling reason why the findings made in the somewhat popular models of type 1 diabetes should not be extended to other autoimmune diseases such as rheumatic diseases, provided that there are clues about relevant antigens that are presented in local lymphoid tissue.

CONCLUDING REMARKS

The described properties of Tregs, i.e. the possibility of generating them extrathymically *in vivo* or *in vitro* with any desired antigen specificity, their ability to co-home with T effector cells into antigen-draining lymph nodes and/or sites of inflammation, their potential to suppress effector cells at early and late stages of differentiation, and last but not least the ability to suppress neighboring T effector cells of any antigenic specificity, make these cells an ideal tool with which to intervene in unwanted immunity in an antigen-specific way. Thus one would hope that eventually the exploitation of evolutionarily selected mechanisms to deal with unwanted immune responses against self will replace indiscriminate immunosuppression by drugs with potentially deadly side effects. This is not to say that such drugs may be completely useless: their transient application may help to set the immune system to a stage where Tregs can be more effective in dealing specifically with unwanted immunity. What should be avoided, however, is the long-term indiscriminate use of the drugs that eventually will ruin the protection against infections and malignant disease afforded by the immune system.

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4

B-cell antigen receptor signaling and autoimmunity

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Introduction • BCR signaling pathway • Anergy as a mechanism for silencing self-reactive B cells: differential B-cell signaling • References

INTRODUCTION

A number of illnesses affecting joints or muscles are associated with antibodies to 'self' molecules and are classified as autoimmune rheumatic diseases. They include rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), antiphospholipid syndrome, polymyalgia rheumatica, systemic sclerosis, Sjögren's syndrome, polymyositis and dermatomyositis, myasthenia gravis, and a spectrum of related syndromes. Altered development and function of B cells may play a prominent role in the development and progression of autoimmune rheumatic disorders, with RA and SLE being the classic and most widely studied.

Loss of self-tolerance leading to production of self-reactive antibodies is integral to the development and progression of RA and SLE. By largely stochastic processes, immunoglobulin (Ig) gene arrangement gives rise to B cells with an enormous range of antigen specificity. Although optimally protective, a disadvantage of such diversity is the potential to generate self-reactive antibodies. Indeed, B cells contribute to the pathophysiology of autoimmune rheumatic diseases in part by production of germ-line encoded and/or somatically mutated self-reactive antibodies.[1–3] The successful treatment of RA, SLE, multiple sclerosis, and Sjögren's syndrome with anti-CD20 monoclonal antibodies that eliminate B cells further supports the key role of B cells in the development of autoimmune rheumatic diseases.[4–8] In animal models, the absence of B cells prevents the spontaneous development of SLE.[9] Interestingly, this does not merely reflect a role for self-reactive antibody production. SLE-associated T-cell accumulation in lymphoid organs does not occur in the absence of B cells, suggesting a role for B cells apart from secretion of self-reactive antibodies in the development of SLE. These findings and those of several other studies suggest that B cells may also be involved in presentation of self-antigen to T cells, or some novel form of regulation of T-cell activation and recruitment.[10,11]

The critical role of B cells in rheumatological diseases has become increasingly evident as a consequence of insights gained from studies of B-cell antigen receptor (BCR) signaling pathways. The BCR plays a key role in B-cell development and function, and has a central role in regulation of self-tolerance. To ensure self-tolerance, self-reactive B cells are efficiently silenced by one of three distinct mechanisms: receptor editing, clonal deletion, or anergy.[12] Studies suggest that a key determinant of the mode of silencing is the strength of BCR signaling and developmental stage.[13] Antigen avidity, i.e valency, affinity, and concentration, as well as involvement of co-receptors and adaptor molecules, play a role in determining signal quality and strength. At extremes, high avidity antigen interactions with immature B cells lead to receptor editing in an anthropomorphic effort to eliminate autoantigen binding activity. Failing this, these cells are

eliminated by apoptotic death, referred to as clonal deletion. Lower avidity interactions with self, particularly in the periphery, lead to anergy wherein cells remain viable for some time and bind antigen yet are unresponsive to immunogenic stimulation.

In this chapter, we have incorporated the most recent and salient findings regarding BCR signaling, its role in the maintenance of self-tolerance and its impact on the development and progression of autoimmune rheumatic diseases, particularly focusing on SLE and RA. We discuss the primary signaling pathways emanating from the BCRs and their downstream effectors (Figure 4.1). Our review is divided into sections addressing (i) signal initiation, (ii) signal propagation and integration focusing on the role of inositol lipids, and (iii) signal modulation with an emphasis on

