Do natural T regulatory cells become activated to antigen specific T regulatory cells in transplantation and in autoimmunity?

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Antigen specific T regulatory cells (T_{reg}) are often CD4^+CD25^+FoxP3^+ T cells, with a phenotype similar to natural T_{reg} (nT_{reg}). It is assumed that nT_{reg} cannot develop into an antigen specific T_{reg} as repeated culture with IL2 and a specific antigen does not increase the capacity or potency of nT_{reg} to promote immune tolerance or suppress in vitro. This has led to an assumption that antigen specific T_{reg} mainly develop from CD4^+CD25^-FoxP3^- T cells, by activation with antigen and TGF-β in the absence of inflammatory cytokines such as IL-6 and IL-1β. Our studies on antigen specific CD4^+CD25^+ T cells from animals with tolerance to an allograft, identified that the antigen specific and T_{reg} are dividing, and need continuous stimulation with specific antigen T cell derived cytokines. We identified that a variety of cytokines, especially IL-5 and IFN-γ but not IL-2 or IL-4 promoted survival of antigen specific CD4^+CD25^+FoxP3^+ T_{reg}. To examine if nT_{reg} could be activated to antigen specific T_{reg}, we activated nT_{reg} in culture with either IL-2 or IL-4. Within 3 days, antigen specific T_{reg} are activated and there is induction of new cytokine receptors on these cells. Specifically nT_{reg} activated by IL-2 and antigen express the interferon-γ receptor (IFNGR) and IL-12p70 (IL12Rβ2) receptor but not the IL-5 receptor (IL5Ra). These cells were responsive to IFN-γ or IL-12p70. nT_{reg} activated by IL-4 and alloantigen express IL5Ra but not IFNGR or IL-12p70. These early activated antigen specific T_{reg} were respectively named Ts1 and Ts2 cells, as they depend on Th1 or Th2 responses. Further culture of Ts1 cells with IL-12p70 induced Th1-like T_{reg}, expressing IFN-γ, and T-bet as well as FoxP3. Our studies suggest that activation of nT_{reg} with Th1 or Th2 responses induced separate lineages of antigen specific T_{reg}, that are dependent on late Th1 and Th2 cytokines, not the early cytokines IL-2 and IL-4.

Keywords: antigen specific T_{reg}, nT_{reg}, Th1-like T_{reg}, Th2-like T_{reg}, immune tolerance

HISTORICAL PERSPECTIVE

Immune tolerance results from a combination of deletion of antigen specific T and B cell clones, anergy, and suppression. Like all biological systems, immunity has in built self-regulation that prevents induction of destructive autoimmunity and controls or limits all immune effector responses against any antigen. While a variety of leukocytes can regulate, this review will focus only on CD4^+ T regulatory cells (T_{reg}).

Since the first description of suppressor T cells, the difference between non-antigen specific T_{reg} that reside in thymus, bone marrow, and peripheral lymphoid tissues, and antigen specific T_{reg} that are present mainly in spleen and tissues, has been appreciated (1–3). This division is consistent with natural T_{reg} (nT_{reg}) and antigen specific T_{reg}. Early studies characterized CD8^+ T suppressor cells, reviewed (4) but this work was discredited (5) and a common view was suppressor T cells did not exist, until the recognition of CD4^+ T_{reg}.

ANTIGEN SPECIFIC CD4^+CD25^+ T_{reg}

Alloantigen specific transplant tolerance was found in the mid 1980s to be mediated by CD4^+ T cells not CD8^+ T or B cells (6–8). In the early 1990s Waldman’s group found CD4^+ T cells from host transplant tolerant animals infect adoptive hosts’ T cells to maintain alloantigen specific tolerance (9).

