On the role of regulatory T cells and microbial products in the control of T and B cell immune responses

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Abstract

Self-nonself discrimination is the basic property of the immune system that allows rejection of pathogens without attacking self-specific structures. Discrimination of self and nonself is based on both structural features of the antigen as well as on the context, in which the antigen is encountered. While specific recognition of nonself-antigens in presence of microbial products induces potent immune responses, several suppressing mechanisms exist that limit immune reactions to specifically recognized antigens in a context devoid of microbial agents.

A prominent example of suppressing mechanisms is regulation of T cell responses by regulatory T cells (Tregs). Treg-mediated suppression is induced upon T cell receptor (TCR) stimulation of Tregs and therefore dependent on Treg specificity. We found that TCRs derived from mouse regulatory and conventional T cells cover a similar spectrum of affinity towards self-antigens, which implies that Tregs express a similar TCR repertoire as conventional T cells. This result suggests that Treg-mediated suppression is not induced by recognition of self-antigen but rather regulated by recognition of the immunological context.

Characterization of Treg function in autoimmune diseases is hampered by the fact that Tregs in an inflamed tissue cannot be discriminated from infiltrating activated conventional T cells. We report that at the site of autoimmune reactions Tregs can be distinguished from activated T cells by the expression of CD27. Using this novel Treg marker we show that the suppressive activity of Tregs isolated from inflamed tissues is not limited in vitro, which precludes a Treg-intrinsic defect. However we have observed that cytokines as IL-7 and IL-15, which are present in the autoimmune inflammatory milieu, potently block Treg-mediated suppression in vitro. These results suggest that in vivo IL-7 and IL-15 may interfere with Treg function at the sites of ongoing autoimmune reactions.

Recognition of a context containing signs of microbial invasion leads to the counterbalancing of suppressing mechanisms and to the induction of potent immune responses. Such a context is characterized by the presence of pathogen-associated molecular patterns (PAMPs) that are recognized by Toll-like receptors (TLRs) expressed on a variety of cell types. We show that TLR triggering is critically required for the induction of productive T-dependent human naïve B cell responses. B
cell receptor (BCR) triggering and T cell help induced initial B cell proliferation but were not sufficient to sustain prolonged survival and accumulation of B cells. Extensive proliferation, isotypic switch and differentiation to Ig-secreting cells were promoted by microbial agents acting on TLRs expressed by naïve B cells upon BCR stimulation. This finding demonstrates that humoral immune responses (as cellular immune responses) are critically dependent on context discrimination via detection of PAMPs.
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<tr>
<td>AID</td>
<td>activation induced cytidine deaminase</td>
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<td>AIRE</td>
<td>autoimmune regulator</td>
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<td>APC</td>
<td>antigen presenting cell</td>
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<td>BCR</td>
<td>B cell receptor</td>
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<td>CFA</td>
<td>complete Freud’s adjuvants</td>
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<tr>
<td>CFSE</td>
<td>Carboxy-fluorescein diacetate succinimidyl ester</td>
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<td>DC</td>
<td>dendritic cell</td>
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<td>IFN</td>
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<td>IL</td>
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<td>JIA</td>
<td>juvenile idiopathic arthritis</td>
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<td>LPS</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>PAMP</td>
<td>pathogen-associated molecular patterns</td>
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<td>PB</td>
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<td>peripheral blood mononuclear cells</td>
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<td>PRR</td>
<td>pattern-recognition receptor</td>
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<td>SF</td>
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<td>regulatory T cells</td>
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Background

Introduction

The function of the immune system is the protection of the organism from invasion of harmful pathogens. The essential prerequisite for this task is the ability to reliably discriminate between self and nonself in order to allow elimination of intruders without attacking self-structures. Self-nonself discrimination occurs at the level of both the antigen and the context, in which the antigen is encountered.

At the level of the antigen, self-nonself discrimination is mediated by specific deletion or inactivation of self-reactive cells. A reduction of the pool of self-reactive cells is primarily achieved by mechanisms of central tolerance. During T-cell development, thymocytes bearing T cell receptors (TCRs) of high-affinity for self-antigens are eliminated through negative selection, while thymocytes with TCRs of low to intermediate affinity to self-antigens are allowed to continue maturation. A key function for negative selection has been attributed to the transcription factor AIRE, which induces the promiscuous expression of tissue-specific antigens by thymic epithelial cells (TECs) and thus enables the thymic deletion of T cells reactive to peripheral antigens. Similarly, maturation of B cells is critically dependent on the avidity of the B cell receptor (BCR) to self-antigens. Developing B cells that recognize self-antigens expressed in the bone marrow are induced to undergo receptor editing and alter their specificity by exchanging the immunoglobulin (Ig) light chain. Immature B cells that do not succeed in converting self-reactive into nonself-reactive surface Ig die by apoptosis through negative selection.

The mechanisms of central tolerance purge the pool of lymphocytes from highly autoreactive cells, but are nevertheless not sufficient to confer absolute protection from autoimmunity, as cells of intermediate affinity towards self-antigens escape negative selection and complete maturation. Indeed, three additional fail-safe mechanisms of peripheral tolerance exist that prevent autoimmunity by controlling self-antigen induced immune responses in the periphery. The first mechanism is based on ignorance of antigens that are expressed at a very low level or that are sequestered from recirculating cells by means of structural barriers. Ignorance is not a permanent way of inducing tolerance as lymphocytes specific for these antigens
Background

persist and pose a potential threat in altered circumstances characterized by increased expression of the antigen or accidental breakage of the structural barrier\(^8\). The second mechanism consists in induction of anergy and deletion of self-reactive lymphocytes that repeatedly encounter their antigen in a non-inflammatory environment. Anergic cells are in a state of hypo-responsiveness to antigenic stimulation, have lost the ability to mount a productive immune response and are prone to die\(^9,10\). The third mechanism to control autoimmunity is imposed by regulatory T cells (Tregs, see below). Tregs suppress CD4\(^{+}\) T cell activation and expansion via direct cell-cell interactions. Elimination of Tregs immediately after birth or inherited lack of Tregs leads to severe autoimmune diseases\(^11\)\(^-\)\(^13\).

The second factor enabling self-nonself discrimination is recognition of the context, in which an antigen is encountered. Context discrimination impacts on the decision whether antigen-specific activation results in productive or in abortive immune responses (Fig. 1). Reliable detection of microbial invasion is enabled by recognition of pathogen-associated molecular patterns (PAMPs), which include a large panel of molecular structures that are characteristic for microbial species while being absent from body tissues. PAMPs are generally recognized by receptors of the innate immune system (see below)\(^14\). Triggering of innate receptors by PAMPs induces in the immune system a state of increased alert, promotes a crosstalk between the innate and the adaptive immune system\(^15\) and provides enhancing stimuli capable of counterbalancing the above-mentioned suppressive mechanisms. For instance in the case of ignored antigens a breakage of the structural barrier does not automatically lead to autoimmunity. Rather it has been shown that autoreactive cells gaining sudden access to their previously sequestered antigen do not start autoimmune reactions unless PAMPs are present. PAMPs induce an inflammatory status of the target organ, which leads to productive T cell priming followed by destruction of the target organ\(^16\). Similarly, for freely accessible antigens the presence of microbial products impacts on the decision whether stimulation of T cells results in activation or anergy\(^17\). Self-antigen presenting immature dendritic cells (DCs), present only in a context devoid of any signs of danger, stimulate T cells in a suboptimal way that results in anergy of interacting self-antigen specific T cells. In contrast, upon detection of a context of danger and uptake of foreign antigen, nonself-antigen presenting mature DCs express costimulatory molecules and are able to induce productive priming of nonself-specific
T cells. And finally, the responsiveness of T cells to Treg-mediated suppression is severely reduced in presence of PAMPs that induce DC maturation. Upon maturation, DCs express costimulatory molecules and produce IL-6 and in this way render T cells insensitive to suppression by Tregs $^{12,18}$.

Thus dual recognition of both structural features of the antigen and the presence of microbial products in the milieu, in which the antigen is encountered, enables reliable discrimination between self and nonself. Suppressing and enhancing mechanisms counterbalance each other in order to modulate antigen-specific activation in periphery. Accurate balance between these opposing forces is of great importance as every disturbance of the optimum can lead to either unsuccessful elimination of invading pathogens or to induction of exacerbated responses leading to autoimmune diseases.

![Diagram](image.png)

**Figure 1. Control of immune responses by recognition of antigen and context.** Combinatorial production of antigen-specific receptors generates a large variety of nonself- and self-specific lymphocytes. This initial pool is purged from highly autoreactive cells by mechanisms of central tolerance. In the periphery, immune responses are controlled by recognition of both the antigen and the context, in which the antigen is encountered. Immune responses to self-antigens in a non-inflammatory context are limited by mechanisms of peripheral tolerance that include ignorance, anergy and Treg-mediated suppression. Inflammation produces enhancing signals capable of counterbalancing the above mentioned suppressing mechanisms and leads to induction of potent immune responses against invading pathogens.
Suppression of T cell responses by naturally arising regulatory T cells

*Naturally arising regulatory T cells*

The first results pointing to a role for T-cell mediated control of T cell responses were obtained from studies on lymphopenia-driven T cell expansion. Reconstitution of athymic nude mice with CD4⁺CD25⁻ T cells resulted in development of severe and multiple autoimmune diseases. In contrast, when total CD4⁺ T cells or CD4⁺CD25⁺ T cells were co-injected, T cells expanded in a controlled way, and mice were protected from autoimmunity. This seminal study first identified the T cell subset characterized by expression of CD4 and CD25 as being responsible for the regulation of T cell responses.

Based on their regulatory function CD4⁺CD25⁺ T cells were defined as naturally arising regulatory T cells. CD4⁺CD25⁺ Tregs constitute 5-10% of circulating CD4⁺ in healthy individuals and are characterized by the expression of several additional surface markers as CTLA-4, GITR, and - at least in mice - neuropilin-1. They show a typical anergic behavior and proliferate only in the presence of strong stimuli and upon addition of exogenous IL-2. CD4⁺CD25⁺ Tregs are not able to produce Th1 or Th2 cytokines upon activation, instead they produce suppressive cytokines as IL-10 and TGF-beta.

CD4⁺CD25⁺ Tregs arise in the thymus, and neonatal thymectomy has been shown to reduce the amount of Tregs in periphery resulting in autoimmune diseases. The master regulator for the induction of the Treg phenotype is the transcription factor Foxp3, and transduction of Foxp3 into naïve T cells induces a Treg phenotype. Besides naturally arising Tregs, other regulatory subsets exist that are induced upon several different in vitro and in vivo treatments.

The main function of Tregs seems to be the limitation of CD4⁺ T cell proliferation by a yet unknown contact-dependent and cytokine-independent mechanism induced upon TCR stimulation of Tregs. Treg suppressor activity has been demonstrated both in vitro, where Tregs efficiently suppress CD4⁺ T cell proliferation and cytokine production, and in vivo, where they control lymphopenia-driven proliferation and expansion of CD4⁺ T cells.
The specificity of regulatory T cells

One issue that has gained much interest is the determination of Treg specificity. As suppressor activity is only induced upon TCR stimulation of Tregs, determination of Treg specificity will lead to a better understanding of the conditions under which Tregs exert their function - an essential prerequisite for the development of therapeutic applications.

The current hypothesis suggests that Tregs may function in the maintenance of self-tolerance by specifically suppressing the activation of autoreactive T cells. Treg could be activated by autologous DC and then suppress the response of nearby conventional autoreactive CD4+ T cells. This theory demands that Tregs can be activated by autologous cells and thus bear TCR of increased affinity for self-antigens

Research on the specificity of Tregs is hampered by the anergic behavior of Tregs that precludes a direct measurement of responses to self-antigen bearing APC. Several attempts were made to directly measure the response of Tregs to autologous APCs in presence of high amounts of exogenous IL-2, which is reported to abrogate their anergic behavior 34-36. Although none of the studies found any substantial increase in activation or proliferation of Treg as compared to normal CD4+ T cells, it is difficult to conclude from these negative results that Treg are not autoreactive.

There is indeed indirect evidence that the development of Tregs might be linked with the expression of TCRs of increased affinity towards self. The development of cells belonging to the Treg lineage seems to be critically dependent on TCR-antigen interaction in the thymus 37-39, mediated by MHC II-expressing thymic epithelial cells (TECs) 40. These observations point to a role for self-antigen in induction of the Treg lineage.

Alternatively, it has been postulated that a selective rather than an instructive process shapes the repertoire of Treg 41. CD4+CD25+ thymocytes have been shown to be much less sensitive to agonist-induced clonal deletion than CD4+CD25- thymocytes suggesting a higher threshold for negative selection in the CD4+CD25+ thymic population. Also this finding implies that the final population of CD4+CD25+ Tregs might contain cells of higher affinity towards self-antigens because of a reduced negative selection process.
A more direct evidence for the prevalence of TCRs of higher affinity towards self in the mature Treg compartment comes from a recent study where a limited number of TCRs from CD4⁺CD25⁺ and CD4⁺CD25⁻ was cloned and introduced into naïve CD4⁺ T cells by retrovirus-mediated gene transfer. When these cells expressing now two TCR were injected into lymphopenic mice, TCRs from CD4⁺CD25⁺ T cells conferred a greater proliferative advantage for expansion of these cells than TCR from CD4⁺CD25⁻, again suggesting a higher frequency of self-reactive TCRs in the Treg population.

But the model of self-reactive Treg that are activated specifically by autologous immature APC and suppress self-antigen induced T cell proliferation is challenged by recent findings. The retroviral transduction of normal naïve CD4⁺ T cells with the Foxp3 transgene resulted in the acquisition of a Treg phenotype that included expression of CD25, anergic behavior and exertion of suppressor activity comparable to naturally arising Tregs. These induced Tregs derived from a normal pool of naïve CD4⁺ T cells that had undergone thymic negative selection, and therefore they were not supposed to be autoreactive - but nevertheless they successfully suppressed T cell responses. This result poses into question the model of self-reactivity in the Treg compartment and postulates that a potential shift in affinity to self-antigens between normal and regulatory T cells is of no importance for the process of suppressing T cell responses.

**Identification and function of regulatory T cells in autoimmune diseases**

Another therapeutically important issue that remains to be addressed is the determination of the role Tregs play in vivo in the control of autoimmune diseases. CD4⁺CD25⁺ cells can indeed be detected in inflamed tissues of patients suffering from autoimmune diseases as juvenile idiopathic arthritis (JIA) and seem to exert some Treg function ex vivo. However several other studies suggested an intrinsic defect in Treg activity as driving mechanism for the development of autoimmune diseases. In addition, the function of Tregs in limiting ongoing autoimmune responses seems to be reduced by a nonresponsiveness of proliferating autoreactive T cells either due to their preactivated state or due to the inflammatory milieu.
containing cytokines as IL-2\textsuperscript{12} or IL-6\textsuperscript{18}. These findings point to a link between Treg activity and development of autoimmune diseases.

Most ex vivo studies on Tregs in autoimmune diseases were performed on CD4\textsuperscript{+}CD25\textsuperscript{+} T cells isolated from the inflamed tissue. But the identification of regulatory T cells in pathologic conditions is complicated by the fact that all Treg markers, including CD25, are also expressed on activated T cells that accumulate at the site of chronic inflammation. It is therefore not possible to discriminate in an ongoing immune response regulatory from activated effector T cells on the base of the known Treg markers, and a possible heterogeneity of the CD4\textsuperscript{+}CD25\textsuperscript{+} subset in inflamed tissues has not been addressed so far.

**Context discrimination by innate immunity and its effect on B cell responses**

**Toll-Like Receptors**

The innate immune system plays an important role in the immediate recognition and elimination of pathogens and acts therefore as a first line of defense against microbial invasion\textsuperscript{14,15}. Moreover, upon activation the innate immune system interacts with the adaptive immune system and delivers essential stimulatory signals for induction and sustaining of adaptive immune responses\textsuperscript{14,15}. As it acts as a potent inductor of adaptive immune responses, the innate immune system must be endowed with the ability to discriminate between self and nonself.