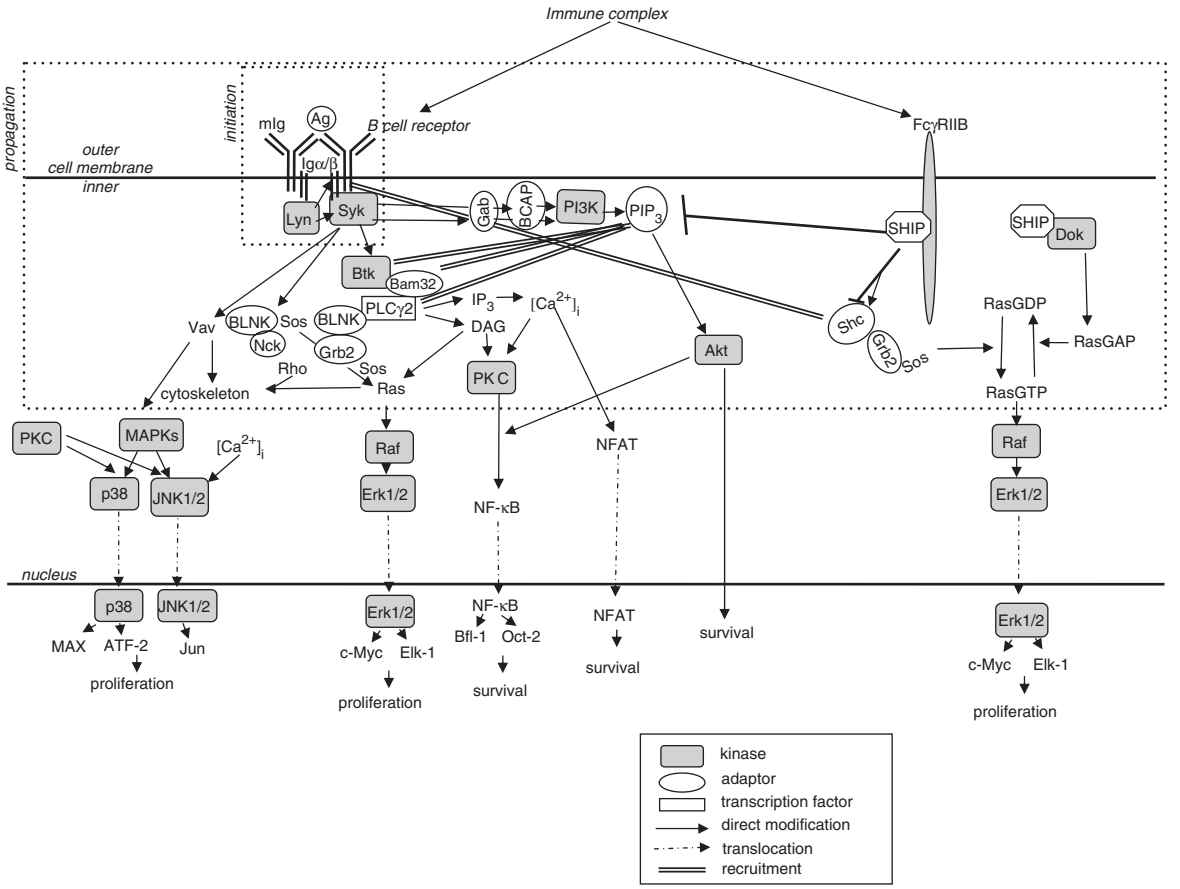

Figure 4.1 BCR signaling cascade: interactions with an inhibitory co-receptor, FcγRIIB. Signal transduction initiates at the cell membrane following ligand-induced aggregation of the membrane immunoglobulin (mIg) and associated Igα/β. Signals are propagated by means of protein phosphorylation, modification, and integration. BCR signaling strength is modulated by activatory and inhibitory co-receptors and their effectors. Finally, activation of transcription factors and gene expression determines B-cell fate. For example, BCR-FcγRIIB co-aggregation leads to inhibition of certain BCR-coupled signaling pathways, terminating cell proliferation, survival and antibody production. Down-regulation of PIP3 levels by FcγRIIB-recruited SHIP is most probably the mechanism underlying the reported inhibition of BCR-mediated activation of Akt, Btk, and PLCγ2, and consequently, the calcium mobilization response. An alternative mechanism of FcγRIIB-SHIP-mediated inhibition involves RasGAP. BCR-FcγRIIB co-aggregation on B cells leads to association of SHIP with the RasGAP-binding protein Dok. Co-aggregation of this complex results in complete inhibition of BCR-induced Erk activation. Please refer to the text for the definitions of abbreviations.

the role of inhibitory receptors and their effectors in prevention of autoimmunity. Our discussion is further refined as we discuss anergy as a mechanism for silencing self-reactive B cells.

BCR SIGNALING PATHWAY

Signal initiation

The B-cell antigen receptor or BCR is a multiprotein structure that is composed of membrane Ig, which serves as the antigen binding subunit, and a non-covalent associated heterodimer composed of Ig-α (CD79a) and Ig-β (CD79b). This complex serves as the signaling subunit. The Ig-α and Ig-β signaling proteins are disulfide-linked heterodimers that contain an *i*mmunoreceptor *t*yrosine-based *a*ctivation *m*otif (ITAM) within each cytoplasmic tail.[14,15] The ITAM is a conserved 18 amino acid motif containing six conserved residues including two tyrosines. The N-terminal of ITAM tyrosine in Ig-α, YEGL, is most strongly phosphorylated upon receptor aggregation and binds to the *src* family protein tyrosine kinases (PTKs) (i.e. Lyn, Fyn, Blk, Lck).[16,17] BCR aggregation results in the phosphorylation of one or more ITAM tyrosines within the Ig-α and Ig-β cytoplasmic tails and this initiates downstream signaling events. Ig-α and Ig-β ITAMs are not equivalent in their contribution to BCR signaling. Several proteins have been shown to associate differentially with Ig-α and Ig-β, which suggests that they may activate distinct downstream pathways.[18] Both Ig-α and Ig-β are essential for BCR chaperone functions, transporting BCRs to the cell surface.[19] Ig-α plays a prominent role in activating PTKs, contains a BLNK docking site, and apparently also contains a negative signaling function.[20–23] Thus, Ig-α and Ig-β are only partially redundant in function and have distinct biological activities.

BCR aggregation activates downstream signaling pathways through the *src* family PTKs and SYK. Following receptor aggregation, initial ITAM phosphorylation of Ig-α/β primarily occurs asymmetrically, with most phosphorylation occurring on the N-terminal or membrane proximal ITAM tyrosines.[23] This phosphorylation is mediated by *src* family PTKs.[17] In part by virtue of their lipid acylation s*rc* family PTKs interact with the non-phosphorylated ITAMs of the resting BCR.[18,24] Maximal receptor signaling requires the binding of phosphorylated ITAMs to *src* family kinase SH2 domains, which amplifies ITAM phosphorylation and the subsequent recruitment and activation of downstream cytosolic tyrosine kinases, such as Syk. Association of BCRs with *src* family PTKs may further be enhanced by the propensity of ligand aggregated molecules, but not monomeric BCRs, to partition into glycosphingolipid-rich microdomains or lipid rafts of the plasma membrane that have been shown to contain increased concentrations of PTKs.[25] Binding and activation of Syk requires recognition of two ITAM phosphotyrosines via its tandem domains.[26,27] The spacing of ITAM phosphotyrosines by ~12 residues is critical for binding Syk's SH2 domains, which are in fixed orientation to one another. Syk activation, and thus Ig-α or Ig-β biphosphorylation, is critical for all downstream signaling.

The ordered dual phosphorylation of ITAMs and activation of Lyn, Syk, and Bruton's tyrosine kinase (Btk, a Tec family PTK) are essential for proper initiation of BCR signal transduction. Deficiencies in any of these result in defective and aberrant B-cell development and function.[28–32] The protein tyrosine kinase Lyn is believed to be primarily responsible for phosphorylating Ig-α/β ITAM tyrosines. Lyn plays a unique role in BCR signaling as it activates both positive and negative signaling circuitry.[33] While the positive role of Lyn is redundant, as demonstrated by normal B-cell development in the bone marrow of Lyn-deficient mice.[34] its inhibitory role in BCR signaling is not. Lyn's inhibitory signaling function depends on its ability to phosphorylate receptors such as FcγRIIB, PIR-B, LMIR, and CD22, as well as the adaptors such as Dok. These inhibitory co-receptors contain *i*mmunoreceptor *t*yrosine-based *i*nhibitory *m*otifs (ITIMs) that recruit phosphatases, such as src homology 2 (SH2) domain-containing inositol 5′-phosphatase (SHIP)-1 and SH2 domain-containing tyrosine phosphatase (SHP)-1.[35–37] Recruited phosphatases suppress BCR signaling by dephosphorylating and deactivating signal transducers.