At that time, we observed that the CD4^+ T cells that transferred antigen specific tolerance rapidly died in vitro (10–12). Death of antigen specific tolerance transferring CD4^+ T cells could be prevented by both stimulation with specific antigen and cytokines provided at that time by supernatant from Concanavalin A stimulated spleen cells. This supernatant was a crude source of IL-2 (12), but is now known to contain a number of cytokines, as well as IL-2. This suggested that the CD4^+ T cells that transfer transplant tolerance were activated cells that may depend on IL-2. We thus examined and found they expressed the IL-2 alpha receptor (CD25) (11). In 1990 we identified alloantigen specific tolerance transferring cells as CD25^+ Class II MHC^+CD45RC^+CD4^+ T cells (11). At that time CD25 was expressed by CD4^+ T cells activated to effect rejection (13), thus we assumed the suppressor cells were derived from specific alloantigen activated CD4^+ T cells. As IL-2 alone only partially sustained the capacity of tolerant CD4^+ T cells to transfer antigen specific tolerance, we concluded other cytokines were required (12). Since we have systematically
examine which cytokines are involved in the maintenance of antigen-specific CD4+CD25+FoxP3+Treg and this is the focus of this review.

**NATURAL Treg**
We also found that normal animals have cells, particularly in thymus and bone marrow, that suppress immune responses in a non-antigen specific manner, and that adult thymectomy depletes these cells, leading to heightened immune responses (14) and greater susceptibility to autoimmunity (15). Alloantigen specific CD4+ T suppressor cells have a different tissue distribution, being greatest in spleen, less in lymph nodes, and not in thymus or bone marrow (7). Further, they do not re-circulate rapidly from blood to lymph, suggesting they re-circulated through peripheral somatic tissue not through lymphoid tissues (7), similar to memory T cells (16), and not like naïve T cells that re-circulate from blood through lymphoid tissues (17). These basic differences in the migration of antigen specific and nTreg can be used to distinguish these cell populations by cell surface markers that direct their migration pathways, reviewed (18).

Later, activated CD4+ T cell in normal animals that expressed CD25 and prevented autoimmunity in neonatal thymectomized mice were described (19). These CD4+CD25+ Treg suppressed in a non-antigen specific manner, and are known as nTreg. nTreg are thymus derived and express FoxP3 (20) that prevents IL-2 induction and induces CD25 expression. FoxP3 expression in mice is a marker of Treg, but in man activated CD4+ and CD8+ T cells transiently express FoxP3 (21) and can be induced to have prolonged expression of FoxP3 (22). IL-2 is essential for survival of nTreg in peripheral lymphoid tissues (23, 24). CD4+ T cell with high expression of CD25, are regulatory, whereas CD4+CD25+ T cells are not regulatory (25).

Natural Treg have low expression of CD127, the IL-7 receptor, which is highly expressed by effector lineage CD4+CD25− T cells (26), albeit activated CD4+ T cells (27), and T follicular helper cells (Tfh) also have low expression of CD127 (28). The survival of nTreg without an immune response is dependent on low levels of IL-2, whereas CD4+CD25+ T cells depend upon IL-7 (29) not IL-2 for their survival without antigen activation. In the thymus IL-2 (30), not IL-7 (31) is critical for production of nTreg, although IL-7 plays a separate role in induction of nTreg in the thymus (32).

The CD4+CD25+FoxP3+ T cells are a heterogeneous group, and include naïve nTreg produced by the thymus, that have TCRs with increased affinity for self either due to thymic selection for self or expansion of self reactive clones in the periphery (33, 34). These naïve nTreg are polyclonal, with a wide repertoire of TCR. In normal immunological naïve hosts, some naïve nTreg with TCR specific for autoantigens, may have contacted antigen and been activated or expanded, to increase the repertoire of autoreactive nTreg. In addition, especially in hosts with acquired immune tolerance, there may be CD4+CD25+ Treg reactive to foreign or alloantigens, that have been expanded and function as antigen specific Treg. These are no longer naïve nTreg. Hosts with established antigen specific tolerance may have a large population of activated Treg with TCR specific for the tolerated antigen that mediate this tolerance, as well as the normal naïve nTreg with a TCR repertoire for self as well as a limited repertoire for other foreign antigens.