Key components of the innate immune system are pattern-recognition receptors (PRRs) that sense the presence of invading pathogens by recognizing conserved PAMPs. The best-characterized member of PPR is the family of TLRs\textsuperscript{14,15} (Fig. 2). Until now there are 11 known TLRs in mammals that detect various pathogen-derived conserved structures as bacterial lipoproteins (TLR2 and 6), dsRNA (TLR3), LPS (TLR4), flagellin (TLR5), ssRNA (TLR7 and 8) and unmethylated CpG-DNA (TLR9). TLR triggering induces signaling through the common adaptor protein MyD88 ultimately leading to the activation of NF-kB. Only TLR3 binding activates an alternative pathway that promotes production of IFN\textbeta and thus induces a different set of genes. Interestingly, TLR4 can signal through both MyD88-dependent and –independent pathways.
Background

TLRs are expressed on a variety of cell types including DC and other cells from the innate (neutrophils, eosinophils, mast cells) and adaptive (B cells, T cells) immune system as well as on stromal cells \textsuperscript{14,15}. Every cell type expresses a characteristic set of TLRs, and this differential expression pattern combined with the narrow ligand specificity of every single TLR and the diverse signal transduction pathways induced by different TLRs allows to evoke a large spectrum of possible responses to a variety of different PAMPs.

Different mechanisms ensure that TLRs are activated only in presence of pathogen-derived structures; these mechanisms are mainly dependent on the exact cellular localization of the TLRs: surface TLRs (2, 4, 5, 6) recognize typical bacterial structures and therefore act via direct self-nonself discrimination. In contrast, TLR 3, 7, 8, 9 localize in intracellular lysosomal compartments and detect nucleic acids that are not necessarily uniquely pathogen-associated structures. In this case, the difference between self and nonself is detected only by the different localization of host- and pathogen-derived nucleic acids, as host-derived nucleic acids normally do not have access to the endosomal compartments \textsuperscript{14,15} (Fig. 2).

**Figure 2. Toll-like receptors.** TLRs are a large family of pattern-recognition receptors that recognize conserved molecular structures of microbial origin. TLRs localize either on the cell surface or in endosomal compartments. Upon activation, most TLRs start signaling cascades via the common adaptor protein MyD88 with the exception of TLR3 and TLR4 that can activate an alternative pathway. TLRs potently stimulate the innate immune system and enable a cross-talk between the innate and the adaptive immune system, which is essential for induction of adaptive immune responses. (Adapted from O’Neill\textsuperscript{51})
The two signal model of naïve B cell activation

According to the current two signal model, naïve B cell activation by T-dependent antigens is induced by the sequential integration of two signals: Activation is initiated by binding of the antigen to the BCR, which triggers a signaling cascade leading to the upregulation of costimulatory molecules on the B cell surface. In addition, stimulated BCRs mediate efficient antigen internalization allowing processing and presentation of antigenic peptides on MHC class II molecules for recognition by specific T cells\(^{52,53}\). At the immunological synapse specific T cells are activated and in turn stimulate B cells via CD40L-CD40 interaction and cytokines\(^{54}\). The current dogma is that the timely integration of BCR stimulation (signal one) and T cell help (signal two) is both necessary and sufficient to drive naïve B cell proliferation and differentiation to Ig secreting plasma cells\(^{52}\) (Fig. 3).

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**Figure 3. The activation of naïve B cells.** Crosslinking of B cell receptor by antigen delivers signal 1 and induces upregulation of MHC II and costimulatory molecules on B cells. Specific T cells are activated at the immunological synapse and stimulate B cells via CD40L and cytokines (signal 2). Timely integration of signal 1 and 2 induces naïve B cell activation, proliferation and differentiation.
**TLR costimulation as a mechanism for induction of B cell responses**

It has been known for long time that mouse B cell responses are massively increased when antigen is administered with pathogen-derived molecular structures as LPS or in CFA containing bacterial components. It has been shown that mouse naïve B cells can respond in vitro to LPS and CpG by undergoing extensive proliferation and differentiation, even in the absence of BCR triggering or T cell help. Indeed mouse B cells constitutively express a variety of TLRs, including TLR4 and TLR9, which are triggered by LPS and CpG.

However, recent studies have revealed important differences in the expression of TLRs between mouse and human B cells. Human B cells express TLR2, TLR6, TLR7, TLR9 and TLR10 but not TLR4 and differ therefore in their spectrum of TLR expression from mouse B cells, which do express TLR4. In addition, TLR expression is differentially regulated in mouse and human B cells. While in mice both naïve and memory B cells express TLRs, constitutive TLR expression is restricted in humans to memory B cells. Human naïve B cells do not express TLR but are able to upregulate TLRs following BCR stimulation. The coupling of BCR stimulation to TLR expression endows the human system with a high degree of specificity since it allows focusing of innate signals specifically on antigen stimulated and memory B cells. The selective responsiveness of memory B cells to TLR agonists has been suggested to be a mechanism to maintain serological memory by induction of polyclonal memory B cell differentiation to plasma cells.

There is growing evidence that TLRs play an important role in induction of mouse B cell responses. Recently it has been shown that particles containing proteins and nucleic acids can induce autoantibody formation by synergistically engaging BCR and TLR9 or TLR7. In addition, TLR signaling supports induction of T-dependent B cells responses in mice. However, it remains to be established whether and how TLR signals contribute to the activation of human naïve B cells.
In this thesis three aspects related to self-nonself discrimination were addressed. In a first part it was determined whether Tregs are self-reactive. We have established a sensitive assay for detection of TCR triggering that can be used for the determination the TCR specificity of anergic T cell. Using this assay the frequency of self-reactive TCRs present in the repertoire of mouse CD4^+CD25^+ regulatory T cells and CD4^+CD25^- conventional T cells was quantified by measuring TCR triggering upon stimulation with self-antigen bearing DCs (see manuscript 1).

Then we have addressed the question of how to define human Tregs in an inflamed tissue using samples of patients suffering from the autoimmune disease juvenile idiopathic arthritis (JIA). We have determined surface markers that allow discriminating in the CD4^+CD25^+ compartment of an ongoing autoimmune response Tregs from activated T cells. By use of this novel Treg marker the function of Tregs in peripheral inflamed tissues was studied (see manuscript 2).

And finally, the impact of TLR-mediated context discrimination on the induction of T-dependent B cell responses in the human system was studied. Using improved methods for human naïve B cell isolation \cite{66} and for induction of cognate interactions between polyclonal human B and T cell populations we have analyzed the effect of PAMPs in presence of BCR triggering and T cell help on activation, proliferation, and differentiation of naïve human B cells (see manuscript 3).
Results

Manuscript 1: No evidence for self-reactivity of CD4+CD25+ T cells

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submitted

Abstract

Naturally-occurring regulatory T cells (Tregs) are able to suppress the responses of normal naïve T cells in vitro and in vivo, and can prevent experimentally-induced autoimmunity in vivo. These properties suggest that Tregs may function in the maintenance of self-tolerance in vivo, by specifically suppressing the responses of autoreactive T cells. This theory demands that Tregs should bear T cell receptors (TCRs) largely specific for self-antigens. Here we quantitatively measure the frequency of self-reactive TCRs expressed by CD25+ Tregs, and by CD25 naïve T cells. We find that the self-reactivities of TCRs from both populations are identical, and that neither contain strongly self-reactive TCRs. This result is difficult to reconcile with an antigen-specific role for Tregs in maintaining self-tolerance.
Introduction

The immune system is able to respond to, and eliminate, organisms expressing foreign antigens, and thereby provide protective immunity. It must simultaneously remain nonresponsive to potentially antigenic self-constituents, in order to avoid autoimmunity. Understanding how this state of self-tolerance is achieved is one of the fundamental goals of immunology.

The theory of clonal selection 67 proposed that lymphocytes bearing self-reactive receptors should be deleted during their development. This was subsequently shown to occur during the maturation of T lymphocytes in the thymus 68-70, and represents the principal means by which self/non-self discrimination is established. However, in spite of this, some T cells with receptors specific for self-antigens do develop 71,72, and yet do not usually cause autoimmunity. There must therefore exist additional ways in which self tolerance is maintained.

Naturally-occurring regulatory T cells (Tregs) have been proposed to fulfill such a role 12. These cells are present as a subpopulation of 5-10% of CD4+ T lymphocytes in normal individuals, and can be identified by their constitutive expression of the IL-2 receptor α chain, CD25 73,74. They are able to suppress the responses of other T cells, both in vitro 34,36,75 and in vivo 73,76,77. Moreover, CD25+ Tregs are able to prevent experimentally-induced autoimmunity in vivo: thymectomy of mice 3 days after birth (at which time CD25+ Tregs have yet to appear in the periphery), or transfer of naïve CD25- T cells from normal mice into lymphopenic recipients (eg nude mice, or Rag- or CD3e-knockout mice), both lead to various organ-specific autoimmune diseases 19,20,22,78,79. Reconstitution of such mice with CD25+ T cells completely prevents disease.

Autoimmune diseases also occur in mice with genetic mutations which prevent the development of regulatory T cells (eg scurfy, IL-2-knockout, and IL-2 receptor [IL-2R]-knockout mice), and can be prevented by transfer of CD25+ Tregs into neonatal mice 24,80-83. These genetic models clearly suggest that CD25+ Tregs are relevant in the physiological (as opposed to experimentally-induced) prevention of autoimmunity, and lead to the question: are Tregs important throughout life, specifically suppressing autoimmunity whilst allowing immune responses to foreign organisms? This proposition implies that most Tregs should express TCRs specific for self-antigens, if they are to avoid suppressing all immune responses 12,84.
A number of studies have addressed the idea that TCR-specificity influences the development of CD25⁺ Tregs:

(i) CD25⁺ cells do not develop in TCR transgenic mice bred onto backgrounds which prevent the expression of endogenous TCRs (eg Rag- or TCR-a-knockouts);²⁰,³⁷,⁸⁵-⁸⁷, implying that the specificity of the transgenic TCR is incompatible with Treg development. In each case, Tregs are able to develop when endogenous TCRs can be co-expressed.

(ii) CD25⁺ thymocytes are preferentially generated when some TCR transgenes are expressed in mice also transgenically expressing their cognate antigen ⁵⁷,³⁹,⁷⁷,⁸⁸,⁸⁹, or after transplantation of thymi expressing the relevant antigen ⁷³. However, the yield of CD25⁺ thymocytes is very low in most cases, and in other cases (including the same TCR transgenics crossed onto different antigen-expressing backgrounds) transgenic T cells are deleted without generating CD25⁺ cells ³⁷,⁸⁹,⁹¹.

(iii) In wild-type mice, although CD25⁺ thymocytes appear to be resistant to deletion by viral superantigens, they are susceptible to normal negative selection by self peptide-MHC complexes ⁴⁰,⁹²-⁹⁴.

These latter qualitative studies do not exclude, however, that there could be a quantitative difference in the sensitivity of CD25⁺ & CD25⁻ cells to negative selection (as proposed by van Santen et al. ⁴¹). Thus, it remains possible, as suggested by several TCR transgenic systems, that TCRs with an increased reactivity to self-antigen could favour the thymic development or survival of CD25⁺ Tregs.

Despite the abundant research on their development, few reports have directly addressed the self-specificity of the TCR repertoire of mature CD25⁺ Tregs. The inherent difficulty of this stems from their anergic behaviour in vitro, which precludes straightforward measurement of their responses to self-antigen bearing APCs. Both Bensinger et al. ⁴⁰ and Pacholczyk et al. ⁹⁵ attempted to circumvent this problem by providing exogenous IL-2, which is known to overcome the anergic behaviour of Tregs in vitro ³⁴-³⁶. Neither group was able to detect substantial proliferation of CD25⁺ Tregs upon stimulation with autologous splenic APCs. The positive control in these experiments was the proliferation of CD25⁺ Tregs derived from mice in which thymic negative selection was completely absent – indicating that at least some self-reactive TCRs must have been purged from the normal CD25⁺ Treg repertoire. However, whether self-reactive cells remained, and to what extent, could not be determined.
Hseih et al.,42 measured the ability of cloned TCR genes to augment the in vivo expansion of naïve CD25+ T cells in lymphopenic mice. They found that TCRs from CD25+ Tregs conferred a greater proliferative advantage than did those from CD25+ T cells – suggesting an increased frequency of self-reactive TCRs. The absolute frequency was not determined, however.

Here we describe the quantitative measurement of the frequencies of self-reactive TCRs present in the repertoires of CD25+ Tregs, and of naïve CD25- T cells.

**Results**

Our experimental strategy was to generate T cell hybridomas by fusion of CD25+ Tregs or naïve CD25- T cells with a TCR-negative thymoma. We could then directly analyse the self-reactivity of hybridomas expressing TCR (the genes for which must have been derived from the fused T cell populations), by stimulation with autologous APCs.

To provide a direct and robust assay for TCR triggering, we used a reporter gene in which green fluorescent protein (GFP) expression is driven by 9 tandem copies of the NFAT (nuclear factor of activated T cells) binding motif derived from the promoter of the human IL-2 gene96; see figure 1a). TCR signaling in transduced cells rapidly (≤20 mins, not shown) leads to NFAT-mediated transcription, and expression of GFP. Importantly, the fraction of GFP-expressing cells is determined by the strength of the TCR stimulus, allowing quantitative comparisons to be made of TCR signaling96; see figure 2a).

Following a similar approach to Sanderson & Shastri97, we introduced the reporter gene into a TCR a/b-negative derivative of the BW5147 thymoma98, and tested transduced clones for induction of GFP expression after activation with PMA + ionomycin (figure 1b). All 40 clones tested showed activation-dependent GFP expression (in the range 30-98% GFP+ cells, not shown), and 10 of these were further screened by fusion with total mouse splenic T cells. Fusion efficiencies ranged from $10^{-3}$ to $10^{-4}$ (not shown), and 15-100% of the resulting hybridomas expressed GFP upon stimulation with anti-CD3e antibodies (figure 1c). None of the parental thymoma clones responded to anti-CD3e, as expected from their lack of TCR expression. One thymoma clone was selected for use in subsequent experiments, based on its high fusion efficiency and on the high frequency of hybridomas generated with inducible GFP expression.
To test the sensitivity of the system, we generated hybridomas from T cells from DO11.10 mice, which carry a TCR transgene specific for a peptide derived from chicken egg albumin (Ova; \(^{99}\)). DO11.10-derived hybridomas expressed GFP upon stimulation by anti-CD3e antibodies, and by Ova-pulsed H-2\(^d\) dendritic cells (DCs), but did not respond to dendritic cells in the absence of Ova (figure 2a). The maximal response was elicited by pre-pulsing DCs with >300mg/ml Ova protein, and induction of GFP expression could still be detected by DCs pulsed with less than 500ng/ml Ova. This range of sensitivity is comparable to that achieved by measuring upregulation of CD69 expression on naïve, \textit{ex vivo} DO11.10 CD4\(^+\) T cells (although in this case the background in the absence of Ova is significantly higher; figure 2b).

We also examined whether the limit of detection would be appreciably increased if DCs were pulsed with Ova, not alone, but as part of a complex mixture of many proteins. We exposed DCs overnight to Ova protein, diluted into a soluble lysate derived from multiple mouse tissues, with an absorbance at 280nm (A\(_{280}\)) of >3 (thus containing very approximately 2-3mg/ml protein). Induction of GFP expression by DO11.10-derived hybridomas could still be measured with Ova concentrations of <500ng/ml, although the fraction of GFP-expressing cells at higher concentrations was significantly reduced (figure 2a). Hence, by extrapolating from these results using DO11.10-derived hybridomas, the system can detect TCRs specific for proteins present at ≥500ng/ml, or at approximately ≥0.025% in a complex mixture.

It was important to establish whether Tregs could generate hybridomas with fusion efficiencies similar to those of naïve T cells. This would exclude the possibility that hybridomas from CD25\(^+\) cells could be predominantly derived from a small subpopulation of contaminating non-Tregs. As a homogeneous source of Tregs, we used
HA-TCR transgenic mice crossed onto the Ig-HA transgenic background, in which expression of the cognate antigen, influenza virus haemagglutinin (HA), is driven by the immunoglobulin k promoter. In these mice all clonotype-expressing CD4+ T cells behave as Tregs. Thus, expression of the clonotypic TCR serves as an independent marker of Tregs and any contaminating CD25- cells in the sorted CD25+ population are unlikely to bias the results, since they function as Tregs, too. We sorted clonotype-positive CD4+ CD25+ and CD25- Tregs from HA-TCR x Ig-HA double transgenic mice, and naïve CD25- T cells from HA-TCR transgenic mice on a wild-type genetic background, as controls. As expected, the naïve CD25- T cells, but not the CD25+ and CD25- Tregs, proliferated in response to soluble anti-CD3e stimulation in vitro (not shown). However, all sorted cell populations generated hybridomas with comparable fusion efficiencies (within a two-fold range; figure 3a, left). In addition, all hybridomas from sorted clonotype-expressing T cell populations continued to express the clonotypic TCR (figure 3a, right), confirming the identities of the originating T cells.