The outcome of BCR signaling is determined by the balance between kinase and phosphatase activity. Thus, Lyn plays a central role in the

equilibrium between activation and inhibition of B-cell signaling pathways, determining, at extremes, B-cell tolerance versus autoimmunity. This latter hypothesis is further supported by studies in which mice overexpressing or deficient in Lyn demonstrated breakdown of self-tolerance, and developed circulating autoantibodies, and lupus-like nephritis.[38–40]

The recruitment and activation of Syk is essential to couple the BCR to downstream signaling events.[41] Studies in Syk-deficient B cells showed a profound defect in BCR-mediated activation of downstream signaling pathways while *src* family PTK activation and Ig-α/β phosphorylation remained intact. Singly phosphorylated ITAMs, or chimeric Ig-α or Ig-β in which one of the tyrosines is absent, do not bind and consequently fail to activate Syk kinase.[26] Thus recruitment of Syk to doubly phosphorylated Ig-α and/or Ig-β ITAMs results in activation of the kinase and initiation of multiple distinct downstream signaling pathways.[42] For example, the activation and recruitment of Lyn and Syk to the BCR complex both precede and influence the activity of Btk, a cytoplasmic tyrosine kinase that is required for the sustained calcium influx that follows B-cell activation.[43]

Loss of function mutations in Btk affects B-cell development and B-cell activation in response to antigen.[44] In humans this type of mutation results in the disease X-linked agammaglobulinemia (XLA).[45,46] This disorder is characterized by the absence of mature B cells in the periphery and a serious deficiency of serum antibodies.[45] In mice, Btk inactivation results in a disorder called X-linked immunodeficiency (*xid*).[47,48] Studies using *xid* mice suggest that disrupting the kinase function of Btk could result in desensitization of B-cell signaling and possibly provide a therapeutic effect in autoimmune disorders, including RA.[49] However, several lines of recent evidence challenge the positive role of Btk in regulation of BCR signaling and suggest that Btk may be required for tolerance. Patients with XLA had increased numbers of self-reactive B cells in the periphery and failed to establish proper B-cell tolerance.[50] Btk-deficient B cells obtained from these patients display unusual Ig light chain repertoires showing impaired secondary recombination regulation, which indicates that receptor editing, one of the mechanisms that normally ensures B-cell tolerance, may be defective. Interestingly, in a recent study conducted by the same group, similar self-reactive B cells were detected in RA patients, suggesting that Btk may be essential for regulation of B-cell tolerance in humans.[51] It is not clear from this study whether occurrence of self-reactive B cells from RA patients was associated with defects in Btk, or other B-cell intrinsic defects, or whether the association between B-cell self-reactivity and Btk deficiency observed in XLA patients was just an outcome of genetic co-segregation with unknown mechanisms. These findings, however, suggest that Btk deficiency may allow the release of self-reactive B cells into the periphery. Studies are ongoing in an effort to delineate the role of Btk as a therapeutic target for treatment of B-cell-mediated diseases.[52]

The propagation of downstream BCR signals requires that a number of effector molecules become activated via tyrosine phosphorylation after the proximal signaling molecules (i.e. Lyn, Syk, Btk) are activated. A second mechanism by which Syk couples the BCR to downstream signal transduction molecules is by its interaction with and subsequent phosphorylation of the adaptor molecule B-cell linker protein (BLNK, also known as SLP-65 or BASH). BLNK acts as a platform for effector molecule assembly and transduces initial BCR-proximal events into several divergent signaling pathways (Figure 4.1).[53–56] Particularly important events are the recruitment and activation of PLCγ2, and elevation in intracellular calcium ($[Ca^{2+}]_i$).

The adaptor molecule BLNK is essential for PLCγ2 recruitment from the cytosol to the plasma membrane and for coupling BCR aggregation to calcium influx.[53,56,57] Syk rapidly phosphorylates BLNK following BCR aggregation and provides a primary docking site for the SH2 domain of PLCγ2, as well as other effector and adaptor molecules involved in BCR signaling.[53–55] For example, phospho-BLNK has been shown to associate with the SH2 domain of Btk, which is significant since dual phosphorylation of PLCγ2 by Syk and Btk is required for optimal activation of PLCγ2. In the absence of BLNK, B cells fail to recruit PLCγ2 to the plasma membrane

and have severely impaired distal BCR signaling.[54] Furthermore, the guanine exchange factor, Vav, and adaptor complex of Grb2/SOS also associate with phosphorylated BLNK and can activate Rac and Ras. BLNK recruits other adaptors, such as Nck, which associates with cytoskeletal elements and has been proposed to connect BCR signaling to morphological reorganization and cellular migration.[58,59] BLNK$^{-/-}$ mice exhibit attenuated but not abolished BCR-mediated calcium mobilization, suggesting that partially redundant mechanisms must exist for BCR-mediated PLCγ2 activation.[60] A potential candidate mediator of this function is cytosolic adaptor Bam32, which contains single pleckstrin homology (PH) and SH2 domains, the latter shown to associate with PLCγ2.[61] Ablation of Bam32 in B cells results in a decreased BCR-mediated calcium influx and proliferation.[62,63] These findings suggest that alternative and often redundant pathways are activated following BCR ligation.

Signal propagation and integration: role of inositol lipids

BCR signal transduction involves a complex network of interactions. For example, BCR-mediated activation of calcium mobilization does not depend solely on the linear activation of Lyn, Syk, Btk, BLNK, and PLCγ2. Inner leaflet membrane phospholipids are of paramount importance to B-cell signaling. Ligation of the BCR leads to the activation of PI-3K, which phosphorylates plasma membrane phosphatidylinositol 4,5-biphosphate [PI(4,5)P2] yielding phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P3]. PI(3,4,5)P3 is critical to retain multiple PH domain-containing cytosolic proteins at the membrane and also to co-localize PH domain containing proteins that may function in same signaling pathway, e.g. Akt (PKB) and phosphoinositide-dependent kinase-1 (PDK-1).[64,65] Although the activation/recruitment of Lyn and Syk to the BCR complex both precedes and influences the activity of Btk, PI(3,4,5)P3 production is a rate-limiting step in Btk function. PI(3,4,5)P3 production is critical for the translocation and activation of Btk, and the subsequent Btk-mediated phosphorylation of PLCγ2. Moreover, the subcellular localization and activity of Btk are regulated by PH domain binding. In mice with X-linked immunodeficiency (Xid) there is a point mutation in the PH domain of Btk which prohibits recruitment to PI(3,4,5)P3 and results in defective BCR signaling and impaired B-cell maturation and responsiveness.[47,66]

PI-3K-dependent activation of PLCγ2 causes the mobilization of calcium from both intracellular and extracellular stores through cleavage of the ubiquitous plasma membrane lipid phosphoinositide PI(4,5)P2 into the second messengers I(1,4,5)P3 and DAG. Pharmacological inhibitors of PI-3K completely abolish BCR-mediated calcium mobilization.[67–69] B cells deficient in effectors involved in PI-3K recruitment, such as CD19, exhibit diminished PI(3,4,5)P3 production, PLCγ2 activation, and calcium mobilization.[69–73]

Elevated $[Ca^{2+}]_i$ levels are required for the activation of certain transcription factors that are necessary for B-cell activation and survival, such as NF-κB and NF-AT.[74–78] DAG activates conventional protein kinase C (PKC) isoforms that regulate the MAPK family (i.e. ERKs, JNKs, SAPKs, p38).[79,80] Following activation of these kinases, different sets of transcription factors are phosphorylated, e.g. Elk-1 and c-Myc by Erk, c-Jun and ATF-2 by JNK, and ATF-2 and MAX by p38 MAPK (Figure 4.1). It is the profile of these activated transcription factors that determines B-cell fate.