**INDUCTION OF Treg FROM CD4+CD25− T CELLS**
CD4+CD25− T cells can be activated by antigen in the absence of inflammatory cytokines, to antigen specific Treg. The first induced Treg (iTreg) described by Weiner are Th3 cells induced by TGF-β in oral tolerance, reviewed (35). Groux et al. described induction of antigen specific Treg by repeated culture of CD4+ T cells with antigen and IL-10, producing Treg cells that suppress via production of IL-10 and TGF-β (36). Tr1 and Th3 cell do not express CD25 or FoxP3 (35, 37).

Induced Treg are derived from peripheral CD4+ T cells that are stimulated by antigen and TGF-β in the absence of inflammation and inflammatory cytokines. These iTreg are induced to express FoxP3, albeit its expression is not stable as the Treg specific demethylation region (TSDR or CBS2) for FoxP3 is not demethylated (38). Both TGF-β which down regulates many genes, and FoxP3 expression which down regulates other genes, are required to induce iTreg from CD4+ T cells (39).

Most attempts to describe Treg oversimplify the complex nature of these cells in vivo, by describing all Treg as one type of cells, or dividing their description into nTreg and iTreg. nTreg remain non-antigen specific polyclonal Treg when cultured with IL-2 alone, whereas antigen specific nTreg are not expanded by IL-2. This and the small frequency of nTreg reactive to a specific antigen has led some to conclude that some, if not the majority, of antigen specific Treg reactive to foreign antigens may be derived from iTreg and not from activation of nTreg (40–43). The lack of a distinct surface marker to distinguish antigen specific Treg produced as iTreg from those derived from nTreg makes determination of the precise contribution of nTreg and iTreg to states of induced tolerance difficult (44, 45).

This review will focus on antigen specific Treg induced from nTreg, not on iTreg. Most of the material presented is derived from murine models. In each section, murine results will be presented first, then any human data will be discussed. At the end of each section, any information on similar cells derived from iTreg will be briefly mentioned.

Our work on Treg has shown that differential cytokine receptor expression is key to the identification of different T cell subtypes, including nTreg (46). This differential expression of cytokine receptors can be used to identify and distinguish a large number of functionally distinct Treg populations and is the major focus of this review.

**ARE THERE ANTIGEN SPECIFIC Treg?**
Acquired or induced immune tolerance is antigen specific, as shown in allograft (6–8, 11) and autoimmune tolerance (47, 48). In autoimmunity induced tolerance is epitope specific (47, 48). The CD4+ T cells that transfer transplant tolerance are alloantigen specific (6–8, 11). Antigen specific Treg not polyclonal nTreg are needed to prevent autoimmunity including myelin basic protein induced EAE (49), type 1 diabetes (50–52), gastritis (53), and peptide specific Treg control EAE induced by that peptide (54).

Animals with tolerance to an antigen or allograft do not have a major increase in CD4+CD25+ T cells, which remain at ratios of approximately 1:10 to CD4+CD25− T cells (55, 56). As these antigen specific Treg represent a fraction of the CD4+CD25+ T cells, they suppress the immune response at ratios well below 1:10,
A key unanswered question is the relationship of naïve non-antigen specific Th cells, including Th1, Th2, Th17, and Tfh cells. The generation of Treg from nTreg that suppress at ratios of <1:10 in an antigen specific manner would be highly desirable. We have described how such antigen specific Treg can be generated from naïve nTreg in vitro with 3–4 days of culture (46).

**IS THERE MORE THAN ONE ANTIGEN SPECIFIC SUBSET OF Treg?**

There is ample evidence that the pathways for activation of nTreg and Treg are multiple and complex, producing antigen specific Treg that control different subpopulations of effector CD4+ cells, including Th1, Th2, Th17, and Tfh cells. The generation of antigen specific Treg from either naïve nTreg or effector lineage CD4+CD25+ T cells, is complex involving activation of antigen specific T cells with antigen in an environment of cytokines that promotes maturation and clonal expansion of these antigen specific Treg. The cytokines that induce these lineages differ and relate to the environment present at the location of activation.