We also compared the antigen sensitivities of HA-TCR-expressing hybridomas derived from Treg or naïve T cells. Both expressed GFP upon anti-CD3e
stimulation, and had near-identical responses to H-2^d^ DCs pulsed with the antigenic HA peptide at all concentrations (figure 3b). Therefore, the nonresponsive character of Tregs is completely lost in Treg-derived hybridomas. In addition, the hybridomas showed clear responses (≈5-8% maximum response; figure 3c) to H-2^d^ DCs pulsed with a soluble tissue lysate (as described above) derived from Ig-HA transgenic mice, further confirming that the system can detect T cell reactivity to self-antigens present in the tissue lysate.

Figure 3. Fusion efficiencies and antigen responsiveness of hybridomas derived from CD25^+^ Tregs and from CD25^-^ naive T cells. A) Fusion efficiencies of sorted clonotype-positive (6.5^+^) CD25^+^ and CD25^-^ CD4^+^ T cells from Ig-HA x HA-TCR double transgenic mice, and of 6.5^+^ CD25^-^ CD4^+^ T cells from HA-TCR transgenic mice (left panel). Level of surface HA-TCR expression (by staining with the anti-clonotype antibody 6.5) on the resulting hybridomas (right panel); the top bar indicates the level of surface HA-TCR expression on hybridomas derived from clonotype-negative (6.5^-^) CD4^+^ T cells from HA-TCR transgenic mice. Error bars indicate the standard deviations of the log(fluorescence) between individual hybridomas. B) Hybridomas derived from clonotype-positive (6.5^+^) CD4^+^ CD25^-^ naive T cells from HA-TCR transgenic mice (filled circles), and CD25^+^ Tregs from Ig-HA x HA-TCR double transgenic mice (empty circles) were stimulated overnight with Balb/c DCs pulsed with HA peptide. The fraction of cells expressing GFP, normalized to the maximum response attained by stimulation of each hybridoma with anti-CD3ε antibodies (% maximum response) is shown. Similar results were obtained for 8 hybridomas (of which one is depicted) per group. C) Hybridomas as in (B) above were stimulated with Balb/c DCs pulsed with a soluble tissue lysate derived from Ig-HA transgenic mice. The data represent the mean response of 4 hybridomas per group.
We next generated hybridomas from CD4+ CD25+ Tregs, and from naïve CD4+ CD25- T cells from the spleens of wild-type C57BL/6 mice. CD25+ and CD25- CD4+ cells were sorted to ≥95% purity (figure 4a), and were assayed for their response to anti-CD3e antibodies in vitro. In keeping with the published reports by1,2, CD25+ cells responded by proliferating vigorously, whereas CD25+ cells did not. Moreover, CD25+ cells suppressed the proliferation of the CD25- cells in a cell dose-dependent fashion (figure 4b). Therefore, the purified CD25+ T cells had the phenotypic and functional characteristics of Tregs.

Prior to fusion, we stimulated both T cell populations with plastic-bound anti-CD3e and anti-CD28 antibodies, in the presence of 100U/ml IL-2. These stimuli have been previously described as sufficient to overcome the anergic state of CD25+ Tregs3. Cells in both populations enlarged to form blasts and proliferated under these conditions. By measuring the dilution of CFSE in identical, labeled cultures, we found that the majority of input cells in both populations were induced to proliferate, although CD25- cells underwent approximately one additional cell division compared with CD25+ cells (figure 4c). Thus, there is no indication that any sub-population of cells is able to preferentially expand in these cultures. In agreement with our previous control experiments, CD25- and CD25+ cells generated hybridomas with similar fusion efficiencies (figure 4d), further arguing that hybridomas from CD25+ cells are not derived from a small number of contaminating, or otherwise abnormal, cells.

In three separate experiments, we generated and analyzed a total of more than 350 hybridomas derived from each of the CD25+ and CD25- cell populations. Upon activation with anti-CD3e antibodies, >98% of hybridomas derived from both populations expressed GFP, and the brightness of GFP-expressing cells was identical (figure 4e). Hence, in accordance with our control experiments (figure 3b), TCR signalling is not impaired in hybridomas derived from CD25+ Tregs. The fraction of cells which could be induced to express GFP in each hybridoma varied from 17-98% (median 71%, not shown), likely reflecting stochastic loss of the chromosomes containing the NFAT-GFP reporter, TCR a or TCR b genes following fusion. In subsequent analyses, therefore, we normalized the fraction of responding cells in each hybridoma to the maximum response attainable using anti-CD3e stimulation.
Results

Figure 4. Generation of hybridomas from CD4+ CD25+ Tregs and from naïve CD4+ CD25- T cells. A) Spleen cells from wild-type C57BL/6 mice were stained with antibodies specific for CD4 and CD25 (left), and CD4+ CD25- naïve T cells (centre) and CD4+ CD25+ Tregs (right) were sorted to 98% and 95% purity, respectively. B) In vitro suppression of proliferation by CD25+ Tregs. CD25+ and CFSE-labeled CD25- CD4+ T cells were mixed, and stimulated for 4 days with anti-CD3e antibodies. The number of cells which had diluted CFSE (ie CD25- cells which had undergone at least one division) is indicated. Error bars indicate the standard deviations of 2-4 replicate cultures. C) Dilution of CFSE labeling in sorted CD4+ CD25- naïve T cells (upper panel) and CD4+ CD25+ Tregs (lower panel) after 3 days activation with plastic-bound anti-CD3e and anti-CD28 antibodies, in the presence of 100 U/ml IL-2. Marker indicates the intensity of CFSE labeling of undivided cells. Precursor frequencies of dividing cells are 96% and 74% of the initial CD25- and CD25+ cell populations, respectively. D) Efficiency of fusion of CD25+ and CD25- CD4+ T cells, expressed as the number of hybridomas generated per input activated T cell. Circles, triangles and squares depict the efficiencies of fusions in different experiments; vertical lines indicate their geometric means. E) Brightness of green fluorescence of CD25+ and CD25- T cell-derived hybridomas, either without stimulation (upper bar), or amongst GFP-expressing cells after overnight activation with plastic-bound anti-CD3e antibody (lower bars). Error bars indicate the standard deviations of the log(green fluorescence) between individual hybridomas.
Results

We measured the self-reactivity of the CD25⁺ Treg- and naïve CD25⁻ T cell-derived hybridomas by stimulating them using syngeneic DCs. The DCs were pulsed overnight with a lysate of multiple mouse tissues, at A⁺280\textgreater 3, to provide them with a source of tissue-specific, as well as ubiquitously-expressed, self antigens. No hybridomas from either population responded strongly (\(\approx 25\%\) maximum response) to this stimulation. The number of hybridomas responding at \(\geq 1\%\) maximum response was \(\approx 3\%-4\%\), and was not significantly different between CD25⁺ and CD25⁻ cells (figure 5a, right). By analysing a sufficient number of stimulated cells from each hybridoma, we were able to detect induction of GFP expression above background (which was less than 0.01% maximum response; see figure 2a) in \(\approx 30\%\) of hybridomas from both groups; however, at no level of response did the fraction of responding hybridomas differ between CD25⁺ and CD25⁻ cells (figure 5a, left). Thus, we find no evidence for self-reactivity of CD4⁺ CD25⁺ T cells.

To attain the maximum sensitivity of detecting tissue-specific self-antigens, we further screened a reduced panel of 48 hybridomas derived from CD25⁺ cells using DCs pulsed separately with each of the lysates from individual mouse tissues (again, at a final A⁺280\textgreater 3). No hybridomas responded at a level higher than that seen with the mixed lysate (not shown).

To ensure that our system could indeed detect self-reactive T cells, we performed additional fusions using CD4⁺ T cells derived from AbEpIi⁻ mice (‘single peptide’ mice); 102. The I-A⁺ MHC class II molecules in these mice are all covalently linked to a single, defined peptide. They are consequently unable to present peptides derived from any other proteins, and thymic negative selection of self-peptide-specific CD4⁺ T cells cannot occur. More than 40% of single peptide-derived hybridomas responded strongly (\(\geq 25\%\) maximum response) to stimulation by I-A⁺ wild-type DCs, and 76% responded at \(\geq 1\%\) maximum response (figure 5b, right). Self-reactive T cells are clearly easily detectable in this system. In contrast, upon stimulation by single peptide-derived DCs, only \(\approx 3\%\) of the hybridomas responded at \(\geq 1\%\), and their response profile was almost identical to that of wild type CD4⁺ T cells responding to wild type DCs (compare triangles in figure 5b to figure 5a).

We also wondered whether CD25⁺ and CD25⁻ T cell populations might differ in their level of alloreactivity. This might be expected if the TCRs of CD25⁺ Tregs did not recognize specific self-peptides presented by MHC molecules, but had a higher
affinity for the invariant backbones of the MHC molecules themselves. Although is hard to imagine how such a scenario might help CD25+ Tregs to discriminate self-from non-self-antigens, we nevertheless examined the anti-H-2d alloreactivity of the hybridomas derived from CD25+ and CD25- cells from H-2b mice. The fraction of hybridomas responding at ≥1% maximum response was ≈10%, significantly higher than that responding to syngeneic APCs, but differed only slightly between CD25+ and CD25- cells (8% and 13%, respectively; figure 5c). We conclude that the frequency of alloreactive TCRs is no higher amongst CD25+ Tregs than naïve CD25- T cells. This result concurs with the studies of Dieckmann et al. 103, who found that allo responses of Tregs were essentially normal, and of Pacholczyk et al. 95, who found no evidence that TCRs used by CD25+ thymocytes bound preferentially to the MHC framework.
Figure 5. Self- and allo-reactivity of hybridomas from CD4+ CD25+ Tregs and from naïve CD4+ CD25- T cells. Hybridomas were stimulated overnight with DCs, and, for each one, the fraction of cells induced to express GFP was determined, normalized to the maximum response attained by stimulation of the same hybridoma with anti-CD3e antibodies (% maximum response). The results shown are representative of those obtained with three independent experiments. Left panels show the cumulative fraction of hybridomas whose response exceeded the level indicated. Right panels show the fraction of hybridomas whose response exceeded 1% of maximum. A) Hybridomas derived from CD25- (circles) and CD25+ (triangles) CD4+ T cells from wild-type C57BL/6 mice were stimulated with syngeneic DCs pulsed with a soluble tissue lysate. B) Hybridomas derived from single peptide mice were stimulated with DCs from either C57BL/6 mice (circles) or from single peptide mice (triangles), pulsed with a soluble tissue lysate as above. C) Hybridomas as in (A) above were stimulated with allogeneic DCs from wild-type Balb/c mice, activated overnight with TNF-α.
Discussion

Subject to a number of caveats (discussed below), our data indicate that CD25⁺ Tregs are not self-specific, or at least that the frequency of strongly self-specific cells in this population is sufficiently small to be undetectable amongst the ≈350 hybridomas analyzed here, and the frequency of weakly self-reactive cells is identical to that of naïve CD25⁺ T cells. Both scenarios are difficult to reconcile with a role for CD25⁺ Tregs in suppressing autoimmunity in an antigen-specific fashion.

Experiments of this type are subject to a number of potential pitfalls: first, CD25⁺ Tregs may be specific for rare, tissue-specific self-antigens, which were not efficiently presented in our in vitro system, but which could nonetheless cause autoimmunity in vivo without control by Tregs. Although we can never rule this possibility out completely, we have quantified the limit of detection of our system using hybridomas of known antigen-specificity (figure 2). We were able to detect an antigenic protein present in a mixture at less than ≈0.025%; thus, the self-antigens recognized by Tregs, if there are any, should all be less abundant than this. While tissue-specific antigens of such rarity certainly exist, many experimentally identified autoimmune target-antigens are expressed at comparatively high levels (eg myelin basic protein \(^{104,105}\), insulin \(^{106,107}\)). So, the absence of CD25⁺ derived hybridomas reacting to self-antigens in our system is not readily explainable by the universally low expression of such antigens.

Second, CD25⁺ Tregs may recognize self-antigens with an affinity too low to be discernable by our assay, but high enough to allow them to suppress unwanted autoimmune responses. Indeed, the level of TCR stimulation required in vitro to elicit suppression by Tregs is considerably lower than that needed for proliferation of naïve CD25⁺ T cells \(^{34}\), and it has been proposed that the avidities of TCRs from CD25⁺ Tregs for self peptide-MHC complexes lie in a narrow range, above that required for positive selection of CD25⁺ T cells, but low enough to escape thymic negative selection \(^{12}\). We have attempted to tackle this issue by exploiting the very low background and high sensitivity of the NFAT-GFP reporter construct. We were able to detect TCR triggering above background in up to 30% of hybridomas in response to self-antigens presented by DCs (figure 5a). The very low level responses of most of these cells is probably well below that required for T cell activation in vivo. In fact, we detected similar responses amongst single peptide-derived hybridomas to single
peptide-derived DCs (figure 5b), corresponding to the recognition of the same peptide-MHC complex responsible for positive selection in the thymus. However, throughout the range of responses, the self-reactivity of CD25+ and CD25- T cells was identical. Hence, if the TCRs from CD25+ Tregs do lie in a window of avidity for self-antigens, it completely overlaps with the avidities of TCRs from naïve CD25- T cells.

Third, as mentioned above, the frequency of self-reactive cells amongst CD25+ Tregs may indeed exceed that amongst naïve CD25- T cells, yet still be so small that such cells are absent from our analysis. Based on our failure to detect any strongly self-reactive cells out of around 350 CD25+ derived hybridomas, such cells must be present at a frequency of less than 1 in 116 (at a 95% confidence level), if at all. If the role of Tregs in normal individuals is to specifically recognize, and suppress autoimmunity against, self-antigens, this would imply that >99% of Tregs are in this respect inert. Worse, they would share with naïve CD25- T cells an equal likelihood of recognizing foreign, pathogen-derived antigens, when encountered for the first time. Since an immune response in this instance is desirable, additional mechanisms would be required to prevent Treg-mediated suppression. It is our opinion that this reductio ad absurdum argues that even a low frequency of self-reactive Tregs is incompatible with their role in antigen-specific prevention of autoimmunity.

The data presented here are seemingly at odds with those of Hsieh et al 42, who concluded that a large proportion of Tregs recognize self-antigens. These authors reintroduced cloned TCRs into naïve T cells (such that the recipient T cells now expressed two TCRs: the endogenous [transgenic] TCR, and the newly-transduced one), and used expansion after transfer into TCR-a-deficient mice as an indirect measure of self-reactivity. Using random pools of ≈10,000 TCRs, they found that those derived from CD25+ cells conferred a 2- to 4-fold greater expansion advantage than did those from CD25- cells, and caused a wasting disease in the recipients. Additionally, by selecting 10 over-represented CD25+ Treg-derived TCRs (the most-reliably skewed to the Treg phenotype) from around 200 sequences, they identified 4 which individually bestowed an in vivo expansion advantage on transduced cells, and one which also responded to autologous APCs in vitro. None of these individual TCRs were described as conferring wasting disease. We feel that the apparent discrepancy between these results and our own is simply in the interpretation. The
expansion advantage imparted by the 10 selected CD25+ Treg-derived TCRs was around 10-fold greater than by the random pool of 10,000. This reveals that the selection criterion indeed enriched for the most-reactive TCRs; the lower bound for the frequency of Treg-derived TCRs conferring an in vivo expansion advantage would then be ≥1 in 51, and only 1 in 202 for TCRs, which lead to wasting disease. These values are close to our own upper bound for the fraction of strongly self-reactive Tregs (≤1 in 116, see above), using a different, more direct, assay. As we argued above, we consider that such a scarcity of self-reactive CD25+ Tregs excludes that self-specificity is important for their function.

Our finding that CD25+ Tregs generally do not bear TCRs specific for self-antigens implies that self-recognition should not be required for their well-documented suppression of experimentally-induced autoimmunity. In fact, there are reports in which this has been shown to be the case. Both Hori et al. and Fontenot et al. generated CD25+ Tregs by Foxp3-transduction of CD25− T cells. These induced CD25+ cells therefore had identical (largely non self-specific) TCR specificities to the CD25− cells from which they were derived. Nevertheless, these cells were still able to suppress autoimmunity brought about by transfer of CD25− cells into lymphopenic mice. In this situation, any preferential ability of Tregs to discriminate self-antigens based on their TCR specificities would have been completely abolished.

What, then, could be the mode of action of CD25+ Tregs in vivo? It is worth noting that most experimental systems in which Tregs provide protection from autoimmunity require the induction of a period of lymphopenia (eg by thymectomy, or transfer of cells into lymphocyte-deficient recipients). A model consistent with these studies is that Tregs act by preventing the dysregulated activation and/or expansion of naïve T cells in such circumstances. Such a notion is supported by experiments indicating that Tregs are able to limit the homeostatic expansion of normal T cells during lymphopenia. Furthermore, Barthlott et al. found that even TCR Tg non-Treg cells of irrelevant specificity could limit the expansion of normal T cells in lymphopenic hosts, and prevented the development of autoimmune disease.