Signal modulation: role of ITIM-containing proteins and their effectors in prevention of autoimmunity

The strength of the BCR signal is determined in part by co-receptors and accessory molecules that either augment or attenuate the potency of the signal. The temporal and spatial regulation of these processes ultimately defines signal quality and quantity.

It is important to note that several studies have demonstrated genetic alterations in BCR co-receptors in patients with autoimmune diseases. For example, in SLE, polymorphisms were identified in the genes that encode FcγRIIB,[81–84] programmed cell death 1 (PD-1),[85] and CD22.[86] Similarly, alterations in the levels of CD19.[87] functional CD45,[88] and SHP-1[88,89] have been observed

in patients with B-cell-mediated autoimmune diseases.

In a simplistic model of BCR signaling, co-receptors can be classified according to whether they increase or decrease the threshold for B-cell activation and survival after co-aggregation. Those that increase the threshold, dampen the immune response, while those that decrease the BCR signaling threshold increase immune responses. Thus, an increase in BCR signaling threshold may result in immunodeficiency, while a decreased BCR threshold may result in autoimmunity. For example, CD45 and CD19/CD21 co-receptor complex decrease BCR signaling threshold and act as positive regulators. Negative regulators include FcγRIIB, CD22, CD72, the paired immunoglobulin-like receptor (PIR-B), and the myeloid-associated immunoglobulin-like receptors (MAIRs or LMIRs), which are characterized by content of ITIM signaling domains.

Most inhibitory receptors recruit SH2 containing phosphatases and function through one of two pathways. ITIMs in FcγRIIB, and MAIR recruit the inositol phosphatase SHIP-1, while those in CD22, CD72, and PIR-B recruit the protein tyrosine phosphatase SHP-1. SHP-1 dephosphorylates proteins in the signalsome thus dampening signaling and SHIP-1 converts PI (3,4,5)P3 to PI(3,4)P2. The inhibitory effects of SHIP-1 can be more global, modulating signaling by distantly stimulated PI3 kinase-dependent receptors, while the inhibitory effect of SHP-1 is localized to the signalsome in which it is engaged. The importance of these inhibitory pathways to autoimmunity is demonstrated by the autoimmune diseases seen in SHP-1 deficient (*moth eaten)* mice.[90] We have observed production of self-reactive antibodies in SHIP-1-deficient mice (K Merrell and JC Cambier, unpublished observations).

A detailed description of co-receptors and cell surface molecules that modulate the BCR signal was reviewed in several recent articles.[33,91,92] Here we will present an overview of the ITIM-containing inhibitory co-receptors including: FcγRIIB, CD22, PIR-B, and MAIRs/LMIRs. We will also discuss the recent findings regarding CD45 and autoimmunity.

IgG-containing immune complexes can co-ligate BCR and the low affinity IgG receptor, FcγRIIB, leading to inhibition of BCR-induced phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P3] accumulation, proliferation, and calcium mobilization.[93] Inhibition through FcγRIIB is primarily mediated by its ITIM region, which recruits SHIP-1.[36,94,95] SHIP-1 degrades PIP3 to PI(3,4)P2 and recruits the adaptor molecule downstream of kinase (DOK), which acts to inhibit other downstream signaling pathways (i.e. Ras/Erk activation) (Figure 4.1).[96] Thus, co-ligation of FcγRIIB with BCR provides a mechanism that may promote deletion of low-affinity self-reactive B cells during high affinity maturation and controls autoantibody production.[96] Conversely, ablation of FcγRIIB renders mice susceptible to experimental autoimmune diseases upon immunization with autoantigens and they spontaneously develop SLE-like syndrome on the C57BL/6 background.[97,98] This spontaneous autoimmunity is strain-specific, e.g. BALB/c/FcγRIIB deficient mice do not show any autoimmunity, suggesting the presence of other genetic factors that influence disease susceptibility. These findings may also imply the existence of other inhibitory mechanisms that play a compensatory role in the regulation of autoimmune diseases in different strains of mice.[98] Indeed, a locus in chromosome 1, which contains the FcγRIIB gene, is associated with autoimmunity in multiple mouse models (i.e. NZB, BXSB).[99,100] Polymorphisms in the transmembrane region of the FcγRIIB gene were identified in a study done in 193 Japanese patients and 303 healthy controls, where homozygosity for I232T polymorphism was significantly increased in SLE patients compared with controls.[82] FcγRIIB-I232T polymorphism was associated with reduced FcγRIIB-mediated inhibition of B-cell proliferation.[83]

Recent work by Okazaki et al., reported that co-deficiency of two inhibitory receptors, FcγRIIB and PD-1, induced an autoimmune disease state, hydronephrosis, accompanied by self-reactive antibody production in BALB/c mice, which was not observed in either FcγRIIB- or PD-1-deficient mice.[101] PD-1 is a type 1 transmembrane protein that belongs to the Ig superfamily and contains cytoplasmic tyrosine residues within a consensus ITIM. Studies have shown that PD-1 provides a signal that

limits response to antigen by recruiting SHP-2.[102] PD-1$^{-/-}$ mice develop lupus-like glomerulonephritis and arthritis on the C57BL/6 background.[103] In humans, gene mapping studies suggested that there was an association between 7 and 12% of SLE patients and a SNP in PD-1.[85] Although the mechanism of FcγRIIB and PD-1 complementarity is not clear, in their study, Okazaki et al. clearly demonstrated that FcγRIIB and PD-1 cooperatively regulate autoimmunity in the mouse, suggesting that some human autoimmune diseases may also be regulated by the combination of dysfunction of human FcγRIIB and PD-1 genes. These findings may suggest that polymorphisms affecting the strength and quality of Ig signaling are important in determining the genetic susceptibility or resistance to autoimmune disease. Predisposition to human autoimmunity occurs when different combinations of susceptibility alleles combine to reach some threshold.

Consistent with these observations, it has been shown that CD72 polymorphisms, which are associated with the relative quantity of an alternative splicing product, and also with the presence of nephritis among the patients with SLE, may modify susceptibility to human SLE through interacting with FcγRIIB.[104] CD72 functions as a negative regulator of BCR signaling.[105] Interactions were also identified between FcγRIIB and CD19, where FcγRIIB-mediated inhibition can be mediated through selective dephosphorylation of CD19 leading to abrogated PI-3K recruitment.[95]

Finally, a recent study by McGaha et al. demonstrated that the partial restoration of FcγRIIB levels on B cells in lupus-prone mouse restored tolerance and prevented autoimmunity.[106] The physiologic consequences of cell-bound IgG and immune complexes are modulated by a balance between activating (i.e. FcγRIA, FcγRIIA, FcγRIII, and FcγRIV) and inhibitory Fcγ receptors and include immune regulatory and inflammatory responses.[98,107–109] B cells express FcγRIIB but not other Fc receptors. Thus, findings from McGaha et al. illustrate an important role for FcγRIIB in regulation of a common B-cell check-point, and suggest that relative changes in its expression can result in either tolerance or autoimmunity.[106] Similar observations were also made for CD22.[87]