Our hypotheses are that: (i) every phase of the immune response is regulated to some degree, and that Treg are integral to control of all immune responses. (ii) All normal immune response, both in vivo and in vitro, are associated with activation of a CD4+ Treg response. (iii) Treg activation is driven by the cytokines present, including those produced by activated effector T cells. (iv) The more advanced or aggressive the immune response, the more potent the Treg that are generated by the cytokines produced, to control the response. We propose there are several levels of regulation by different functional subclasses of CD4+ Treg that are induced and activated by the ambient cytokines. Some of these separate Treg lineages and types are described in Table 1.

**WHY ARE ANTIGEN SPECIFIC Treg HARD TO IDENTIFY?**

A key unanswered question is the relationship of naïve non-antigen specific Treg generally described as nTreg, to antigen specific Treg. In particular whether antigen specific Treg are derived from nTreg or a product of activation of effector lineage CD4+CD25+ T cells, now known as iTreg (62). Whilst some conclude that antigen specific Treg are mainly iTreg, this review will examine the pathways by which nTreg can be activated to antigen specific Treg, raising the possibility that activation of nTreg may be the dominant source of antigen specific Treg.

Our thesis is based on our findings that antigen specific Treg die in vitro and in vivo, unless stimulated by specific antigen and cytokines produced by activated effector cells during immune response to the antigen (10–12). This makes identification of antigen specific Treg very difficult, unless they are re-exposed to specific antigen and the cytokines they depend upon. Further, antigen specific Treg do not require IL-2, and in fact may be killed by IL-2 (12). Thus most current protocols for the ex vivo expansion of nTreg will not promote antigen specific Treg.

**ANTIGEN SPECIFIC Treg EXPRESS CELL SURFACE MARKERS OF ACTIVATED T CELLS**

Activated Treg express different cells surface markers to nTreg. As examples nTreg express CD45RA and are CD44hi, whereas activated Treg express markers of memory cells, being CD45RO+ and CD44hi. CD45RC is a marker of an activated Treg (11). Class II MHC is only expressed by activated Treg and is a marker of these cells in man (63) and rats (11) but not in mice. nTreg express CD62L and re-circulate from blood to lymph, whereas activated Treg lose expression of CD62L and migrate through peripheral tissue not through lymphoid tissues in murine (64, 65) and humans (66). In naïve CD4+CD25+ Treg, CD62L+ not CD62L− Treg suppress GVHD (67, 68). Expression of CCR4 and CCR7, which facilitate migration to lymphoid tissues are expressed by nTreg but not antigen activated Treg (69). Activated Treg migrate to sites of inflammation and express E/P selection (70) and chemokine receptors (65, 71) that will direct them to the site of inflammation that they are programed to control (18). Thus, Treg effective against Th1 responses express CXCRC3 (72), those effective against Th2 express CCR8 (73), those for Th17 express CCR6 (74), and those for Tfh express CXCR5 (75).

**ACTIVATION OF Treg TO EXPRESS TRANScription FACTORS AND CYTOKINES OF Th LINEAGES, MAKING Th-LIKE Treg THAT SUPPRESS THE RELEVANT Th RESPONSE**

Cytokines normally associated with induction and function of Th1, Th2, Th17, and Th CD4+ T cells are now found to play a key role in the induction, maintenance, and function of activated Treg. Transcription factors that were considered the master regulators of Th responses, play an essential role in activated Treg function, including T-bet the Th1 transcription factor (76), GATA3 the Th2 transcription factor (77), and RORyt the Th17 transcription factor (78). There is plasticity in Th cell lineages, in that various lineages can at time express transcription factors and cytokines not classical for the lineage (79). Epigenetic modification of transcription factor genes and miRNA expression contribute to stability of a lineage, but this can be broken, discussed by O’Shea and Paul (79). CD4+CD25+FoxP3+ Treg can express Th effector lineage transcription factors, together with FoxP3, thereby retaining Treg capacity.

**ACTIVATION OF Treg IN ASSOCIATION WITH Th1 RESPONSES**

In our studies, culture of nTreg with a specific alloantigen and either IL-2 or IL-4 induce antigen specific Treg within 3–4 days of culture (46). They suppress the capacity of naïve CD4+ T cells to proliferate in vitro to specific donor at 1:32–64 and to effect rejection of specific donor grafts at 1:10 (46), whereas nTreg only fully suppress at 1:1, both in vivo and in vitro (46, 57, 59). In an autoimmune model, antigen specific Treg were also induced in vitro by culture with specific autoantigen and IL-2 that prevented disease in vivo (unpublished results). No other Th1 or Th2 cytokines promote proliferation of nTreg including IFN-γ, IL-12p70, IL-12p40, IL-5, IL-13, nor did TGF-β, and IL-10 (46).