At first glance, this hypothesis seems to be ruled out by the occurrence of autoimmunity in mutant mice with a genetic block in the generation of CD25+ Tregs.
In these mice, disease arises during normal development, without requiring the experimental induction of lymphopenia. However, Min et al., have shown that the normal neonatal environment supports lymphopenia-driven T cell proliferation and expansion. It is conceivable that CD25+ Tregs are important at this period of life, non-specifically restraining the proliferation and activation of naïve, potentially-autoreactive T cells. In adults, in whom the peripheral lymphoid compartment is ‘full’, this suppression mechanism would not ordinarily be invoked. There are data which support this idea: several groups have have depleted CD25+ cells in vivo in adult mice using anti-CD25 antibodies, but this treatment did not result in autoimmunity (, ; the resulting depleted cells were able to confer autoimmunity after transfer into lymphopenic recipient mice, indicating that Tregs had indeed been functionally eliminated). In contrast, depletion of CD25+ cells in neonatal mice did cause autoimmunity, with a similar rate of incidence as that following day 3 thymectomy.

We would like to stress that our finding that naturally occurring CD25+ Tregs are largely not self-specific by no means precludes their potential therapeutic use, nor does it argue that antigen-specific Tregs cannot be artificially generated for clinical or other purposes. It does cast a doubt, though, on theories in which the physiological role of Tregs is to suppress autoimmunity, but allow immunity, in an antigen-specific fashion.
**Materials and Methods**

*Generation of NFAT-GFP transduced fusion partner cells.* The minimal human IL-2 promoter, containing three NFAT binding sites, was cloned by polymerase chain reaction from the NFAT-LacZ plasmid (provided by N. Shastri), and three copies were tandemly inserted upstream of the GFP coding sequence in a self-inactivating retroviral vector (derived from pSir [Clontech]). Upon infection and reverse transcription, the 5’ LTR of this vector is replaced by the mutant 3’ LTR in which the enhancer elements (located at positions 198-298 in the MoMLV LTR) have been deleted: thus, expression of GFP is driven only by the internal, NFAT-binding site-containing promoter. Retrovirus-containing supernatant was collected following transfection of Ecotropic Phoenix packaging cells (provided by G. Nolan), and used to spin-infect a-b- BW5147 cells. Infected cells were cloned, and screened for expression of GFP following activation using 100nM phorbol myristylate acetate (PMA) + 1mg/ml ionomycin.

*Hybridoma generation.* Sorted splenic T cells were activated with plastic-bound anti-CD3e (145.2C11) and anti-CD28 (37.51) antibodies in the presence of 100U/ml mouse IL-2 (produced in transfected X63 cells) for 2-3 days. Equal numbers of activated T cells and the NFAT-GFP transduced fusion partner were then fused using PEG-1500, and plated at limiting dilution in the presence of 100mM hypoxanthine, 400nM aminopterin, and 16mM thymidine (HAT). Fusion efficiencies represent the number of HAT-resistant clones generated per input activated T cell.

*Isolation and assay of CD25+ Tregs.* Splenic T cells were stained with anti-CD4 (GK1.5) and anti-CD25 (PC61), and sorted into CD4+CD25- (naïve T cells) and CD4+CD25+ (Tregs) populations. To assay in vitro suppressor activity, 20,000 naïve T cells were CFSE-labeled, mixed with various numbers of Tregs, and stimulated for 4 days with 2000 immature DCs and 100ng/ml soluble anti-CD3e (145.2C11) in round-bottomed wells. The number of dividing CD25+ T cells (indicated by dilution of CFSE) was determined by flow cytometry.

*Activation using dendritic cells.* DCs were generated from mouse bone marrow cells by culture for 2 weeks in GM-CSF (produced in transfected X63 cells, and used at 1/50 dilution of the supernatant; 117. One day before use, they were activated by addition of 20ng/ml mouse TNF-a (R&D systems), or a soluble lysate from mouse tissues (see below) at a final A280=3. Both treatments resulted in DC
maturation, revealed by upregulation of MHC class II molecules (not shown). For stimulating DO11.10-derived and HA-TCR transgenic-derived hybridomas, soluble Ova or HA peptide (corresponding to amino acids 110-119 of influenza HA; SFERFEIFPK) were added at the time of activation. To measure alloreactivity, Balb/c-derived DCs (H-2\(^d\)) were used to stimulate C57BL/6-derived hybridomas (H-2\(^b\)-restricted). Soluble lysates were produced from mouse brain, gut, heart, kidney, liver, lung, lymph node, spleen and thymus by mechanical disruption in phosphate buffered saline (PBS), followed by centrifugation to remove insoluble material, and used either individually or mixed together at equal A\(_{280}\). Activated DCs were washed and cultured overnight with an equal number of hybridomas or naïve CD4\(^+\) T cells. The percentage of GFP-expressing hybridomas, or of CD69\(^+\) T cells (after staining with the anti-CD69 antibody H1.2F3 [Pharmingen]) was determined by flow cytometry.

**Mice.** C57BL/6, Balb/c, DO11.10 \(^{99}\), HA-TCR transgenic \(^{100}\), Ig-HA x HA-TCR double transgenic \(^{38}\) and AbEpIi (‘single peptide’ mice; \(^{102}\) mice were maintained under SPF conditions, and used at 8-12 weeks of age. DO11.10, HA-TCR transgenic and Ig-HA x HA-TCR double transgenic mice were on an inbred Balb/c background; single peptide mice were on an outbred C57BL/6 x 129/Sv background, with an H-2\(^b\) MHC haplotype. All experiments were performed with the approval of the Swiss Veterinary office.
Manuscript 2: Coexpression of CD25 and CD27 identifies Foxp3+ regulatory T cells in inflamed tissues

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*These authors contributed equally to the work


Abstract

A better understanding of the role of CD4⁺CD25⁺ regulatory T cells in disease pathogenesis should follow from the discovery of reliable markers capable of discriminating regulatory from activated T cells. We report that the CD4⁺CD25⁺ population in synovial fluid of juvenile idiopathic arthritis (JIA) patients comprises both regulatory and effector T cells that can be distinguished by expression of CD27. CD4⁺CD25⁺CD27⁺ cells expressed high amounts of Foxp3 (43 % of them being FoxP3⁺), did not produce IL-2, IFN-γ or TNF, and suppressed T cell proliferation in vitro being, on a per cell basis, 4-fold more potent than the corresponding peripheral blood population. In contrast, CD4⁺CD25⁺CD27⁻ cells expressed low amounts of Foxp3, produced effector cytokines and did not suppress T cell proliferation. Following in vitro activation and expansion regulatory but not conventional T cells maintained high expression of CD27. IL-7 and IL-15 were found to be present in synovial fluid of JIA patients and when added in vitro abrogated the suppressive activity of regulatory T cells. Taken together these results demonstrate that, when used in conjunction with CD25, CD27 is a useful marker to distinguish regulatory from effector T cells in inflamed tissues and suggest that at these sites IL-7 and IL-15 may interfere with regulatory T cell function.
Introduction

There is now clear evidence that a distinct population of naturally occurring regulatory T cells, which can be identified by the constitutive expression of CD4 and CD25, plays an essential role in controlling autoimmunity. Regulatory T cells are generated in the thymus or in periphery and, once activated, suppress other T cells by an as yet uncharacterized contact-dependent, cytokine-independent mechanism. A functional result of suppression is impaired production of IL-2, although evidence has been provided that an initial IL-2 production by responder cells is necessary for expansion of CD4+CD25+ T cells and induction of their suppressor function. The suppressor function of regulatory T cells can be relieved by exogenous IL-2 that acts on both regulatory and responder T cells and by IL-6 that blocks suppression at the level of responder cells.

The development and function of regulatory T cells is critically dependent on the transcriptional repressor Foxp3. Mice and humans that lack Foxp3 die from severe autoimmune diseases, while transduction of Foxp3 in naïve CD4+ T cells is sufficient to convert these cells into regulatory T cells. Since Foxp3 is the master control gene, it is in principle the most specific marker for regulatory T cells. However, the facts that Foxp3 is expressed exclusively intracellularly and that reliable reagents for staining are not yet available prevent its use for the identification and isolation of regulatory T cells.

CD25 is the hallmark antigen of regulatory T cells in mice and humans. In normal conditions, CD25 appears to identify a relatively homogeneous population of anergic regulatory T cells, although some heterogeneity may exist. For instance it has been reported that among CD4+CD25+ T cells those expressing CD103 or CD62L are more suppressive than their negative counterparts. Other useful markers of regulatory T cells under normal conditions include GITR, CTLA-4 and, in mice, neuropilin-1.

There is growing interest in the identification of regulatory T cells in various pathological conditions and recent studies indicate that CD4+CD25+ cells with regulatory function can be indeed detected in inflamed tissues. However, the identification of regulatory T cells in an ongoing immune response or in inflamed tissues in complicated by the fact that all the above markers, including CD25, are also expressed on activated T cells. A possible heterogeneity of the CD4+CD25+ subset...
in inflamed tissues has not been addressed so far and the problem therefore remains how to discriminate in an ongoing immune response regulatory from activated effector T cells.

Here we report that CD27 is stably expressed on regulatory T cells and can be used in conjunction with CD25 expression to discriminate in inflamed synovia regulatory T cells, expressing high amounts of Foxp3 and endowed with potent suppressive activity, from Foxp3− effector T cells devoid of suppressor activity. We also show that IL-7 and IL-15 are present in synovial fluid and in vitro abrogate the suppressive function of regulatory T cells.
**Results**

CD4⁺CD25⁺ T cells from synovial fluid of JIA patients express high amounts of Foxp3 and show suppressor activity in vitro.

Mononuclear cells were isolated from both synovial fluid and peripheral blood of 15 JIA patients (7 with polyarticular and 8 with oligoarticular disease course) and analyzed for the expression of CD4 and CD25 (Fig. 1A,B). The percentage of CD25⁺ cells within the CD4⁺ population ranged from 4 to 11.6 (median, 8.6) in peripheral blood, a value comparable to that found in healthy donors (not shown), while in synovial fluid it was significantly higher ranging from 6.5 to 35.2 (median, 12.3) (Fig. 1B).

![Figure 1](image-url)

**Figure 1.** CD4⁺CD25⁺ T cells are enriched in synovial fluid of JIA patients, express high amounts of Foxp3 mRNA and efficiently suppress proliferation of CD4⁺CD25⁻ autologous T cells. A) PBMC and SFMC from the same JIA patient were stained for CD4 and CD25. B) Percentages of CD4⁺CD25⁺ in PBMC and SFMC in 15 JIA patients. *P* value determined by Wilcoxon rank test. C) PBMC and SFMC were sorted based on expression of CD4 and CD25, and analyzed for expression of Foxp3 mRNA relative to 18S rRNA by quantitative real-time PCR. One representative experiment out of 10. D) Proliferation of 1.5 x 10⁶ CFSE-labeled PB CD4⁺CD25⁻ T cells stimulated by anti-CD3 and DCs in the presence of equal numbers of autologous CD4⁺CD25⁺ T cells (open histograms) or CD4⁺CD25⁻ T cells (filled histograms) isolated from PBMC or SFMC. The total number of responder T cells that had performed one or more cell divisions was determined by CFSE dilution analysis. The percentage of suppression was calculated from the number of dividing responder cells in presence of CD4⁺CD25⁺ T cells as compared to their number in presence of CD4⁺CD25⁻ control cells. One representative experiments out of 5.
CD4+CD25+ and CD4+CD25− T cells were isolated from PB and SF of the same JIA patient and analyzed for expression of the transcription factor Foxp3 using quantitative real-time PCR. Foxp3 mRNA was higher in CD4+CD25+ as compared to CD4+CD25− cells in both PB and SF (Fig. 1C). However, the amount of Foxp3 mRNA was much higher in the two populations isolated from SF than in those isolated from PB (Fig. 1C). These findings suggest that synovial CD4+CD25+ T cells may be activated in vivo and that some regulatory T cells may be present within the CD4+CD25− subset. Indeed, upon in vitro stimulation PB CD4+CD25+ regulatory T cells, and to a lower extent CD4+CD25− T cells, rapidly upregulated Foxp3 mRNA (Supplementary Fig. 1).

![Supplementary Figure S1. Transient upregulation of Foxp3 mRNA in CD4+CD25+ and CD4+CD25− cells upon TCR stimulation. A) CD4+CD25+ T cells were sorted from peripheral blood and stimulated by plate-bound CD3 (filled squares) or CD3 plus CD28 antibodies (filled circles). B) CD4+CD25+ regulatory T cells (filled squares), CD4+CD45RO+ memory T cells (open squares) and CD4+CD45RO− naïve T cells (open diamonds) were stimulated by plate-bound CD3 antibodies. Foxp3 mRNA was determined at the time indicated by real-time PCR and normalized to the amount of 18S rRNA in each sample.](image)

To examine the suppressive activity in vitro, SF and PB CD4+CD25+ T cells were added at a 1 to 1 ratio to cultures of CFSE-labeled autologous PB CD4+CD25− responder T cells stimulated by DCs and anti-CD3. As control, responder T cells were cultured in presence of unlabeled CD4+CD25− T cells. Four days later cultures were analyzed by FACS and the total number of responder T cells that had performed more than one cell division was determined by CFSE dilution analysis. The percentage of suppression was calculated from the number of dividing responder cells in presence of CD4+CD25+ T cells compared to their number in presence of CD4+CD25− control cells. As shown in Fig. 1D, CD4+CD25+ T cells isolated from PB or SF potently suppressed T cell proliferation (93% and 98.3% inhibition, respectively). We conclude that synovial fluid contains a large population of CD4+CD25+ T cells characterized by high expression of FoxP3 and suppressor activity in vitro.
**Results**

CD27 discriminates between regulatory and activated T cells in synovial fluid.

Since CD25 is an activation marker, the pool of CD4^+CD25^+ cells in SF may contain not only regulatory T cells but also activated T cells. To dissect a possible heterogeneity, we separated SF CD4^+CD25^+ T cells according to the expression of several cell surface markers and used Foxp3 mRNA to monitor the presence of regulatory T cells. Subsets defined by presence or absence of CTLA-4, CD62L, CD69 and GITR contained comparable amounts of Foxp3 mRNA (Fig. 2A). Similar data were obtained using CCR4, VLA-4 and CD103 (data not shown). Thus, these markers, known to be expressed on circulating regulatory T cells^{103,124-126,135-137}, did not segregate with FoxP3 expression in cells of inflamed synovia.

CD27 is a TNFR-family member that is expressed on naïve and subsets of memory T cells and is lost on terminally differentiated effector T cells^{138,139}. Since the latter are highly enriched in SF of adult rheumatoid arthritis and JIA patients^{140,141}, we asked whether CD27 may discriminate regulatory from activated effector T cells in inflamed joints. Indeed, in some synovial samples we noted that a high proportion (up to 50%) of CD4^+CD25^+ T cells were CD27^- (Fig. 2B and Table II). CD4^+CD25^-CD27^- cells were also found in PB of patients and healthy adults although at lower frequency (mean 15.6%, range 8.3-33 in 14 patients and 7.9%, range 2.6-11.3 in 5 controls).

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**Figure 2.** CD27 identifies Foxp3 expressing cells within CD25^+ and CD25^- synovial CD4^+ T cells. A) CD4^+CD25^+ SFMC were stained with antibodies to CTLA-4, CD62L, CD69 and GITR. Positive and negative subsets were sorted and analyzed for Foxp3 mRNA. Mean±SD of three separate experiments. B) PBMC and SFMC from patient 12 were stained with antibodies to CD4, CD25 and CD27. The histograms show the expression of CD27 on gated CD4^+CD25^- or CD4^+CD25^+ T cells. C) SFMC were sorted according to the expression of CD4, CD25 and CD27, and analyzed for expression of Foxp3 mRNA. For comparison the value in CD25^-CD27^- was set to 100. Mean±SD of 3 separate experiments.
The four subsets of CD4+ cells identified according to the expression of CD25 and CD27 were isolated from SF and PB of JIA patients and from PB of adult healthy donors and tested for Foxp3 mRNA expression. FoxP3 mRNA was at least 10 fold higher in CD4+CD25−CD27+ cells as compared to CD4+CD25+CD27− cells (Fig. 2C and Supplementary Fig. 2). Low amounts of Foxp3 mRNA were also found in CD4+CD25−CD27− T cells, and no Foxp3 was detected in CD4+CD25−CD27− cells.