CD22 is a B-cell-specific inhibitory co-receptor that belongs to the Ig superfamily, and contains seven Ig-like domains and three cytoplasmic ITIMs. CD22 regulates BCR signaling through recruitment of SHP-1 to its ITIM motifs.[110] Activation of SHP-1 regulates the strength of the BCR-induced calcium signal.[111] In this manner, CD22 is thought to control signaling threshold of B cells, preventing overstimulation. CD22$^{-/-}$ mice show higher BCR-mediated calcium signaling, and their B cells show evidence of basal activation, such as expression of activation markers, and increased sensitivity to apoptosis.[112,113] CD22$^{-/-}$ mice may develop high affinity autoantibodies.[112] Also, CD22$^{-/-}$ mice show characteristic changes in B-cell maturation, such as a higher proportion of mature, follicular cells,[114] and a reduced number of marginal zone B cells in the spleen,[115] thought to be direct consequences of increased signaling. However, the effect of CD22 deficiency on BCR signaling is sensitive to the strain of the mice used,[116,117] suggesting a role of other genetic factors in CD22-mediated modulation of BCR signaling.

Recent studies suggested that one of these regulatory factors for CD22 may be its own ligand, namely, sialic acid α2-6 linked to galactose (Siaα2-6Gal). Siaα2-6Gal is a glycan that specifically binds to CD22 *in vivo*.[118,119] The interaction of CD22 with its ligand modulates its activity as a negative regulator of BCR signaling.[120] For example, lupus-prone mice, whose B cells have lower expression of CD22 ligand than those of wild-type mice, have reduced production of autoimmune antiboby.[121] Inhibition of CD22–ligand interactions or the absence of ligands decrease SHP-1 recruitment and increase calcium influx, enhancing BCR signaling.[120,122,123] These studies suggest that CD22 regulates B-cell function *in vivo* in a ligand-dependent manner, with mechanisms still under investigation.[124,125] Interestingly, Siaα2-6Gal is typically found on N-linked glycans of glycoproteins, including those involved in BCR signaling, such as CD45, and IgM.[126–128] Both IgM and CD45 were shown to be CD22 binding partners.[129] Recently, an open-label pilot study of anti-CD22 (epratuzumab) in the treatment of active SLE showed some B-cell depletion but no consistent changes in autoantibody levels.[130] The role of CD22 ligands

in regulation of BCR signaling and utility for therapeutic applications are yet to be determined.

CD45 is a receptor-like protein tyrosine phosphatase that establishes the sensitivity of the BCR to stimulation. Both CD45-deficient mice and humans develop severe combined immunodeficiency (SCID) with defects in B-cell development and function. B cells from CD45-deficient mice are hyporesponsive to BCR stimulation and display reduced calcium responses, demonstrating a positive regulatory role for CD45 in BCR signaling.[131,132] In part, this is accomplished by maintaining an adequate supply of BCR-associated *src* family kinases.[133] CD45 can also negatively regulate signals emanating from BCR. Acting in opposition to CD45 is Csk, which functions to phosphorylate the C-terminal inhibitory tyrosine of the *src* family PTKs, keeping them in a 'repressed' state.[134] Whether CD45 positively and negatively regulates protein kinase phosphorylation depends upon its subcellular localization relative to its substrate and the phosphorylation state of the protein kinases.[135] Recent studies showed that introducing a point mutation into the CD45 juxtamembrane wedge (CD45 E613R) abolished the inhibitory effect of CD45.[136] The analogous point mutation introduced into the germ-line of mice leads to lymphoproliferative disorder and a lupus-like autoimmune disease and autoantibody production.[137] CD45 E613R-mediated negative regulation was also suggested by a recent study, where CD45 E613R B cells were hyperproliferative and have augmented calcium responses.[138] Thus, CD45-deficient and CD45 E613R mice reflect the positive and negative regulatory role of CD45 on B-cell function, with mechanisms still under investigation.

The paired Ig-like receptors (PIRs) and the myeloid-associated immunoglobulin-like receptors (MAIRs or LMIRs) are transmembrane glycoproteins that play a role in BCR regulation. They exist in activating and inhibitory isoforms and are often expressed in pair-like fashion on the same cell.[139,140] The expression of the inhibitory isoform, PIR-B, can have an attenuating effect on BCR signaling, while the activating form, PIR-A, appears to function independently of the BCR.[141,142] PIR-B contains multiple ITIMs, which are constitutively phosphorylated and associated with SHP-1 in B cells.[143,144] The inhibitory form of MAIR, MAIR-I, contains ITIM sequences in its cytoplasmic tail that can recruit SH2-domain containing inhibitory effectors, like SHIP, although much more needs to be worked out regarding MAIR signaling and regulation of the BCR signal.[145]

ANERGY AS A MECHANISM FOR SILENCING SELF-REACTIVE B CELLS: DIFFERENTIAL B-CELL SIGNALING

It was recently estimated that 50–75% of newly produced B cells are self-reactive and must be silenced by tolerance mechanisms.[13,146] Evidence of receptor editing is seen in ~25% of peripheral B cells,[147] and 10% of B cells appear to be silenced by deletion.[148] The remaining self-reactive cells are presumably silenced by other mechanisms, e.g. deletion or anergy. It is also clear that self-reactive B cells develop by somatic mutation during the germinal center response. These are likely silenced by anergy or clonal deletion.

Anergy is a reversible state of unresponsiveness determined by the binding of cognate self-antigen.[149] It is the consequence of reception of signal one (antigen) without signal two (cognate T-cell help, Toll-like receptor agonists). Thus, anergy can be prevented by provision of T-cell help immediately following exposure to antigen.[150,151] The reversibility of anergy suggests that continuous presence of the antigen in the microenvironment is essential to maintain unresponsiveness. Based on the assumption that some self-antigens are tissue-specific, it is reasonable to suggest that loss of anergy *in vivo* could result from the lodging of self-reactive B cells to anatomical sites free of self-antigen. Such a situation could lead to restoration of responsiveness and activation by cross-reactive immunogens, leading to autoimmunity. Understanding molecular mechanisms involved in anergy may provide insights to target autoimmune diseases.

Anergic B cells provide a particularly interesting example of differential BCR signaling leading to altered physiologic responses. Anergic cells persist in the periphery without deletion and receptor editing. Instead, they become refractory to further BCR stimulation.[152] This refractoriness is multifactorial, which begins with a decrease in

the strength of association between mIgM and Ig-α/β.[153] This may result in decreased Ig-α/β, and Syk phosphorylation upon BCR aggregation.[152,154] Anergic cells also exhibit chronic low level increases in intracellular free calcium but are unable to further elevate intracellular calcium upon BCR aggregation.[155] Normally, stimulation of B cells leads to the calcium-dependent activation of NF-AT and NF-κB, both necessary for B-cell activation and survival.[74–76] However, in anergic cells, altered calcium levels result in constitutive NF-AT activation but impaired NF-κB activation.[77,156] Alteration in transcription factor activation in anergic cells may cause their shortened lifespan.