With CD4+CD25+ T cells from animals with tolerance to a fully allogenic graft, we found that IL-2 or IL-4 induces proliferation to self, specific donor, and third party alloantigen. Proliferation of these Treg to specific donor, and not to self or third party, is
### Table 1 | Subclasses of CD4+ T cells with regulatory function.

| [A] PRESENT TO CONTROL AUTOIMMUNITY IN NORMAL HOSTS |
|-----------------------------------------------|
| *nT*<sub>reg</sub>  | Produced in thymus and released into periphery, prevent activation of destructive autoimmune responses. Absence of *nT*<sub>reg</sub> due to neonatal thymectomy (19), lack of IL2, CD28, or FoxP3 (223) leads to widespread autoimmunity. Expression of CTLA4 is required for function of *nT*<sub>reg</sub> (224). These cells will control low level immune responses, and suppress at a ratio of 1:1 with more aggressive immune responses (58) including fully allogeneic responses (57, 59). They inhibit antigen presenting cells by direct contact and act in peripheral lymphoid tissues not at sites of inflammation. |

**Induced *T*<sub>reg</sub>** generated when antigen is presented in a non-inflammatory environment, when TGFβ is present in the absence of activated antigen presenting cells and inflammatory cytokines such as IL-1β and IL-6. This produces additional *T*<sub>reg</sub>, that are antigen specific to prevent induction of autoimmune response, in situations where self antigen is released due to non-inflammatory tissue injury such as trauma, ischemia, or chemical injury of tissue as well as in normal tissue re-modeling and failed or incomplete apoptosis, reviewed (225). In these circumstances TGFβ produced to promote repair of tissue also induces *T*<sub>reg</sub> to prevent unwanted and unnecessary autoimmune responses. Their survival is ephemeral if there is repair of tissue, but they may be further activated if inflammation supervenes.

**Th3 and Tr1 cells** produced in mucosal sites, in response to antigens that penetrate the mucosa. There is abundant IL-10 and IL-10 family of cytokines, as well as TGFβ at these sites, that promotes tolerance induction to normal mucosal flora and oral antigens to prevent local and unwanted immune responses and inflammation that would disrupt the mucosal integrity. They are essential to the preservation of mucosal integrity and act by production of TGFβ and IL-10 that in turn promotes induction of more Th1 and Tr1.

| [B] PRESENT AFTER ACTIVATION OF AN IMMUNE RESPONSE TO A SPECIFIC ANTIGEN |
|-----------------------------------------------|
| **Antigen Activation of *nT*<sub>reg</sub>** by inflammatory immune responses with cytokines produced early after activation of effector CD4+ T cells. The best described is the effects of high concentrations of IL2, inducing expansion of *nT*<sub>reg</sub> in the presence of a specific antigen. IL-4 also can induce activation of antigen specific *T*<sub>reg</sub> from *nT*<sub>reg</sub>, Th1 and Th2 responses induce expansion of antigen specific *T*<sub>reg</sub>, respectively called Ts1 and Ts2 cells, that control responses other that of the inducing response. This contributes to polarization to one response, for example Th2 cytokine activated *nT*<sub>reg</sub> inhibit Th1 and Th17 responses. |

**Activation of antigen specific activated *nT*<sub>reg</sub>** by cytokines produced late in an ongoing immune response. This induces the *T*<sub>reg</sub> to express cytokines and transcription factors of the activated Th cells, so the *T*<sub>reg</sub> become Th-like and express the transcription factor and late cytokines of that Th lineage.

**Conversion of activated effector cells to regulatory cells**

(i) Activated *T*<sub>reg</sub> infecting activated T cells, via IL35/IL-10 (226) or surface TGFβ (227) to a regulatory T cell phenotype and function.