Supplementary Figure S2. CD27 identifies Foxp3 expressing cells within peripheral blood CD4+CD25+ cells from JIA patients and adult healthy donors. PBMC from two JIA patients (A) and 4 adult healthy donors (B) were sorted according to the expression of CD4, CD25 and CD27 and analyzed for expression of Foxp3 mRNA. For comparison the value in CD25+CD27− was set to 100. Values represent mean ± SD.

To estimate the frequency of Foxp3 expressing SF T cells within the four subsets we used a sensitive PCR method to detect Foxp3 in replicate samples containing limiting numbers of T cells (5 cells per sample). The highest frequency of FoxP3+ cells (41.6%) was found in the CD4+CD25−CD27+ subset, while the CD4+CD25+CD27− and the CD4+CD25−CD27+ subsets showed much lower frequencies (6.2% and 2.7%, respectively) and the CD4+CD25−CD27− subset was negative (Fig. 3). Considering that frequencies are underestimated, these results suggest that CD27 marks a homogeneous population of Foxp3 expressing cells in inflamed synovia.
GITR and CTLA-4 were expressed at comparable levels on CD4⁺CD25⁺CD27⁺ and CD4⁺CD25⁺CD27⁻ cells, whereas CD62L and CCR4 were expressed on a higher proportion of CD27⁺ cells (Supplementary Fig. S3).

In addition, upon TCR triggering CD4⁺CD25⁺CD27⁺ T cells did not express IL-2, TNF or IFN-γ mRNAs whereas CD4⁺CD25⁺CD27⁻ cells expressed high amounts of cytokine mRNAs (Supplementary Fig. S4). Taken together these findings are consistent with the notion that CD4⁺CD25⁺CD27⁺ cells are regulatory cells while CD4⁺CD25⁺CD27⁻ represent activated effector cells.

To directly test the suppressive function, the four subsets identified by the expression of CD25 and CD27 were isolated by cell sorting from synovial fluid and added to cultures of CFSE-labeled PB CD4⁺CD25⁻ T cells. As shown in Fig. 4A, suppressive activity was restricted to the CD4⁺CD25⁺CD27⁺ subset. The other 3 subsets either did not interfere or even enhanced T cell proliferation (Fig. 4A). synovial fluid CD4⁺CD25⁺CD27⁻ T cells were, on a per cell basis, 4-fold more potent as compared to CD4⁺CD25⁺CD27⁻ T cells isolated from peripheral blood of the same patient (Fig. 4B). We conclude that based on four different criteria (expression of FoxP3 mRNA, expression of surface markers, lack of cytokine production and
suppression of T cell proliferation) CD27 expression in the context of CD25 expression allows discrimination of regulatory from activated/effector T cells in inflamed joints.

**Figure 4. CD27 identifies potent suppressor cells within CD4+/CD25+ synovial T cells.** A) Proliferation of CFSE-labeled CD4+/CD25+ peripheral blood T cells stimulated by anti-CD3 and DCs in the absence (open histogram) or presence (filled histogram) of equal numbers of the indicated autologous CD4+ T cells isolated from synovial fluid. The percentage inhibition was calculated as in Fig. 1. Comparable results were obtained using synovial T cells isolated from patients #14 (shown) and #2, 5 and 7. B) Proliferation of CFSE-labeled CD4+/CD25+ peripheral blood T cells in the presence of serial two-fold dilutions of autologous CD4+/CD25+/CD27+ T cells from peripheral blood (open circle) or synovial fluid (filled square). Shown is the total number of responder T cells that had undergone more than one cell division. Means±SD of triplicate cultures. Comparable results were obtained with samples from patients #15 (shown) and #5 and 7.
CD27 is retained on regulatory T cells following activation and expansion.

It is known that CD27 is lost upon terminal differentiation of conventional CD4⁺ T cells. To investigate whether regulatory T cells may lose or retain CD27 expression upon activation, we isolated CD4⁺CD25⁺CD27⁺ regulatory T cells and CD4⁺CD25⁻CD27⁺ naïve and memory T cells from PB (Fig. 5A). Cells were labeled with CFSE and stimulated with anti-CD3 in the absence or presence of exogenous IL-2 or anti-CD28. As shown in Figure 5B and C, CD4⁺CD25⁻ T cells proliferated extensively and downregulated CD27 expression as a function of cell division. In contrast, CD4⁺CD25⁺ regulatory T cells proliferated poorly in the absence of IL-2 whereas they performed several cell divisions when exogenous IL-2 or costimulation was provided. Remarkably, in both conditions proliferating regulatory T cells retained high expression of CD27. We conclude that CD27 is a stable marker of regulatory T cells that is retained following activation and expansion.

Figure 5. CD27 is stably expressed in proliferating regulatory T cells whereas it is downregulated on activated non-regulatory T cells. A) CD4⁺CD25⁺CD27⁺ regulatory T cells and CD4⁺CD25⁻CD27⁺ T cells (comprising naïve and memory cells) were sorted from peripheral blood to >99% purity. B) Cells were labeled with CFSE and stimulated with plastic-bound CD3 antibodies in the absence or presence of IL-2 or CD28 antibodies. CD27 expression was measured as a function of cell division on day 5 (left panel) and day 10 (right panel). One representative experiment out of three performed.
Different proportions of CD4^+CD25^+ subsets in JIA patients with polyarticular or oligoarticular disease.

Some of the patients in this study had the disease limited to a few joints (persistent oligoarticular), whereas others showed a more aggressive polyarticular involvement (extended oligoarticular, polyarticular and systemic) (Table I and II). While the percentage of total CD4^+CD25^+ cells did not differ significantly between the two groups, the relative proportion of CD27^+ and CD27^- cells within the CD4^+CD25^+ subset in affected joints was significantly different (Fig. 6A). Patients with polyarticular disease showed higher proportion of CD27^- activated/effector cells and slightly lower proportion of CD27^+ regulatory T cells as compared to patients with oligoarticular disease that showed a significantly higher ratio of regulatory to activated cells (Fig. 6B).

**Figure 6.** Differential distribution of CD25^+CD27^- regulatory and CD25^+CD27^- activated/effector cells within synovial CD4^+ T cells of JIA patients with oligoarticular or polyarticular disease course. A) Percentages of total CD4^+CD25^+ T cells and CD27^+ and CD27^- subsets in 13 JIA patients with oligoarticular disease course (white bars) and 12 JIA patients with polyarticular disease course (grey bars). B) Ratio between regulatory and activated/effector cells in oligoarticular (white bars) or polyarticular (grey bars) JIA patients. Boxes contain values falling between the 25th and 75th percentiles. Whisker lines that extend from the boxes represent the highest and the lowest values from each subgroup. The lines in the boxes represent median values. *P* value determined by Mann Whitney U test. See also Table II for data from individual patients.
IL-7 and IL-15 are present in synovial fluid and limit the suppressor activity of regulatory T cells.

Biopsies of synovial tissues were obtained from 3 JIA patients. When analyzed by immunohistochemistry, regulatory T cells co-expressing CD4, CD25 and CD27 were found in lymphoid aggregates (Fig. 7).

To investigate the possibility that cytokines that are present in the inflamed tissue may interfere with regulatory T cell function, we tested a large panel of recombinant cytokines for their capacity to block the suppressive activity of regulatory T cells in vitro. The activity of regulatory T cells was strongly reduced in the presence of IL-2, IL-7 and IL-15 and virtually abolished when IL-7 and IL-15 were added together (Fig. 8A). In contrast, the pro-inflammatory cytokines IL-6, IL-12, TNF and IFN-γ, as well as another γ-common dependent cytokine, IL-4, were ineffective while TGF-β and IL-10 showed direct suppressive activity even in the absence of regulatory T cells (Fig. 8A). Remarkably, IL-7 and IL-15 were detected in the synovial fluid of JIA patients, IL-7 being significantly higher in patients with...
polyarticular as compared to patients with oligoarticular disease (Fig. 8B). Taken
together these results suggest that in inflamed tissues IL-7 and IL-15 may substantially
limit the suppressor function of regulatory T cells.

Figure 8. IL-7 and IL-15 counteract the suppressor activity of regulatory T cells and are present in
synovial fluid of JIA patients. A) Proliferation of CFSE-labeled CD4+CD25+ peripheral blood T cells
stimulated by TSST-pulsed DCs in the absence (open histograms) or presence (filled histograms) of equal
numbers of the CD4+CD25+ T cells. The cultures were set up in medium alone (no cytokines) or in medium
supplemented with the indicated cytokines. B) IL-7 and IL-15 were quantified by ELISA in sera and synovial
fluid from 15 oligoarticular (white bars) and 15 polyarticular (grey bars) JIA patients and in sera from 12 age-
matched healthy controls (black bars). Boxes contain values falling between the 25th and 75th percentiles.
Wiskard lines that extend from the boxes represent the highest and the lowest values from each subgroup. The
lines in the boxes represent median values. P value determined by Mann Whitney U test.
### Table I. Clinical characteristics of JIA patients included in the study

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RF: rheumatoid factor; SF: synovial fluid; ESR: erythrocyte sedimentation rate; NSAID: non steroidal anti-inflammatory drugs; MTX: methotrexate; CS: corticosteroids, CyA: Cyclosporin A.
Table II. Distribution of CD27 in synovial CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells subsets in JIA patients with oligoarticular and polyarticular disease course.

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a) Percentages of total CD4⁺ synovial fluid (SF) T cells;
b) Percentage of CD27⁻ cells within CD4⁺CD25⁺ cells;
c) Percentage of CD27⁻ cells within CD4⁺CD25⁻ cells.


**Discussion**

In this study we addressed the problem of identifying naturally occurring regulatory T cells in inflamed tissues. We found that in synovial fluid of JIA patients CD27 expression can be used to discriminate, within the CD4^+^CD25^+^ subset, regulatory T cells from activated effector T cells. CD27 is expressed on regulatory T cells in both peripheral blood and synovial tissues and is retained by these cells following activation and clonal expansion *in vitro* whereas CD27 is absent on effector T cells and is rapidly lost on CD27^+^ naïve and memory T cells following activation \(^{142,143}\). Thus, although CD27 is not a specific marker for regulatory T cells, the differential regulation of expression in regulatory and conventional T cells makes it a suitable marker for the identification of regulatory T cells in inflamed tissues.

The intensity of CD25 expression is considered a reliable marker for regulatory T cells in peripheral blood \(^{16}\). However, this may not be sufficient to identify regulatory T cells in inflamed tissues. Indeed a recent study demonstrated that CD25^{dim} cells from synovial fluid express Foxp3 and have suppressive activity \(^{46}\). In this regard, we found that CD27 is expressed not only on all CD25^{bright} cells, but also of a sizeable proportion of CD25^{dim} cells and that CD25^{dim}CD27^+^ and CD25^{bright}CD27^+^ T cells express comparable amounts of Foxp3 mRNA (C.R.R., unpublished data). We conclude that the combination of CD25 and CD27 allows identification of most of regulatory T cells while effectively excluding effector T cells.

Growing evidence over the past few years indicates that CD27, as well as other members of the TNFR family, such as OX40 (CD134) and 4-1BB (CD137), plays an important role for the effective generation of many types of T cell responses \(^{144}\). It remains to be established what role CD27 may play in regulatory T cell function and whether sustained expression of CD27 on regulatory T cells contribute to their maintenance *in vivo*.

Using limiting dilution analysis we could estimate that 41% of CD4^+^CD25^+^CD27^+^ T cells isolated from inflamed joints express Foxp3. The possibility of isolating a rather homogeneous population of regulatory T cells from inflamed tissues was instrumental to establish their relative potency as compared to regulatory T cells from peripheral blood. A direct comparison of regulatory T cells
from synovial fluid and peripheral blood of the same patient revealed that synovial regulatory T cells expressed much higher levels of Foxp3 and were on a per cell basis 4-fold more potent in suppressing T cell proliferation than peripheral blood regulatory T cells. As activation by anti-CD3 and IL-2 increases both Foxp3 expression and suppressor function of peripheral blood regulatory T cells ⁵⁰, these findings suggest that the increase potency of synovial regulatory T cells is a consequence of their activated state in vivo.

The relevance of discriminating regulatory from effector T cells in inflamed tissues is underlined by our finding that in the joints of JIA patients up to 50% of CD4⁺CD25⁺ T cells were CD27⁻ effector T cells and that the ratio of regulatory to activated T cells was higher in patients with oligoarticular disease course than in those with severe polyarticular disease course. Since the contamination of effector T cells can be substantial and varies with disease state it will be important to reassess the presence of regulatory T cells in other pathological tissues ⁴³,⁴⁵.

Although regulatory T cells can be found in tissues undergoing chronic inflammation it remains to be established whether they exert their function in vivo. Besides possible intrinsic defects in regulatory T cells ⁴⁷-⁴⁹, there may be several mechanisms that limit the efficacy of regulatory T cells in peripheral inflamed tissues. For instance, in vitro preactivated T cells become resistant to suppression and this resistance is dependent on the strength and duration of the stimulus ⁴⁵,⁵⁰. We also found that naïve T cells become completely refractory to suppression 24 hours after TCR stimulation (C.R.R., unpublished). In addition, exogenous signals such as those provided by GITR-L and IL-6 render responsive T cells resistant to suppression mediated by CD4⁺CD25⁺ regulatory T cells ¹⁸,¹⁴⁵. We screened a large panel of cytokines for their capacity to relieve suppression in vitro and found that IL-7 and IL-15, and more effectively a combination of the two, can counteract the suppressor function of regulatory T cells. Based on these results and on the finding that both IL-7 and IL-15 can be detected in the joint fluid of JIA patients, we suggest that in target tissues the function of regulatory T cells may be substantially limited by these cytokines and that therapies that aim at neutralizing such cytokines may not only decrease bystander T cell activation but also reconstitute the suppressor function of regulatory T cells.
**Materials and methods**

*Patients.* Twenty-five consecutive JIA patients diagnosed according to ILAR Durban’s criteria were included in the study. All patients had active disease and underwent synovial fluid aspiration for steroids injection. To be included in the study, patients with persistent oligoarticular course should have presented a disease duration longer than 1 year. In all cases, a steroid injection in the same joint in the previous 6 months was considered as an exclusion criterion. The patients tested in the functional studies were under NSAID and/or methotrexate treatment. The main clinical and laboratory features and the ongoing treatment at the moment of the study are reported on Table I. Approval for the study was obtained from the institutional medical ethics review board.

*Media and reagents.* The medium used throughout the experiments was RPMI 1640 supplemented with 10% fetal calf serum, 1% Glutamax, 1% nonessential amino acids, 1% pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin (all Invitrogen), 5x10⁻⁵ M 2-mercaptoethanol (Merck). Recombinant human IL-6, IL-7, IL-10, IL-12, IL-15, IFN-γ, TNF, and TGF-β were purchased from Pharmingen. IL-2 and IL-4 were produced in our laboratory using the myeloma-based expression system.

*FACS analysis.* The following monoclonal antibodies were used: mouse CD4-FITC, CD4-APC, CD27-FITC, CD27-PE, CD62L-PE, CD69-PE, CTLA4-PE, CCR4-PE (all from Becton Dickinson), CD25-FITC (Dako), CD25-PE (Miltenyi Biotec), and GITR-PE (R&D Systems).

*Cell isolation.* Peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) were isolated by Ficoll-Hypaque (Sigma) density gradient centrifugation. Following staining for various surface markers, subpopulations of CD4⁺ T cells were sorted by FACS Vantage (Becton Dickinson). Purity of cell preparations was typically >97%. Monocytes were isolated from PBMC by positive selection using CD14-microbeads (Miltenyi). The purified monocytes were cultured for 3-5 days in RPMI-10% FCS containing 50 ng/ml GM-CSF (Leukomax, Novartis) and 1000 U/ml IL-4.

*Proliferation assay.* Peripheral blood CD4⁺CD25⁻ T cells were labeled with 0.5 µM CFSE (Molecular Probes) for 8 minutes at room temperature. After quenching of the labeling reaction by addition of RPMI-10% FCS, cells were washed extensively. Cells (1.5x10⁶) were cultured alone or together with different numbers of
unlabeled regulatory or control cells in the presence of $10^3$ immature DCs and either 2 ng/ml TSST (Toxin Technology) or 0.25 ng/ml CD3 antibodies (supernatant from clone OKT3-3). Proliferation was measured on day 4 on a FACSCalibur (Becton Dickinson) using propidium iodide (Sigma) to exclude dead cells. In some experiments T cells were stimulated with plate-bound CD3 antibodies (2 µg/ml, from clone TR66) in the absence or presence of recombinant IL-2 (100 U/ml) or CD28 antibodies (2 µg/ml, Pharmingen).