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5

Macrophages in rheumatoid arthritis

Peter LEM van Lent and Wim B van den Berg

Introduction • Resident intima macrophages in rheumatoid arthritis • Differentiation and function of macrophages in RA synovium • Activation of synovial macrophages • Macrophages and joint destruction • Depletion of type A intima cells inhibits onset of arthritis • Final remarks • References

INTRODUCTION

Rheumatoid arthritis (RA) is characterized by chronic inflammation in multiple joints and concomitant destruction of cartilage and bone. Macrophages play a crucial role in both the inflammatory process and tissue destruction.[1–3] Macrophages become activated by the RA process in the synovial tissue, either directly through stimulation with bacterial or viral triggers, or indirectly through T- and B-cell-mediated events. The latter responses can be directed to joint-specific autoantigens, but may also include reactions to persistent viral and bacterial elements. Although RA has been considered an autoimmune process, a crucial autoantigen has not been defined and it seems more likely that multiple candidate triggers are involved. This argues for general therapeutic approaches at a downstream level, making activated macrophages an obvious target.

RA is a systemic disease, with its main expression in body compartments that are surrounded by a synovial lining layer, containing large amounts of macrophages. Such compartments include diarthrodial joints and precipitation of the RA process in such areas underlines the crucial role of tissue macrophages in disease onset. During active arthritis monocytes infiltrate from the blood into the synovium, differentiate into mature macrophages, and form the dominant cell type in the inflamed synovium. However, synovial lining macrophages remain a crucial source of inflammatory mediators and contribute significantly to local cytokine and chemokine production. Of great interest, RA synovial macrophages appear to express deranged levels of Fcγ receptors, and proof is accumulating that an aberrant reaction of macrophages to immune complexes, leading to prolonged activation, contributes to increased and prolonged release of proinflammatory and cartilage destructive cytokines. Therapeutic approaches targeting the macrophage itself or its dominant proinflammatory mediators have already been shown to be efficient in the treatment of RA. Inhibition of the macrophage-derived master cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1 created a major breakthrough in the treatment of this crippling disease. Insight into mechanisms of macrophage activation and mediators involved in that process may provide novel targets for further optimization of therapy.

RESIDENT INTIMA MACROPHAGES IN RHEUMATOID ARTHRITIS

The inside of diarthrodial joints, the preferential site for development of RA, is lined by a layer of cells, usually one to three cells in thickness, which is called the intima. This layer contains two types of cells, the fibroblast-like type B cell and the macrophage-like type A cell, which interdigitate

using cytoplasmic processes.[4] These cells are enclosed within a matrix, probably produced by the lining cell itself, containing collagen type IV, forming a covalently stabilized polygonal framework and a second interlocking polymer network of laminin. Immunohistologic investigations have shown that three of the four constituents forming a basement membrane (collagen type IV, heparan sulfate, proteoglycan, and laminin) are present but that entactin, a sulfated glycoprotein that connects laminin and type IV collagen, is absent. The intima lining sits on compact loose connective tissue bearing a vascular plexus that gives a close contact with the blood vessels. The origin of the type A cell is probably a monocyte, as shown in elegant studies using mice with the Chediak Higashi syndrome. Monocytes of these mice that contain crystals were transferred to control mice and kinetic studies showed accumulation of crystal-containing type A cells in the lining layer.[5] These cells are constantly replaced via the circulation, although the turnover is slow. After selective removal of type A cells in the intima of mice, it takes more than 30 days before the lining cell layer returns to normal levels.[6]

As a first sign of onset of arthritis, intima cells become activated. Intima cells form a strategic barrier within the joint. Substances leaking from the joint, bacterial infections, or immune complexes formed within the synovial fluid first meet this layer and the abundance of receptors expressed by type A cells leads to phagocytosis and activation of these cells. Moreover, this layer lies just above the vascular plexus in the synovium, which also makes these cells very accessible for substances arriving via the bloodstream. Immunolocalization studies have shown that phagocytic intima cells express many proinflammatory factors like cytokines IL-1α, TNF-α, IL-6, IL-15, IL-18, IL-32,[7,8] and chemokines like IL-8 or MCP-1, but also growth factors like GM-CSF and TGF-β.[9] As type A cells produce various chemokines, these cells are involved in attraction of inflammatory cells during the onset of arthritis and probably also in arresting of inflammatory cells within the synovium during the chronic phase.

DIFFERENTIATION AND FUNCTION OF MACROPHAGES IN RA SYNOVIUM

Activation of the lining layer directs the influx of inflammatory cells, such as polymorphonuclear leukocytes (PMNs), lymphocytes (T and B cells), and large amounts of monocytes (Figure 5.1).

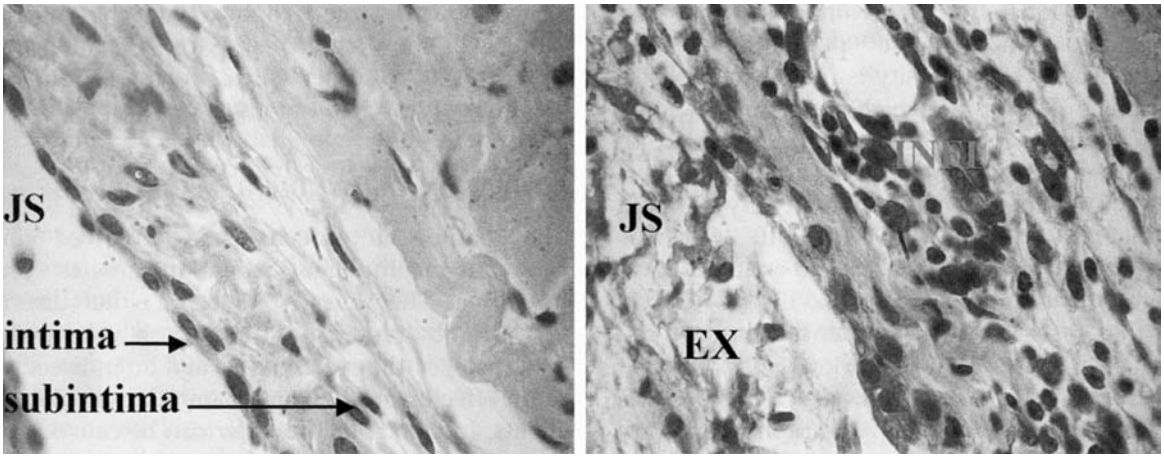

Figure 5.1 Synovial lining layer in knee joints of normal (A) and arthritic (B) mice. JS, joint space; Ex, exudate; Infl, infiltrate. Original magnification ×400. Hematoxylin/eosin staining.