(ii) Persistent activation of effector lineage induces them to produce IL-10 and dampen their own response as was described some 20 years ago (228–230).

Promoted by IFN-γ, IL-12p70, and IL-5, but not TGF-β, IL-12p40, IL-10, or IL-13 (Hall et al., unpublished data). These cytokines became candidates for the promotion of survival of alloantigen specific CD4+ *T*<sub>reg</sub> in vitro, where we had not yet identified the specific cytokines involved (12). We had shown that antibody blocking IFN-γ (12) IL-5 and TGFβ (55) does not prevent transfer and maintenance of tolerance by CD4+ T cells from tolerant animals, however. Polyclonal activation of *nT*<sub>reg</sub> was induced by self antigen and IL-2 or IL-4, and with an antigen proliferation of *nT*<sub>reg</sub> induced by IL-2 or IL-4 was further increased (46).

This led us to examine if there are two pathways for activation of antigen specific *T*<sub>reg</sub>, one promoted by Th1 cytokines and the other by Th2 cytokines (46). We identified separate pathways for Th1 and Th2, and called the early Th1 activated *T*<sub>reg</sub>, Ts1 cells, and the early Th2 activated *T*<sub>reg</sub>, Ts2 cells. The characteristics of these cells are summarized in Table 2, which also shows that Ts1 and Ts2 cells are an intermediate step in the activation of antigen specific *T*<sub>reg</sub> and that they can be further activated by late Th1 and Th2 cytokines to more potent Th1-like *T*<sub>reg</sub> (*Figure 1*) or Th2-like *T*<sub>reg</sub> (*Figure 2*).

**IL-2 and ANTIGEN ACTIVATION OF *nT*<sub>reg</sub>**

In cultures of naïve CD4+CD25+FoxP3+ *T*<sub>reg</sub> with allo or autoantigen and IL-2, we found that within 2–4 days there was a change in phenotype of the cells, see Table 2. Their expression of mRNA for interferon-γ receptor (IFN-γR) increases (46) and the receptor for IL-12p70 (IL-12Rβ2) is induced, whereas the receptor for IL-5 (IL-5Rα) is not induced. There is also enhanced expression of mRNA for IL-5 and reduced expression of IFN-γ. Other cytokine expression remains unchanged, with no IL-2, and similar expression of IL-4, IL-10, and TGFβ to that of fresh naïve *nT*<sub>reg</sub>. Foxp3 expression is maintained in the majority of cells, and there is no induction of T-bet or GATA3. These changes are not observed when *nT*<sub>reg</sub> are cultured with IL-2 and self antigen, suggesting these changes occur related to activation of antigen specific *T*<sub>reg</sub>. We called these cells Ts1 (46).

Ts1 cells are more potent than *nT*<sub>reg</sub> in suppression in vitro, as they fully suppress naïve CD4+ T cells proliferation in MLC at 1:32–1:64 (46), whereas *nT*<sub>reg</sub> only fully suppress MLC at 1:1 or greater (59), Evidence that antigen specific *T*<sub>reg</sub> are activated is that Ts1 cells suppress specific donor allograft rejection mediated by naïve CD4+ T cells at a ratio of 1:10 (46), whereas naïve *nT*<sub>reg</sub> only suppress rejection at 1:1 (37), and Ts1 cells do not suppress third party rejection at 1:10 (46). The animals where Ts1 suppressed rejection, develop tolerance to the allograft and after 150 days have CD4+ CD25+ Foxp3+ T cells that expressed IFN-γR and IL-5, consistent with these Ts1 cells retaining their phenotype over a long period and being key to the maintenance of tolerance.