**Real time PCR.** For quantitative assessment of relative mRNA levels, total RNA was prepared from sorted subpopulations using Trizol LS reagent (Invitrogen) according to the manufacturer’s instructions. RNA was then reverse transcribed using M-MLV RT reverse transcription kit with random hexamer primers (Invitrogen). The relative level of FoxP3 mRNA in each subset was determined by real-time PCR on an ABI PRISM 7700 sequence detector (Applied Biosystems) using the Assay-On-Demand product for FoxP3 detection (Hs00203958_m1) and universal PCR master mix (both from Applied Biosystems). The obtained values were normalized to the amount of 18S rRNA (4310893E, Applied Biosystems) present in each sample. For detection of relative cytokine mRNA levels, sorted T cell subsets were stimulated for 6 hours with 50 nM phorbol 12,13-dibutyrate (PdBu) (Sigma) and 1µg/ml anti-CD3 (clone TR66) before extraction of total RNA. Cytokine mRNA levels were determined using Applied Biosystems products for IL-2 (4309882P), TNF (Hs00174128_m1), and IFN-γ (4327052F).

**Cytokine detection assays.** IL-7 and IL-15 were measured using commercial ELISA (R & D Systems) in the sera and synovial fluid of 30 JIA patients (15 with oligoarticular course and 15 with polyarticular course) and in sera of 12 age-matched controls that were obtained for routinely preoperative examination before minor surgery. The assay detection limit was 0.1 pg/ml for IL-7 and 2 pg/ml for IL-15.

**Five-cell PCR.** For determination of Foxp3 mRNA at a 5-cell level, the four CD4⁺ T cell subsets were first sorted by flow cytometry. From each of the purified subpopulations, 5-cell aliquots were then resorted directly into wells of a 96-well conic plate. The subsequent procedures for cDNA preparation and nonspecific cDNA amplification were carried out as described by Bigouret et al. ¹⁴⁷. One µl of the nonspecifically amplified cDNA was used to amplify Foxp3 cDNA with 0.5 µM of the specific primers Foxp3-F (CACCTACGCCACGCTCATC) and Foxp3-R.
(ACTCAGGTTTGGCGGATG) (both from Microsynth) in presence of 1.5 mM MgCl\textsubscript{2}. As a control, the expression of CD3 was assessed using the primers CD3 S1 (CGTTGTTTCCCCCTTTTTTCTT) and CD3 AS1 (GATTAGGGGTAGGGAGTG) (Microsynth). The program used for amplification was 3 min at 94°C; 30 sec at 94°C, 30 sec at 58°C, 30 sec at 72°C, 40 cycles.

**Immunohistochemistry.** Tissue specimens were prepared for immunohistochemistry according to standard technique. Briefly, specimens were fixed in 10% formalin for 4 hours, dehydrated and embedded in paraffin. Paraffin serial sections were stained for 30 minutes at room temperature with mouse antibodies to CD4 (4B12), CD25 (25C04), CD27 (137B4) (from Neomarkers), CD20 (L26), CD3 (polyclonal antisera) (from Dako) followed by anti-mouse Ig antibody conjugated to peroxidase labelled-dextran polymer (EnVision, Dako) and chromogenic diaminobenzidine substrate (Dako). Slides were counterstained with Mayer’s hematoxylin. For double immunofluorescence, secondary labeling was performed, for 30 minutes at room temperature with Alexa Fluor 594 goat anti-mouse IgG2b (Molecular Probes) and, subsequently, with Alexa Fluor 488 goat anti-mouse IgG1 (Molecular Probes) to label CD25 and CD27, respectively.

**Statistical analysis.** Differences in the percentages of matched peripheral blood and synovial fluid CD4\textsuperscript{+}CD25\textsuperscript{+} T cells were analyzed by the Wilcoxon matched pairs signed rank test. Differences in the percentages of CD4\textsuperscript{+}CD25\textsuperscript{+}CD27\textsuperscript{+} and CD4\textsuperscript{+}CD25\textsuperscript{+}CD27\textsuperscript{−} and in the amounts of IL-7 and IL-15 in oligoarticular and polyarticular JIA patients were analyzed by Mann-Whitney U test.
Manuscript 3: Toll-like receptor stimulation as a third signal required for activation of human naïve B cells.

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Abstract

According to the current model, naïve B cell activation is dependent on the sequential integration of two signals: B cell receptor (BCR) crosslinking by antigen, followed by cognate interaction with helper T cells through an immunological synapse. Using an improved method to purify human naïve B cells we found that BCR stimulation and T cell help induced initial cell division but were not sufficient to promote survival and differentiation thus leading to abortive proliferation of naïve B cells. Extensive B cell proliferation, isotypic switch and differentiation to immunoglobulin (Ig)- secreting cells was induced by addition of microbial products that trigger any of the Toll-like receptors (TLRs) that are upregulated in naïve B cells upon BCR triggering. TLR agonists acted directly on B cells and were required irrespective of the nature of the T helper cells present. Supernatants of dendritic cells (DC) stimulated by DC-specific TLR agonists were also capable of enhancing B cell responses although to a much lower and variable extent. These results indicate that human naïve B cell activation is critically dependent on innate stimuli acting optimally on TLRs expressed by B cells. The coupling of BCR stimulation to TLR expression endows the human system with a high degree of specificity since it allows focusing of innate signals only on antigen stimulated B cells.
**Introduction**

Naïve B cell activation is initiated by binding of antigen to the BCR. Triggered BCRs initiate a signaling cascade that leads to upregulation of costimulatory molecules and mediate efficient antigen internalization followed by processing and presentation of antigenic peptides on MHC class II molecules for recognition by specific T cells \(52,53\). At the immunological synapse specific T cells are activated and in turn stimulate B cells via CD40L-CD40 interaction and cytokines \(54\). The current dogma is that the timely integration of BCR stimulation (signal one) and T cell help (signal two) is both necessary and sufficient to drive naïve B cell proliferation and differentiation to Ig secreting plasma cells \(52\). Compared to naïve B cells, memory B cells have looser triggering requirements. For instance human memory B cells can be readily activated in the absence of BCR stimulation by bystander T cell help and cytokines \(60,148,149\).

It has been known for long time that mouse naïve B cells can respond to LPS and CpG by undergoing extensive proliferation and differentiation, even in the absence of BCR triggering or T cell help \(55,56\). Indeed mouse B cells constitutively express a variety of TLRs, including TLR4 and TLR9, which are triggered by LPS and CpG \(15,57\). There is growing evidence that TLRs play an important role in B cell physiology. Recently it has been shown that particles containing proteins and nucleic acids can induce autoantibody formation by synergistically engaging BCR and TLRs \(61-63\). However remains to be established whether the BCR-TRL co-engagement is a general requirement for B cell activation also in the presence of T cell help.

TLRs are differentially expressed in human and mouse B cells. In contrast to mouse B cells, constitutive TLR expression is restricted to human memory B cells that express TLR2, TLR6, TLR7, TLR9 and TLR10 but not TLR4 \(58,59\). Consequently TLR agonists can directly activate human memory but not naïve B cells and it has been suggested that this selective responsiveness may be a mechanism to maintain serological memory \(60\). It was also shown that human naïve B cells upregulate TLRs following BCR stimulation \(58\) but it was not established whether and how TLR signals contribute to human naïve B cell activation.

To define the triggering requirements of naïve B cells it is essential to identify pure populations and to measure their response in a quantitative fashion. In previous studies human naïve B cells were isolated according to the expression of IgD \(150,151\) or
In this study we used an improved method of naïve B cell isolation using a novel marker, the ABCB1 transporter, that precisely identifies human naïve B cells\textsuperscript{66}. We show that proliferation and differentiation of human naïve B cells require a combination of three signals: 1) BCR triggering, 2) cognate T cell help, and 3) TLR stimulation.

**Results**

**CpG synergizes with BCR triggering and T cell help in activation of human naïve B cells.**

We recently reported that the ABCB1 transporter is expressed on human naïve B cells and is absent on transitional and memory B cells \textsuperscript{66}. We used this marker to isolate highly pure naïve B cell populations identified as CD19\textsuperscript{+} CD27\textsuperscript{-} IgG/A\textsuperscript{-} ABCB1\textsuperscript{+} cells (Fig. 1A). Naïve B cells were labeled with CFSE and cultured in the presence of F(ab')\textsubscript{2} fragments of anti human Ig (anti-Ig) and autologous CD4\textsuperscript{+} T cells in the presence of the bacterial superantigen TSST as a source of cognate T cell help. As shown in Figure 1B (upper panel) anti-Ig or T cell help alone failed to induce B cell proliferation, whereas combination of anti-Ig and T cell help induced B cell proliferation as measured by CFSE dilution after 5 days. At this time point naïve B cells had undergone up to 3-4 divisions, but proliferating B cells failed to accumulate and died after approximately 4 divisions, as detected by the accumulation of propidium iodide positive cells that had diluted CFSE (Fig.1C). The TLR9 agonist CpG 2006 had only a marginal effect on naïve B cells when added alone or in combination with anti-Ig or T cell help (Fig. 1B, lower panel). Remarkably, however, the combination of anti-Ig, T cell help and CpG led to extensive B cell proliferation and accumulation of large numbers of B cells that had undergone more than 7 divisions. Thus, CpG potently synergized with BCR triggering and T cell help in induction of human naïve B cell proliferation.

CpG might synergize in the induction of B cell response by enhancing the capacity of B cells to stimulate T cells and therefore by boosting T cell help. This possibility was ruled out by the finding that T cell proliferation was comparable in the presence or absence of CpG (data not shown) and by the finding that the synergistic effect of CpG was observed also when soluble CD40L was used as a source of T cell help.
help (Fig. 1D). We conclude that sustained B cell proliferation is critically dependent on microbial stimuli acting on TLR9 expressed by activated naïve B cells.

**Results**

A) For isolation of human naïve B cells, peripheral blood CD19+ B cells were labeled with MitoTracker Green FM (MT), stained for lineage (CD3, CD14, CD16), CD27, and surface IgG and IgA, and sorted by FACS to a purity of > 99.99%. Upper panel, before sort; lower panel, after sort. B) CFSE-labeled naïve B cells were stimulated with: i) F(ab’)_2-fragments of goat anti-human Ig (anti-Ig), ii) autologous CD4+ T cells in presence of TSST (T help) and iii) CpG 2006 in various combinations as indicated and analyzed for proliferation on day 5. Events were acquired for a fixed time to allow a direct quantitative comparison of B cell proliferation in the different conditions. One representative experiment out of 20. C) CFSE-labeled naïve B cells were stimulated with anti-Ig, T help and analyzed on day 5. Proliferation profiles of live cells (empty) and dead cells (gray) were determined according to propidium iodide (PI) staining. Shown is one representative experiment out of six. D) CFSE-labeled naïve B cells were stimulated with anti-Ig, CpG and either T cell help or recombinant soluble CD40L and IL-2. Shown is one representative experiment out of five.
The requirements for CpG are observed irrespective of the source of T helper cells.

We considered the possibility that the requirement for CpG in the induction of human naïve B cell proliferation might be limited to conditions where T cell help is suboptimal. Indeed, it has been shown that B cell helper activity is a specialized function of a subset of memory CD4+ T cells identified by the expression of CXCR5, also called follicular helper T cells (T_{FH})\textsuperscript{154-156}. We therefore compared naïve and memory subsets of CD4+ T cells for their capacity to trigger naïve B cell proliferation in the presence or absence of CpG. In the absence of CpG, none of the T cell subsets was capable of sustaining B cell proliferation and effector memory T cells (T_{EM}) even appeared to kill naïve B cells (Fig. 2). In contrast, in the presence of CpG all subsets (with the exception of CD25\textsuperscript{+} regulatory T cells) induced substantial B cell proliferation, the highest level being that induced by T_{FH}. We conclude that naïve B cell activation requires TLR stimulation irrespective of the nature of T helper cells.

![Figure 2. The requirements for CpG are observed irrespective of the source of T helper cells.](image)

Naïve B cells were labeled with CFSE and stimulated as indicated with anti-Ig, CpG and different subsets of autologous CD4+ T cells in presence of TSST. Proliferation was determined on day 5 by FACS analysis. CD4+ T cell subsets were isolated from peripheral blood as follows: naïve (T_{naïve}) CD4+CD45RO\textsuperscript{−}CCR7\textsuperscript{+}, central memory (T_{CM}) CD4+CD45RO\textsuperscript{−}CCR7\textsuperscript{−}CCR5\textsuperscript{−}, follicular help (T_{FH}) CD4+CD45RO\textsuperscript{+}CCR7\textsuperscript{−}CCR5\textsuperscript{+}, effector memory (T_{EM}) CD4+CD45RO\textsuperscript{+}CCR7\textsuperscript{−}, regulatory T cells (T_{reg}) CD4+CD45RO\textsuperscript{−}CD25\textsuperscript{+}. Shown is one representative experiment out of four.
BCR stimulation can be delivered transiently, while T cell help and TLR stimulation need to be sustained.

We next investigated the temporal requirements for naïve B cell activation. Naïve B cells were cultured in the presence of anti-Ig, T cell help and CpG. The stimuli were removed after 24 hours and the cells were recultured in the absence or presence of the same stimuli alone or in combination (Fig. 3A). Removal of the stimuli after 24 hours virtually abolished the B cell response measured on day 5. Addition of T cell help or CpG partially restored the response when added alone and completely restored it when added together. In all cases the presence of anti-Ig did not show an additive effect. Furthermore, the anti-Ig signal could be temporally dissociated from T cell help and TLR signal as shown by the fact that naïve B cells exposed to a short pulse of anti-Ig became capable of responding to a subsequent addition of T cell help and CpG (Fig. 3B). We conclude that signal one can be delivered even by a short stimulation and prepare naïve B cells for subsequent stimulation by T cells and TLR agonists that need to be sustained in time.

**Figure 3.** BCR triggering, T cell help and TLR9 stimulation have diverse functions in human naïve B cell activation. A) CFSE-labeled naïve B cell were cultured with anti-Ig, T cell help and CpG. Stimuli were either left in culture (black profile) or removed after 24h. The cells were then recultured in medium alone or in the presence of the indicated stimuli in the absence (open profiles) or the presence of anti-Ig (filled profiles). Cell proliferation was analyzed on day 5. Shown is one experiment out of three. B) CFSE-labeled naïve B cells were cultured for one day in medium with or without anti-Ig. Cells were then washed and cultured in presence or absence of T cell help and CpG for additional 4 days. One representative experiment out of four.
TLR agonists provide signal three either directly on B cells or indirectly through DC activation.

Upon BCR triggering naïve B cells upregulate several TLRs. We therefore tested an array of TLR agonists for their capacity to promote B cell proliferation when combined with anti-Ig and T cell help. All agonists of TLRs expressed by activated human B cells were capable of synergizing with BCR triggering and T cell help (Fig. 4A). In contrast, poly(I:C) and LPS failed to provide such signals, consistent with the absence of TLR3 and TLR4 in human B cells. Extensive B cell proliferation was observed also in the presence of E. coli DNA or live bacteria that contain agonists of TLR2 and TLR9.

**Figure 4.** TLR agonists provide signal three either directly on B cells or indirectly through DC activation A) CFSE-labeled naïve B cells were cultured in the presence of anti-Ig, T cell help and an array of TLRs agonists including E. coli DNA and intact bacteria. Shown is the proliferative response on day 5 in one representative experiment out of six. B) Immature monocyte-derived DCs were either left untreated or activated with LPS or poly(I:C). Supernatant was collected after 24h and added at an optimal concentration (10%) to naïve B cells in presence of anti-Ig and T cell help. Shown is the relative increase of proliferating B cells that had undergone more than four divisions in supplemented cultures compared to control cultures (medium). Data represent mean +/- stdev of 4 experiments using DC supernatants from 12 donors.
While poly(I:C) and LPS were not able to directly stimulate B cells, we noticed that the supernatant of poly(I:C)- or LPS-activated DCs was able to synergize with anti-Ig and T cell help in induction of B cell proliferation. However the effect was variable and much lower as compared to the effect of direct TLR stimulation (Fig. 4B). To identify a putative activity in these supernatants we tested the effect of neutralizing antibodies against a variety of cytokines and found that anti-IL-6 and anti-IL-12 abolished the stimulatory effect of supernatants of LPS-activated DCs, while antibodies to IL-10 had no effect (Supplementary Fig. 1A). Furthermore, addition of recombinant IL-6 and IL-12 was able to increase proliferation of B cells triggered by anti-Ig and T cell help (Supplementary Fig. 1B).

![Graphs showing the effect of LPS-DC supernatants on B cell proliferation with and without anti-IL-6, anti-IL-10, anti-IL-12, and anti-IL-6/12.](image)

We conclude that signal three can be delivered to B cells by a variety of microbial products acting directly on the TLRs expressed on B cells or indirectly through activation of DCs and release of cytokines, primarily IL-6 and IL-12.