During RA, a number of alterations in the synovial membrane are observed. Synovial lining cells increase many-fold. Type A macrophages still form the predominant population in the hypertrophied intima, approaching 50–70% of cells.[4] Superimposed on this is a highly vascular subintima filled with mononuclear cells, including T and B cells and large numbers of macrophages, often forming aggregates around the blood vessels. Most of the macrophages are thought to stem from monocytes which have infiltrated into the joint, where they diffentiate into macrophages.[10] A small proportion may be derived from locally dividing mononuclear phagocytes. Chemokine receptor expression is different on RA monocytes in peripheral blood and synovial fluid (significantly higher CCR3, 4, and 5 levels in synovial fluid). CCR1 and CCR2 seem to be crucial for monocyte recruitment. CCR3 and CCR5 may play a role in monocyte/macrophage tissue migration or retention. Therapeutic application of chemokine inhibitors seems hampered by redundancy.[11] *In vivo*, generation of monocytes is controlled by various growth factors including IL-3, GM-CSF, and M-CSF. These factors are abundantly present in the RA joint, and are potent stimulators of $CD34^+$ stem cells, which have been found to infiltrate the joints. As such, local production and maturation may contribute to the total macrophage cell mass.

Monocyte differentiation into macrophages in the RA synovium is highly versatile. Many differentiation stadia are found, reflecting various subpopulations of cells that are probably involved in different aspects of immune and effector mechanisms. Some of the maturation stages are now identified by CD markers, as listed in Table 5.1. It is a recent finding that an unexpectedly large subpopulation of $CD68^+$ macrophages express DC-SIGN, a receptor which normally is expressed only on dendritic cells (DCs).[12] DC-SIGN is a crucial receptor involved in the initial interaction with ICAM-3-containing naive T cells, which are abundantly present in RA synovia, and blockade of DC-SIGN prevents binding and subsequent antigen presentation. It may suggest that these DC-SIGN-positive macrophages contribute to local immune activation, apart from the scant numbers of fully matured DCs.

Table 5.1 CD markers on human tissue macrophages

Functional aspects	*CD markers*
Adhesion and migration	CD33, CD169, CCR2, CCR5
Cytokine receptors	CD25, CD119, CDw121b, EMR-1
Fcγ and complement receptor (CR)	CD16, CD32, CD64, CD23
Microbial pattern recognition receptors	CD11b, CD204, CD68, CD14, CD206
T-cell activation	MHC class II

Differences between type 1 and type 2 cytokine polarized macrophages

	Type 1	*Type 2*
Adhesion/migration	CCR-5	CCR-2
Microbial pattern recognition receptor	CD206 Mannose R	CD206++ Mannose R

Expression of different surface markers probably has consequences for macrophage effector function, ranging from more proinflammatory to anti-inflammatory activity. Such a mixture of cell types was found earlier in the chronically inflamed lung, where proinflammatory and suppressor macrophage populations were identified.[13] This diversity is in line with findings in RA synovia. Only a limited number of $CD68^+$ cells produce TNF and IL-1, whereas others produce none or even anti-inflammatory cytokines like IL-10 and TGF-β. Further research into the identification of cell surface markers akin to various subgroups of macrophages is warranted, as it may provide targets for more selective anti-inflammatory therapy.

Normal tissue macrophages and young monocytes that have recently immigrated into normal tissues are quiescent. In an activated state, as found in the synovium of RA patients, macrophages acquire multiple functions. Under conditions of cell stress, macrophages produce alarmins, or damage-associated molecular pattern proteins (DAMPS).

Important members of DAMPS are S100 proteins, characterized by calcium binding motifs, and to date more than 20 members have been described. S100A8 and A9, formerly called MRP 8 and 14, are not only markers of activation, but also display prominent proinflammatory activity when released.[14,15] S100 A8/9 induced marked TNF and IL-1 production and expression of S100A8/9 is seen at sites of joint erosion.

Activated macrophages also elaborate chemokines involved in PMN, monocyte, and T-cell migration. Integrins and vascular cell adhesion molecules (VCAMs) are up-regulated under the influence of IL-1,TNF-α, and interferon (IFN)-γ release. Moreover, reactive oxygen and nitrogen intermediates are produced, eliciting local tissue damage. Production of cytokines like platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and TNF-α enhance the growth and proliferation of lining macrophages through paracrine interaction with the fibroblast-like lining cells. Activated macrophages also release angiogenesis-promoting factors like TGF-β, angiotropin, and vascular endothelial growth factor (VEGF), responsible for neovascularization and further increase of the subintimal layer.

Apart from a role in synovial activation and growth, matured macrophages may function as antigen-presenting cells (APCs), initiating local antigen-specific T- and B-cell responses, and herein amplifying immune-mediated macrophage activation. Moreover, macrophages producing TNF, IL-1, and destructive enzymes will contribute to cartilage erosion. The ultimate fate of macrophages in the RA synovium is not known but a large proportion of the $CD68^+$ lining cells show signs of apoptosis.[4] A minority may traffic to other sites like remote secondary lymphoid organs.

ACTIVATION OF SYNOVIAL MACROPHAGES

The pathogenic mechanisms involved in synovial macrophage activation are as yet unknown. Theoretically, there is either direct activation by phlogistic stimuli such as bacteria or viruses, or the system is turned on indirectly, as an effector mechanism of immune-mediated events. In principle, the latter can be caused by T- and B-cell-mediated recognition of exogenous antigens reaching the joints, including bacteria and viruses, or by immune responses to joint-specific autoantigens (Figure 5.2). Chronicity of the process of macrophage activation may be due to persistence of stimuli, which is obvious in the case of autoantigens, and/or deranged responsiveness of the cells, acquiring tumor-like properties. In particular, viral stimuli have been suggested to be involved in the latter process, although a viral contribution to chronicity of RA is still to be proven.

Endogenous bacterial fragments enter the joint as a continuous process and, when poorly degraded by the macrophages, do form an obvious persistent stimulus for macrophage activation. It was identified that bacterial DNA fragments bearing a CpG motif are powerful stimulants of macrophages.[16] More recent developments provided further insight into receptors involved in cell activation by environmental stimuli. At present up to 10 TLRs (Toll-like receptors) are described. Bacterial cell wall fragments stimulate TLR2,[17,18] lipopolysaccharide (LPS) interacts with TLR4, and viruses mainly trigger TLR3 and 7. CPG motifs trigger TLR9. Additional diversity in response patterns is created by receptor cross-talk and differential use of adapter molecules. The TLR4 receptor is intriguing since it is not only stimulated by LPS but also by breakdown fragments of connective tissue components. This pathway stimulates TNF and IL-1 production and links tissue damage as a sustaining factor of chronic joint inflammation. Regulation of tolerance to these persistent triggers is a delicate

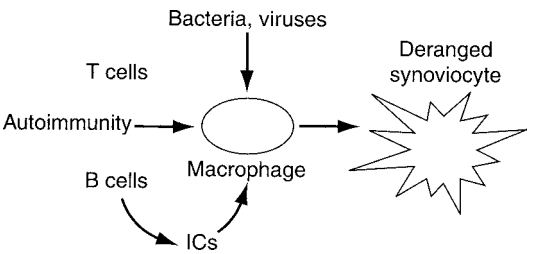

Figure 5.2 Stimuli involved in synovial macrophage activation.

process and disturbances in receptor activation may underlie autoimmune responses.[19] In fact, deranged TLR4 signaling and excessive cytokine production have been demonstrated in RA patients.[20]

When T-cell tolerance against bacterial fragments is lost, T cells are turned on locally and their products activate the macrophage. As a further element of local immune events, antibodies can be generated, forming immune complexes at the site and stimulating macrophages through their Fc receptors. In principle, any protein antigen reaching the joint in sufficient quantities and retained in avascular joint structures, either due to charge-mediated binding or antibody-mediated trapping, may function as a persistent trigger. As such, the difference between autoantigens of joint structures or endogenous and exogenous proteins sticking to joint structures is mainly semantic, although it may be argued that regulation of tolerance is different.