In other hosts with transplant tolerance, we identified CD4+ CD25+ Foxp3+ T cells that expressed IFN-γR and IL-5, that in vitro respond to specific donor and not third party when IFN-γ...
Table 2 | Summarizes the differences in Th1 and Th2 activated Ag specific T_{reg} and nT_{reg}.

| Gene expression | nT_{reg} | Subclasses of Ag specific CD4^{+}CD25^{+} T regulatory cells |
|-----------------|----------|---------------------------------------------------------------|
|                 | Th1 induced | Th2 induced |
|                 | Ts1 | Th1-like T_{reg} | Ts2 | Th2-like T_{reg} |
| IFNγR          | + | +++ | ++ | - | ? |
| IL12Rβ2       | - | + | ++ | ++ | - |
| IL5Ra          | - | - | - | +++ | ? |
| IL4Ra          | - | ++ | ? | + | ? |
| IL2            | - | - | - | - | - |
| IFNγ         | +/+ | + | +++ | +++ | ? |
| IL4           | ++ | ++ | ? | ++ | + |
| IL5           | - | + | ++ | ++ | - |
| IL10          | ++ | ++ | ? | + | + |
| TGFβ          | +++ | +++ | +++ | +++ | + |
| FoxP3         | +++ | +++ | +++ | +++ | + |
| Tbet          | - | - | + | ? | ? |
| GATA3         | - | - | - | - | ? |
| IRF4           | ? | ? | ? | ? | +++ |
| STAT1         | - | - | + | ? | ? |
| Chemokine ligand Receptors | | |
| CCR4           | ? | ? | ? | ? | ? |
| CCR7           | ? | ? | ? | ? | ? |

is present (Hall et al., unpublished data). Further the capacity of tolerant CD4^{+} T cells to transfer tolerance is maintained in vitro by culture with specific donor and IFN-γ not IL-2 (Nomura et al., unpublished data). We suggest that these T_{si} maintain alloantigen specific tolerance but are dependent on production of IFN-γ by Th1 cells.

In an autoimmune model we have also generated antigen specific T_{si} cells in vitro by culture of nT_{reg} with IL-2 and autoantigen. These T_{si} are induced to express IFNγR and IL-2, and suppressed the autoimmunity in an antigen specific manner (Tran et al., unpublished data).

We suggest induction of T_{si} cells is a key step in induction of antigen specific tolerance to Th1 responses. T_{si} would be promoted by the IFN-γ produced by an ongoing Th1 response, after they stop producing IL-2, which is an early Th1 cytokine. T_{si} cells may in part account for the paradoxical anti-inflammatory effects of IFN-γ, reviewed (80, 81).

IFN-γ AND ACTIVATION OF ANTIGEN SPECIFIC T_{reg}
IFN-γ is better known as a pro-inflammatory cytokine, but also has well described effects that control immune responses. IFN-γ directly inhibits Th2 and Th17 cell development, but promotes Th1 responses, including B cell isotype switching, macrophage activation, and cytotoxic T cell development. Activation of the Th1 lineage depends upon IFN-γ activating STAT1, which induces the Th1 transcription factor T-bet, which in turn regulates IFN-γ production by Th1 cells. Once CD4^{+} T cells are activated to a Th1 lineage, they cannot be converted to a T_{reg} lineage (82). IFN-γ is key to CD8^{+} T cell mediated rejection (83, 84) and to allograft vasculopathy (85–87). IFN-γ also activates macrophages to M1 cells and promotes Ig switching to a complement fixing isotypes. IFN-γ promotes MHC class I and II expression on inflamed tissues such as a during rejection (88). By induction of MHC class I, IFN-γ protects allografts from CD8^{+} T perforin/granzyme mediated rejection (84, 89–91).

IFN-γ can limit inflammation (92). IFNγR deficient mice have increased severity and reduced recovery from EAE (93, 94). IFN-γ induces iNOS to produce NO, which limits inflammation (95–98). IFN-γ treatment inhibits GVHD (99). CD8^{+} T cells deficient in IFN-γ mediate more severe GVHD, indicating IFN-γ produced by these cells inhibits the CD8^{+} T cell response by inhibiting proliferation and promoting cell death. CD8^{+}CD45R0 T cells induced to express IFN-γ, in turn induced indoleamine 2,3-dioxygenase (IDO), and accounts for promotion of indefinite allograft survival after blocking the CD40–CD40L interaction (100).