**TLR stimulation is required for induction of class switch and plasma cell differentiation.**

Class switch recombination requires the B-cell-specific activation-induced cytidine deaminase protein (AID), which initiates this reaction through its single-stranded DNA-specific cytidine deaminase activity. Although anti-Ig and T cell help were able to promote naïve B cell proliferation by day 4, the divided cells failed to upregulate AID mRNA (Fig. 5A). In contrast, AID mRNA was markedly upregulated on day 4-B cells stimulated by anti-Ig, T cell help and CpG. Furthermore, surface IgG or IgA were detectable by day 6 in a fraction (5-15%) of B cells primed...
by the three stimuli (Fig. 5B). Thus, in these culture conditions, isotype switch is critically dependent on TLR stimulation.

The differentiation of B cells to plasma cells is an irreversible process mediated by X-box binding protein-1 (Xbp-1)\(^{158}\). The addition of CpG to naïve B cell cultures stimulated with anti-Ig and T cell help was essential for the sustained upregulation of Xbp-1 mRNA (Fig. 5C) and for the differentiation of naïve B cells to antibody-secreting cells (Fig. 5D). We conclude that TLR stimulation plays an essential role also in induction of isotypic switch and B cell terminal differentiation.

![Figure 5](image.png)

**Figure 5. TLR stimulation is required for induction of isotypic switch and differentiation to Ig-secreting cells.** A) and C) Naïve B cells were left untreated (open circles), stimulated with anti-Ig and T cell help (open triangles); anti-Ig and CpG (open square); anti-Ig, T cell help, and CpG (filled square). On the indicated days, living B cells were isolated by cell sorting and mRNA levels for AID (A) or Xbp-1 (C) were determined by quantitative real-time PCR and normalized to the amount of 18S rRNA. One experiment out of two. B) CFSE-labeled naïve B cells were stimulated as indicated and stained for surface IgG (upper panel) or IgA (lower panel) on day 6. Shown is one representative experiment out of ten. D) Cultures were set up as above and immunoglobulin secreting cells (ISC) were determined by ELISPOT on day 7. IgM, filled bars, IgG, hatched bars. IgA, open bars. Data represent mean +/- stdev of eleven experiments.
Results

Discussion

We have shown that TLR stimulation provides a third signal that synergizes with BCR triggering (signal one) and T cell help (signal two) resulting in the induction of sustained proliferation and differentiation of human naïve B cells. While signal one and two together are sufficient to drive initial proliferation of naïve B cells, they fail to sustain B cell expansion and the proliferating B cells die after a few divisions. TLR stimulation rescues proliferating B cells that progressively switch isotype and differentiate to plasma cells, two events that are known to be division dependent. TLR stimulation augments the frequency of naïve B cells that enter the first division (as shown by a reduced peak of undivided cells) and promotes at the same time accumulation of cells at late divisions. This finding is consistent with the report that CpG provides signals that promote cell cycle entry and cell survival.

The mechanism by which TLR signaling interplays with signals transduced by BCR or CD40 remains to be determined. We have shown however that there are different temporal requirements for delivery of the three signals. While signal one can be delivered by transient BCR triggering, signals two and three have to be sustained in time in order to induce maximal clonal expansion. This observation is consistent with the notion that BCR triggering leads to upregulation of costimulatory molecules and TLRs and suggests that a main function of BCR triggering is to enable B cell responsiveness to T cell- and innate immunity-derived signals.

All agonists that trigger TLRs upregulated on activated human naïve B cells were capable of providing signal three. The fact that these stimuli were effective only when given together with signal one and two underlines a remarkable difference between human and mouse B cells. Indeed, mouse naïve B cells constitutively express TLR4 and TLR9 and can be polyclonally stimulated by the cognate agonists, while human naïve B cells respond to TLR agonists only after initial BCR stimulation. Thus the coupling of BCR stimulation to TLR expression endows the human system with an additional degree of specificity, since it focuses innate signals only on antigen stimulated B cells.

Another difference between human and mouse B cells is the spectrum of TLRs expressed. For instance, while mouse B cells express TLR4 and respond to LPS, human B cells do not express TLR4 and responsiveness to LPS is restricted to
DCs\textsuperscript{58,59}. We exploited this fact to show that human DCs stimulated by LPS release in the culture supernatant cytokines, primarily IL-6 and IL-12, which sustain naïve B cell activation. A positive effect of DCs and IL-12 on human B cell activation have been previously reported\textsuperscript{161}.

It is intriguing that similar effects on B cell proliferation and survival could be induced by both TLRs and cytokine receptors, which engage different signaling pathways. Redundant mechanisms promoting survival of dividing cells might be the basis of this biological effect. It should be noticed however that the stimulatory capacity of TLR-activated DCs is quantitatively much lower and more variable as compared to direct stimulation of TLRs on B cells. It remains to be established whether the nature of signal three (type of TLR or cytokine) may impact on the quality of the B cell response generated, for instance in terms of isotypic switch or memory B cell differentiation\textsuperscript{162}.

In conclusion, we demonstrate that human naïve B cells (as naïve T cells) have very strict requirements for priming. The essential necessity for a TLR-derived third signal for naïve B cell activation extends to the human system the recent notion that mouse B-cell responses are controlled by TLR\textsuperscript{65}. Our results imply that TLR agonists can amplify and sustain specific B-cell responses induced by conventional T-dependent antigens and strongly support the concept for inclusion of selected TLR agonists in vaccine formulations\textsuperscript{163}. 
Materials and methods

Media and reagents. The medium used throughout was RPMI 1640 supplemented with 10% FCS (Hyclone), 1% Glutamax, 1% nonessential amino acids, 1% pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin, 5x10^{-5} M 2-mercaptoethanol (all Invitrogen). Recombinant human IL-6 and IL-12 were purchased from BD Biosciences.

FACS analysis. The following monoclonal antibodies were used: mouse anti-human CD3-PC5, CD4-PC5, CD4-APC, CD14-PC5, CD16-PC5, CD27-PE, CD86-PE, purified CXCR5, BrdU-PE, Annexin V-PE (all from BD Biosciences), CD45RO-FITC (Beckman Coulter) goat anti-human IgG-PE, IgG-Cy5, IgA-Cy5 (all from Jackson ImmunoResearch), mouse anti-human CD25-PE (Miltenyi), rat anti-human CCR7 (clone 3D12, kindly provided by M. Lipp, Berlin-Buch, Germany).

Cell isolation. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. CD4^+ T cells and CD19^+ B cells were isolated by use of CD4 and CD19-microbeads (Miltenyi), respectively, according to the manufacturer’s instruction. B cells were labelled with 25 nM MitoTracker Green FM (Molecular Probes), a substrate for ABCB1, for 25 minutes at 37°C and washed two times. Following staining for lineage (CD3, CD14, CD16), CD27, and surface IgG and IgA, naïve B cells were double sorted by FACSaria (Becton Dickinson) with a purity of >99.9%. Beads-purified T cells were stained for expression of CD4, CD45RO and in some experiments also for CD25, CCR7, and CXCR5 and sorted by FACSaria. Purity of T cell preparations was typically >98%. Monocytes were isolated from PBMCs by positive selection using CD14-microbeads (Miltenyi). The purified monocytes were cultured for 3-5 days in RPMI-10% FCS containing 50 ng/ml GM-CSF (Leukomax, Novartis) and 1000 U/ml IL-4. Monocyte-derived DCs were then washed twice, seeded at 1x10^6/ml and stimulated with 100 ng/ml LPS (E.coli 055:B5 LPS, Sigma) or 20 µM poly(I:C) (Amersham) for 24h.

Proliferation assay. Peripheral blood CD3^−CD14^−CD16^−CD19^+CD27^−IgG^−IgA^−ABCB1^+ naïve B cells were labeled with 0.5 µM 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) for 8 minutes at room temperature. After quenching of the labeling reaction by addition of RPMI-10% FCS, cells were washed extensively. Cells (1x10^6) were cultured in 96 well round-bottom
culture plates with different combinations of the following stimuli: 2.5 µg/ml F(ab’)2 anti-human IgM/IgG/IgA (Jackson ImmunoResearch), 1µg/ml CD40 ligand plus 1 µg/ml enhancer (Alexis), 2x10^4 irradiated (20 Gy) T cells in the presence of 0.5 ng/ml TSST (Toxin Technology), 2.5 µg/ml CpG oligodeoxynucleotide 2006 (5’-TCGTCGTTTTGTCGTTTTGTCGTT-3’, Microsynth), 2.5 µg/ml R848 (GLSynthesis), 10µg/ml MALP-2, 1 µg/ml Pam3CSK4, 1 µg/ml flagellin (all from InvivoGen), 100 ng/ml LPS, 20µM poly(I:C), 10 ng/ml IL-6/IL-10/IL-12, or 24 h DC supernatant at a final dilution of 1:10. Bacterial genomic DNA was isolated from E.coli DH5α (Invitrogen) using Trizol LS reagent (Invitrogen) according to the manufacturer’s instructions and used at 10µg/ml for B cell stimulation. Live E.coli DH5α were cocultivated with B cells at a ratio of 100:1 in medium without penicillin/streptomycin. Proliferation was measured on day 5 on a FACSCalibur (BD Biosciences) using PI (Sigma) and CD3-PC5 to exclude dead cells and remaining T cells. Acquisition was done for a fixed time to allow quantitative read-out.

Real time PCR. For quantitative assessment of relative mRNA levels, total RNA was prepared from FACS-sorted populations using Trizol LS reagent according to the manufacturer’s instructions. RNA was then reverse transcribed using M-MLV RT reverse transcription kit with random hexamer primers (Invitrogen). The relative mRNA levels were determined by real-time PCR on an ABI PRISM 7900HT sequence detector (Applied Biosystems) using the Assay-On-Demand product for AID (Hs00221068_m1) and Xbp-1 (Hs00231936_m1) together with universal PCR master mix (4352042) (all from Applied Biosystems). The obtained values were normalized to the amount of 18S rRNA (4310893E, Applied Biosystems) present in each sample.

ELISPOT. Ig-secreting cells (ISC) were detected by ELISPOT on day 7. Filter plates (Millipore MAIPS4510) were coated with 5 µg/ml purified goat anti-human IgM, IgG, or IgA (Southern Biotechnology) and subsequently blocked with PBS containing 1% BSA (Sigma). Serial dilutions of cells were added to the plates and incubated overnight at 37°C. Plates were then washed with PBS/0.05% Tween 20 (Fluka) and incubated with biotinylated isotype-specific secondary antibodies (Southern Biotechnology) followed by streptavidin-horseradish peroxidase (Sigma). The assay was developed with 3-amino-9-ethylcarbazole (AEC, Sigma) as chromogenic substrate.
Addendum: methods developed for the study

In vitro system for quantification of suppressive activity

Suppressive activity of regulatory T cells is generally determined by measuring the incorporation of radioactive $^3$H-thymidine during DNA replication in cultures containing conventional T cells together with different numbers of Treg in presence of irradiated APC. As Tregs are anergic and assumed not to proliferate under such conditions, measured total $^3$H-thymidine incorporation is interpreted as due to proliferation of only conventional T cells. $^3$H-thymidine incorporation determines suppression during the final hours of the experiment and therefore represents a snapshot of the end-point. It is not possible to track the overall outcome of suppression nor is it possible to obtain qualitative information about the proliferating T cells as the number of cell divisions they have undergone, the fraction of cells that proliferate, or the viability of cells in culture.

To allow a more detailed analysis of regulatory T cell activity, we aimed to set

Figure 1. CFSE-based tracking of responder cell division in cocultures of CFSE-labeled conventional and unlabeled regulatory T cells detects suppressor activity. A) 1.5 x 10^5 CFSE-labeled conventional CD4+ T cells were cocultivated with the indicated number of Treg in presence of 1000 immature DC and 0.25 ng/ml anti-CD3 in triplicate cultures. Responder cell proliferation was determined on day 4 by FACS analysis as in Fig.1. Depicted is one representative plot per condition showing responder cell proliferation in absence (open histograms) or presence (filled histograms) of Treg. B) Responder cells that had undergone one or more cell divisions were quantified in each condition. Depicted is the mean +/- sdev of triplicate cultures. C) The percentage of inhibition by decreasing numbers of Treg was calculated using as a reference the proliferation of responder cells in absence of Treg (defined as 0 % inhibition).
up a novel in vitro system for quantification of suppression by human Tregs by use of CFSE dilution analysis. We labeled conventional T cells with CFSE, cocultivated these “responder cells” with Tregs and immature DC in presence of soluble anti-CD3 (clone Okt3). Responder cell proliferation was analyzed by FACS on day 4 by excluding PI* dead cells and CFSE Tregs. Acquisition was done for a fixed time on a fixed volume to allow quantitative comparison of the different conditions. The number of responder cells that underwent one or more cell divisions was determined in each condition, and the extent of suppression was quantified by calculating the percent reduction in cultures containing Tregs as compared to control cultures without Tregs (figure 1). Tracking of responder cell proliferation by CFSE dilution analysis sensitively detected dose-dependency of suppression by Tregs, also at very low numbers of Tregs as at a ratio of 1:64.

To confirm the reliability of the established CFSE-based suppression assay, we determined the suppressor activity of Tregs in parallel using both $^3$H-thymidine incorporation and CFSE-dilution as read-out (figure 2). Results obtained from both assays were comparable although remarkably the CFSE-based assay was consistently more sensitive as shown by an increase of suppression at a 1:1 ratio (95% suppression by CFSE dilution analysis versus 83% suppression by $^3$H-thymidine incorporation) and a decreased number of Tregs required for 50% inhibition of responder cell proliferation (1:3 for $^3$H-thymidine incorporation and 1:7 for CFSE dilution analysis).

![Figure 2. Detection of suppressor activity by CFSE dilution analysis and by $^3$H-thymidine incorporation output similar results.](image-url)

For detection by CFSE dilution analysis (black square) $1.5\times10^4$ CFSE-labeled CD4+ responder T cells were cocultivated with titrated numbers of autologous Treg in presence of iDC and anti-CD3. Cultures were analyzed on day 4 by FACS analysis, and numbers of responder cells that had undergone one or more cell divisions were quantified for every condition. For detection by $^3$H-thymidine incorporation (black triangle) $1.5\times10^4$ unlabeled CD4+ responder T cells were cocultivated with titrated numbers of autologous Treg in presence of iDC and anti-CD3. After 84h of cultivation cultures were pulsed for 12 hours with 1 $\mu$Ci/well $^3$H-thymidine. Proliferation was measured using a liquid scintillation counter. Percent inhibition was determined by calculating the reduction of proliferation in cultures containing Treg as compared to cultures without Treg.

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This increase in sensitivity could be either due to determination of overall suppression instead of a final snapshot or due to tracking of only responder cell proliferation while excluding contaminating Treg proliferation.

As most research on Tregs is done in the mouse system, we aimed to determine whether a similar approach could be used also for detection of suppression activity by mouse Treg. CD4+CD25- and CD4+CD25+ T cells were sort purified from BALB/c splenocytes. CD4+CD25+ responder T cells were labeled with CFSE and cultured with titrated numbers of CD4+CD25+ Treg in presence of syngeneic iDC and soluble anti-CD3 (clone 145-2C11). Proliferation of responder T cells was determined on day 4 by FACS analysis by excluding PI+ dead cells and CFSE- Treg. As shown in figure 3 the reduction of responder cell proliferation by mouse Treg was 80-90% at 1:1 ratio with 50% reduction at ratio of 1:8, which is similar to values reported in literature.

We conclude that the CFSE-based in vitro suppression assay is well suited to characterize in a sensitive way quantitative and qualitative aspects of in vitro regulation of T cell responses in the mouse and the human system.
Determination of TCR specificity of anergic T cells

For the determination of TCR specificity of Treg we had to establish a method, by which we could overcome the anergic state of Treg and easily detect TCR signaling. Our strategy was to generate T cell hybridomas by fusion of purified populations of Treg or conventional T cells, which were preactivated for 3 days with anti-CD3 and anti-CD28 in presence of IL-2, with TCR-negative thymomas (BW5147 derivatives containing GFP under the control of NFAT as a reporter gene) using standard protocols for PEG-mediated fusion followed by selection in HAT medium. The resulting hybridomas bear TCR of initial T cells but have lost anergic behavior and can be assessed directly for TCR specificity by determination of GFP-expression upon TCR stimulation.