Animal model studies have identified a number of potential autoantigens, including cartilage-derived collagen type II, proteoglycan, GP-39, citrullinated proteins, and even the ubiquitously expressed enzyme GPI (glucose phosphate isomerase), showing cartilage-adhering potential.[21–23] There is reason to believe that the antigen causing RA might be associated with cartilage, since removal of cartilage at joint replacement is sufficient to silence such a joint, without the need of synovectomy. Nevertheless, it seems unlikely that one particular autoantigen is at the base of RA pathology and a multiple trigger concept is more obvious. This leaves us with therapeutic options that interfere with general elements of immune functions, such as suppressive T-cell cytokines. Attempts to use joint-specific antigens to induce tolerance and to generate bystander suppression of nonrelated T-cell responses were successful in animal models, but convincing effects and therapeutic applicability in RA patients have yet to be shown.

Efforts to treat RA by depleting CD4 T cells, using monoclonal antibodies or immunotoxins, have been disappointing and questioned the relevance of T cells. However, it is now clear that different subsets of T cells exist, ranging from IFN-γ- and IL-17-producing effector cells to regulatory T cells, and more selective targeting of subsets seems warranted. The recent development of therapeutic targeting of the T-cell activation marker CTLA-4 looks promising and underlines the importance of T cells in RA.

T cell macrophage activation and regulating cytokines

The belief in T-cell activation of macrophages was reduced by the difficulty of finding significant amounts of IL-2 or IFN-γ in inflamed RA synovia. However, the recent identification of IL-17 as a pathogenic mediator of a dinstinct subset of Th17 cells[24,25] and its clear presence in many RA patients[26,27] boosted renewed interest. This revival in thinking is strengthened by the old finding of virtual absence of the counteracting cytokine IL-4. IL-17 itself stimulated the production of IL-1 and TNF-α by human macrophages and synovial fibroblasts and amplified the effect of IL-1 and TNF-α on synoviocytes. Furthermore, data from animal models support the arthritogenic potential of this cytokine. When IL-17 is overexpressed in the joints of mice with experimental collagen type II arthritis (CIA), it strongly aggravates joint inflammation and cartilage destruction, independent of IL-1.[28,29] In addition, it enhances immune complex-mediated arthritis and renders the arthritis independent of TNF. Blockade of IL-17 in classic CIA significantly ameliorated the disease and combined TNF/IL-17 neutralization was superior.

A further argument for IL-17 and T-cell involvement is the abundance of IL-15 in RA synovia. This cytokine is produced by macrophages and is a major stimulus of T-cell activation. Such IL-15-exposed T cells become TNF-producing cells and are potent activators of macrophage TNF production, in an IL-17- and cell–cell contact-dependent fashion.[30–32] Intriguingly, apolipoprotein A-I blocks contact activation and seems a natural regulator.[33]

Additional cytokines involved in boosting T-cell responses are IL-12 and IL-18.[34,35] IL-12 and IL-18, in particular, are found in significant quantities in RA synovia and are products of

activated macrophages. Although IL-18 alone is not a potent maturation factor, it markedly synergizes with IL-12 in Th1 maturation. Both mediators are induced in macrophages by bacterial activation and this provides the intriguing possibility that bacteria are not only phlogistic triggers but also amplify autoimmune responses in the joint through release of IL-12 and IL-18 (Figure 5.3). It may fit with the often suggested relationship between bacterial infections and arthritis. Apart from septic arthritis, arthritis occurs in patients with Lyme disease and infections of the throat and the gastrointestinal tract. In animal models IL-12 was shown to promote an acute, nondestructive joint inflammation to a chronic, destructive process. Early neutralization of IL-12 as well as IL-18 markedly reduced autoimmune collagen type II arthritis, but also nonimmune Zymosan arthritis, underlining that these cytokines are both immune-potentiating as well as directly proinflammatory.[36–38] However, when neutralization is done in established stages of arthritis, opposite effects are noted. With the identification of IL-23 further insight is now provided. IL-23 knockout (KO) mice are protected from disease, whereas selective IL-12 KO mice exhibit more severe disease.[39] It is becoming clear that not IL-12, but IL-23, is the main driving force of Th17 cells. In fact, IL-12/IFN-γ could mediate regulatory functions in a ying-yang relationship with IL-23/IL-17. IL-6, formerly seen as a driver of the Th2 pathway and responsible for inhibiting excessive development of the Th1 population, is now considered a major driver of Th17 differentiation, with IL-23 as a maturation factor. This would fit well with the marked therapeutic effect of IL-6 neutralization in RA trials.

Macrophage activation induced by immune complexes

One of the characteristic features of RA is the presence of high titers of autoantibodies. Impaired B-cell responses have been found within RA synovium and may be caused by impaired antigen presentation or clonal deletion. Autoantibodies are released in large amounts and target many antigens, forming immune

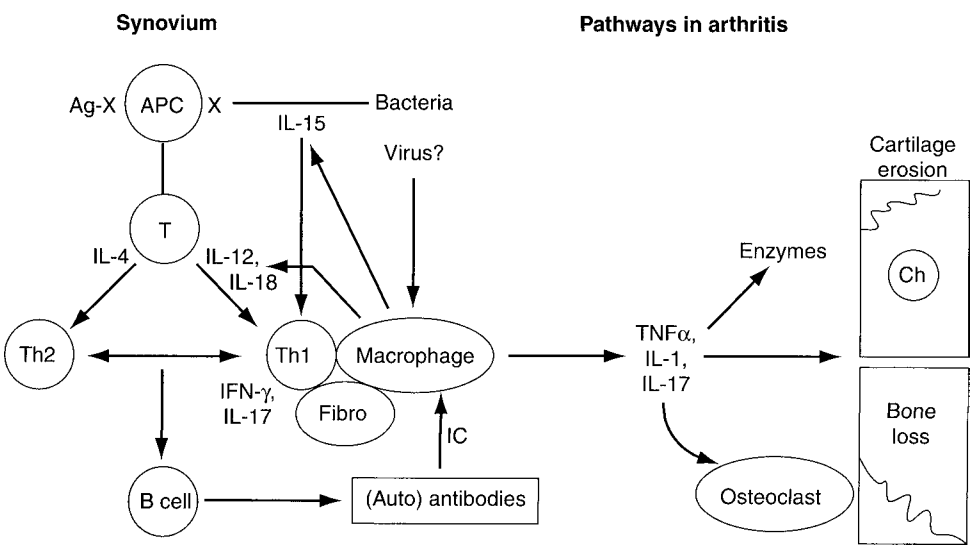

Figure 5.3 Cytokines in synovial activation and tissue destruction.