Eight GFP-transfected thymoma clones were tested for their ability to fuse with mouse CD4+ T cells. The obtained hybridomas were screened for expression of GFP upon TCR stimulation by plate-bound anti-CD3 (figure 4). The thymoma clone 7.21 was chosen as fusion partner for further experiments as it gave rise to hybridomas expressing high levels of GFP upon TCR stimulation.

**Figure 4. Evaluation of potential fusion partners.** CD4+ mouse T cells were activated in vitro and fused by PEG with the indicated GFP-transfected thymoma-derivates at a 1:1 ratio. Single clones were expanded in HAT-medium for 2 weeks. Hybridomas were then stimulated overnight with plate-bound anti-CD3 and analyzed for GFP-expression by FACS. Depicted are the overlays of all TCR-stimulated hybridomas derived from the indicated thymomas.
More than 98% of the obtained hybridomas responded to anti-CD3 stimulation by expression of GFP. The percentage of GFP$^+$ cells in anti-CD3 stimulated hybridomas, defined as maximal response, varied between single hybridomas between 0-100% (figure 5). Hybridomas responding with a maximal response of less than 20% were not included in the analysis. The maximal response was identical in populations derived from Treg and from conventional T cells, which implies that the anergic state of Treg does not impact on the responsiveness of hybridomas.

To test the responsiveness of such hybridomas to self-antigens we used T cells from the AbEpli- mouse (“single peptide mouse” $^{102}$), in which I-A$^b$ MHC II molecules are covalently linked to a single defined peptide and therefore thymic negative selection to self-antigens cannot occur. I-A$^b$ MHC II wild-type and single peptide mouse-derived iDC were CMTMR-labeled and pulsed overnight with a lysate of multiple mouse tissues to provide a source of tissue-specific as well as ubiquitously-expressed self-antigens. Hybridomas were then cocultivated with the self-antigen presenting DC and analyzed for the expression of GFP. To account for the observed large differences in maximal responses, results were expressed in percent of maximal responses. When stimulated with single peptide mouse-derived DC, on which the initial T cells were negatively selected in thymus, no hybridomas responded strongly (>25% maximal response) and less than 5% responded weakly (>1% maximal response). In contrast, when stimulated with syngeneic wild-type DC, more than 40% of single peptide-derived hybridomas responded strongly to self-antigenic stimuli, and more than 75% responded weakly (figure 6, see manuscript 1).
We deduce that the system we have established allows detecting of TCR stimulation by determination of GFP expression in activated hybridomas. Self-reactive T cells are easily detected with this system. As T cell hybridization overcomes the anergic state of regulatory T cells, we have developed a tool to directly compare the extent of TCR triggering in pools of peripheral Treg and normal T cells upon stimulation with self-antigen.
Gene expression analysis on populations of limiting cell numbers

Current methods for determination of gene expression on a transcriptional level base on the measurement of mRNA content in bulk populations by quantitative PCR. Heterogeneity of populations in terms of differential gene expression in individual cells cannot be assessed by use of these methods. In complex tissues as the sites of autoimmune reactions that contain heterogeneous populations of cells in different activation states, such methods are therefore only of limited use. To address the question of how to distinguish Treg from activated T cells in a pool of CD4^+CD25^+ T cells from inflamed tissue, we aimed to establish a sensitive method for characterizing gene expression in populations of limiting cell numbers. To establish this tool we used a modified protocol based on^{147}. 

In short, cells were sort-purified and deposited at limiting cell numbers into 96-well V-bottom culture plates containing combined lysis buffer and cDNA mix solution. Following reverse transcription, cDNA was ethanol precipitated in order to eliminate inhibiting components of lysis buffer/cDNA mix. Tailing of cDNA with homopolymeric dA by use of TdT allowed subsequent nonspecific amplification by oligo-dT primers. Detection of FoxP3 cDNA was performed either by conventional semiquantitative PCR or by quantitative real-time PCR; as internal control for quantification of total cDNA per sample we determined the amount of CD3 and beta-actin cDNA respectively.

To determine the minimal amount of cells necessary for obtaining reliable signals, CD4^+ T cells from healthy donors were sort-purified and deposited at one or five cells per well. Samples were processed as described, and the quantity of beta-actin mRNA in each sample was determined by quantitative real-time PCR. Figure 7 shows the amount of beta-actin transcripts in samples containing 1 or 5 cells each. The difference in beta-actin signal was roughly 7 fold (5.1x10^{-12} for 1 cell and 3.7x10^{-11} for 5 cells per sample).

Figure 7. Determination of minimal cell number required for reliable gene expression analysis. Five replicates of samples containing one or five cells each were analyzed for beta-actin expression by quantitative real-time PCR. Shown is the intensity of the beta-actin signal per sample. Red dotted line: mean of five replicates.
for 5 cells) thus reflecting the expected five-fold difference from the cellular input. However, while all samples scored positive for beta-actin signals when 5 cells were analyzed together, one out of 5 was negative when only one cell was detected. In addition, Ct values were all higher than 35 cycles for single cell analysis thus increasing the risk of low reproducibility. To avoid signals near detection limits, subsequent analysis was performed on populations of 5 cells each.

Next we applied 5-cell PCR to the analysis of CD4^+CD25^+CD27^− Treg from peripheral blood (PB) or synovial fluid (SF) of JIA patients. Determination of highly expressed genes as CD3 was performed by semiquantitative PCR and revealed 100% positive samples from both PB and SF (figure 8a). Similarly expression of beta-actin could be detected by real-time PCR in all samples (data not shown). These results indicate a solid functioning of the method.

To measure cDNA levels of genes expressed at a low level, Foxp3 cDNA was measured by semiquantitative PCR in the same samples. While Foxp3 cDNA could be detected in all samples extracted from SF, no expression was detected in PB-derived samples (figure 8b). To increase the sensitivity of the assay, we determined FoxP3 cDNA levels by real-time PCR and were able to detect low Foxp3-expression also in PB CD4^+CD25^+CD27^−; the difference in expression level between the corresponding subsets in PB and SF was around 2000 fold (figure 9). Measurement of cDNA levels by real-time PCR therefore increases the sensitivity of the assay over several log scales.

In conclusion, our results demonstrate that 5-cell-PCR is a sensitive tool for analysis of both high-level and low-level gene expression in populations of limiting cell numbers.

![Figure 8](image)

**Figure 8. Detection of high-level gene expression in samples of limiting cell numbers by semiquantitative PCR.** A, B) Gene expression of CD3 (A) and Foxp3 (B) was analyzed by semiquantitative PCR in eight replicates of five cells each isolated from PB (upper panel) and SF (lower panel). Gene-specific PCR products were visualized on a 3% agarose gel.

![Figure 9](image)

**Figure 9. Detection of low-level gene expression by quantitative real-time PCR.**

Eight replicates of PB and SF Treg containing five cells each were analyzed for Foxp3 expression by real-time PCR. Shown is the intensity of the Foxp3 signal in each sample. Red dotted line: mean of eight replicates.
Addendum

In vitro culture system for delivery of cognate T cell help to polyclonal B cell populations

In vitro cultivation of human naïve B has been observed to be difficult because of low responsiveness of naïve B cells to widely used polyclonal B cell stimuli as CD40L or CpG. An obvious explanation for this finding is that processes occurring during T-dependent B-cell activation are mimicked in a suboptimal way by the current in vitro cultivation systems. For antigen binding (signal one) good mimicry can be achieved by use of anti-Ig antibodies that crosslink surface Ig on B cells. In contrast, exact mimicry of T cell help (signal two) is difficult because of the polyclonality of human T and B cells, which precludes cognate interaction of antigen-presenting B cells with the corresponding specific T cells. Artificial systems as soluble CD40L or CD40L transfectants deliver only a limited set of signals, while additional signals delivered by activated T cells as cytokines or other interacting surface molecules cannot be induced. In addition the use of – in most cases nonhuman – CD40L transfectants bears the risk of inducing uncontrolled xenospecific B cell responses. Finally irradiated CD40L transfectants that die during cultivation increase the amount of genomic DNA released into culture. As mammalian genomic DNA has been shown to stimulate TLR9 61,63,164 such transfectants might additionally deliver signals activating innate immunity.

We thus aimed to establish a novel culture system that allows inducing cognate interactions between autologous polyclonal human T and B cell populations. We hypothesized that crosslinking of MHC II on B cells with TCRs by superantigens as TSST could induce B cell-T cell interactions similar to cognate interactions. To test this prediction we cultured human naïve B cells with anti-Ig and CpG in presence or absence of autologous T cells and TSST. The presence of either T cells or TSST alone did not influence proliferation of B cells in response to BCR triggering and TLR9 stimulation. In contrast, the addition of T cells in presence of TSST augmented the number of proliferating B cells in culture in a dose-dependent way (figure 10). We deduce that TSST induces potent interaction between polyclonal human B cells and T cells.
To compare the effect of direct cognate T cell help with currently used culture systems mimicking T cell help, we cultured human naïve B cell with anti-Ig and CpG in absence or presence of autologous T cells plus TSST or soluble CD40L. We found that cognate T cell help increased the number of proliferating B cells in culture and induces accumulation of B cells that have undergone more divisions as compared to stimulation with purified CD40L (figure 11). We conclude that direct cognate T-B cell interaction is superior to stimulation of B cells with commercial CD40L.

Figure 10. TSST induces potent interaction between polyclonal human B and T cell populations. A) 1.5x10⁴ CFSE-labeled human naïve B cells were cultured with 2.5µg/ml anti-Ig and 2.5µg/ml CpG in absence or presence of 0.5 µg/ml TSST and 0.5x10⁴ autologous T cells. Proliferation was determined on day 5 by FACS analysis using PI and anti-CD3 to exclude dead cells and T cells. Acquisition was done for a fixed time on a fixed volume to allow quantitative read-out. B) 1.5x10⁴ CFSE-labeled human naïve B cells were cultured with 2.5µg/ml anti-Ig, 2.5µg/ml CpG and 0.5x10⁴ autologous T cells in presence of the indicated concentrations of TSST. Proliferation was determined on day 5 by quantitative FACS analysis.

Figure 11. Stimulation with T cells is more potent than stimulation with CD40L. CFSE-labeled human naïve B cells were cultured with anti-Ig and CpG in absence (black histogram) or presence of either T cells plus TSST (empty histogram) or soluble CD40L (grey histogram). Proliferation was determined by quantitative FACS analysis on day 5.
Conclusions

Balancing forces of suppressing and enhancing nature determine the response of the immune system. In this thesis work two issues related with suppression of T cell responses by Tregs were addressed, namely the specificity of Tregs for self and their identification in inflamed tissues. In addition it was established that microbial products acting on TLRs provide essential enhancing signals required for induction of naïve B cell responses.

Lack of autoreactivity among regulatory T cells

The current theory of Treg activity predicts that Tregs are of increased self-specificity, become activated by immature self-antigen presenting DC and suppress activation of conventional T cells by these iDC. Their mode of action is therefore expected to be self-antigen-specific. Recent studies however show that transduction of normal, nonselself-specific T cells with the Foxp3 transgene induces Tregs with comparable suppressive activity as naturally arising Treg - a finding that stands in contradiction to the previously mentioned model and suggests that self-specificity is no requirement for Treg activity.

The extent of self-specificity of TCRs derived from Tregs or conventional T cells was determined using T cell hybridization as a way to overcome Treg anergy. Analysis of more than 350 TCRs from both T cell populations revealed no TCRs of high self-reactivity. In contrast large numbers of highly self-reactive TCRs were detected in hybridomas derived from single peptide mouse T cells that did not undergo negative selection for self-antigens. These results suggest that Tregs are not of increased self-specificity as compared to conventional T cells. Moreover, the very low frequency of weakly self-reactive Tregs renders it rather unlikely that self-reactivity is of essential importance for Treg function.

This finding implies that Tregs and conventional T cells express similar TCR repertoires and recognize similar antigens. As a consequence the hypothesis arises that T cell activation is automatically followed by induction of a Treg-mediated negative control and is only overcome in case of microbial danger. The control of Treg function may therefore be rather context-dependent than self-antigen-dependent.
Identification of regulatory T cells by coexpression of CD25 and CD27

The function of Tregs in the control of ongoing autoimmune reactions is only poorly characterized, mainly because of a lack of Treg-specific surface markers: in an inflamed tissue CD4⁺CD25⁺ Treg cannot be discriminated from activated conventional T cells, which express the same surface markers as Tregs, and thus Treg-specific analysis is precluded in such settings. The discovery of reliable markers capable of discriminating Tregs from activated T cells in such conditions is therefore essential for a better understanding of the role of Tregs in autoimmune diseases as well as for the development of therapeutic applications.

By dissecting the CD4⁺CD25⁺ compartment isolated from synovial fluid of JIA patients into further subpopulations we have found that in inflamed tissue co-expression of CD25 and CD27 defines Foxp3⁺ cells with suppressive activity, while CD25⁺CD27⁻ cells are devoid of Foxp3 and regulatory activity. In the resting state a large fraction of CD4⁺ T cells express CD27; CD27 expression per se is therefore not specific for Treg. Upon activation however, only regulatory but not conventional T cells maintain high expression of CD27. This differential regulation of CD27 in Tregs as compared to conventional T cells allows discriminating the two populations in complex inflamed tissues.

Using this novel Treg marker it was shown that Tregs isolated from the site of autoimmune reaction are fully functional and suppress T cell proliferation in vitro. A cell-intrinsic defect of Tregs as autoimmunity-promoting factor can therefore be excluded. In contrast, we have observed that inflammatory cytokines as IL-7 and IL-15, which are present in the milieu of autoimmune disease, potently block Treg-mediated suppression in vitro. The in vivo concentration of these cytokines correlated with the severity of the disease, which underlines the importance of Treg activity on the control of disease progression and suggests a mechanism, in which the inflammatory milieu itself potentiates the autoimmune disease by inhibiting Treg-mediated suppression.
Microbial products deliver an essential third signal required for induction of human naïve B cell responses

Enhancing mechanisms capable of overcoming suppressing activity are generally induced upon detection of microbial invasion and are therefore linked with recognition of a context of microbial danger. Context discrimination, mediated by TLRs, determines the stimulatory capacity of DC and is therefore essential for induction of productive T cell responses.

In this thesis work we show that TLR triggering is also critically required for the induction of B cell responses. TLR stimulation provides a third signal that synergizes with BCR triggering (signal one) and T cell help (signal two) resulting in the induction of sustained proliferation and differentiation of human naïve B cells. While signal one and two together are sufficient to drive initial proliferation of naïve B cells, they fail to sustain B cell expansion and the proliferating B cells die after a few divisions. TLR stimulation rescues proliferating B cells that progressively switch isotype and differentiate to plasma cells.

TLR stimulation supports B cell responses by multiple pathways: it augments the frequency of naïve B cells that enter the first division and promotes accumulation of cells at late divisions. TLR triggering was effective only when given together with signal one and two, which underlines a remarkable difference between human and mouse B cells. Indeed, mouse naïve B cells constitutively express TLR4 and TLR9 and can be polyclonally stimulated by the cognate agonists, while human naïve B cells respond to TLR agonists only after initial BCR stimulation. The coupling of BCR stimulation to TLR expression endows the human system with an additional degree of specificity by focusing innate signals only on antigen stimulated B cells.

In conclusion, we demonstrate that human naïve B cells (as naïve T cells) have very strict requirements for priming. Besides recognition of the specific antigen and cognate interaction with helper T cells, naïve B cell activation needs a third signal, which is induced upon recognition of a context that signals microbial invasion. This finding demonstrates that context discrimination via TLR triggering plays a general role in induction of immune responses and acts on both the humoral and cellular branch of the immune system.


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References


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Professional experience

07 – 10 2000 Semester project supervised by R. M. Ruprecht, Dana Farber Cancer Institute (DFCI), Boston, Massachusetts, USA. „Construction of codon-optimized HIV-1 env and SIV gag-pro genes for use as a DNA vaccine, and generation of SHIV clade C envelope challenge viruses“

01 – 07 2001 Diploma thesis supervised by A. Trkola, H. Hengartner and R. Zinkernagel, University Hospital, Zurich, Switzerland. „CC chemokine-induced HIV-1 inhibition: Delineating basic mechanisms and role in pathogenesis“

08 2002 - 03 2006 Ph.D. thesis supervised by A. Lanzavecchia and A. Rolink, Institute of Research in Biomedicine, Bellinzona, Switzerland. „On the role of regulatory T cells and microbial products in the control of T and B cell immune responses“
Languages

German: Native language
English: Oral and written fluent
French: Oral and written fluent
Italian: Oral and written fluent
Spanish: intermediate knowledge of oral and written

Prizes

2002 medal of ETH for an outstanding diploma thesis
2002 Willi Studer Prize for the best diploma in biology at ETH

Publications