IFN-γ is also important in the generation and function of CD4^{+}CD25^{+} T_{reg} that mediate allograft tolerance (101) and prevents immune destruction of tumors (102). In vitro, IFN-γ promotes induction of alloantigen specific CD4^{+}CD25^{+}FoxP3^{+} T_{reg} that prevent rejection (103). This work by Wood’s group in Oxford identifies that naïve CD4^{+} T cell cultured over a period of time in MLC supplemented with IFN-γ, produces antigen specific T_{reg} that can prevent rejection (41, 103–107). Whether IFN-γ induces T_{reg} or expands nT_{reg} or a combination of both is unclear. One possibility is that nT_{reg} are initially activated by IL-2 produced by the activated CD4^{+}CD25^{+} T cells to induce antigen specific T_{si} cells, that in turn are activated by IFN-γ to expand and maintain the antigen specific T_{reg} (as shown in Figure 1), while a variety of factors such as IFN-γ induction of NO or IDO by antigen presenting cells or IFN-γ promotion of antigen specific T_{reg} may reduce the growth of the effector lineage. IFN-γ inhibits induction of T_{reg} from CD4^{+} T cells (82), whereas other report IFN-γ is key to induction of CD4^{+}CD25^{−} T cells to T_{reg} that suppress autoimmunity in IFN-γ deficient mice (108).

Th1-LIKE T_{reg}
Th1-like T_{reg} were first described in 2004 associated with a polarizing Th1 response to ovalbumin (109). Ovalbumin specific T_{reg} are induced from CD4^{+}CD25^{−} T cells by mature CD8α^{+} DC that produced both IL-12 and IL-10 that are required to induce Th1-like T_{reg} (109). These Th1-like T_{reg} express both FoxP3 and the Th1 transcription factor T-bet, as well as ICOS, IFN-γ, and IL-10. The Th1-like T_{reg} suppressed Th1 inflammation in vivo (109). In cancer, Th1-like T_{reg} expressing FoxP3, helios, T-bet, IFN-γ, CXCR3 suppress Th1 responses and are associated with infiltrating Th1 effector cells, probably impairing tumor immunity (110). T-bet expression is required for full T_{reg} function, as T-bet deficient nT_{reg} do not fully control autoimmunity in FoxP3 deficient scurfy mice (72).

T_{reg} induced by activation with a specific alloantigen become FoxP3^{+}IFN-γ^{+} and suppress in an antigen specific manner (111). Human iT_{reg} that express T-bet, IFN-γ, and CXCR3 are CD4^{+}CD25^{+}FoxP3^{+} T cells and suppress (112). Th1-like IFN-γ producing CD4^{+}CD25^{−}FoxP3^{+} T_{reg} are present in the blood.
of multiple sclerosis and renal transplant patients during active immune responses (113, 114). Th1-like Treg can be induced by IFN-γ, IL-12, or IL-27 and each may be a separate lineage, albeit they all express FoxP3, T-bet, STAT1, IFN-γ but not IL-2.

**IFN-γ PROMOTES Th1-LIKE Treg**

Thymus derived nTreg activated in a Th1 environment initially by IL-2, can be further activated by IFN-γ inducing STAT1 to promote expression of the Th1 transcription factor T-bet (115). Absence of STAT1 results in impaired CD4+CD25+ Treg development and increases host susceptibility to autoimmunity (115). These STAT1/T-bet/FoxP3+ Treg control Th1 responses and express CXCR3, which promotes their migration to sites of Th1 inflammation (72). IFN-γ induces T-bet+CXCR3+ Treg that inhibit Th1 responses in the periphery (116). Collectively these studies confirm IFN-γ can act on Treg to increase their effectiveness in controlling Th1 responses, albeit excessive activation by IFN-γ can reduce their suppressive capacity and may convert them to effector Th1 cells.

**IL-12 PROMOTES Th1-LIKE Treg**

IL-12p70 is a hetero-dimer composed of p35 and p40 that is produced by APC not T cells (117). IL-12 is a pro-inflammatory cytokine that enhances Th1 (76, 118), cytotoxic CD8+ T (119), and NK (120) cell responses by increasing IFN-γ (121). IL-12p70 acts by binding to a high affinity receptor, which is a hetero-dimer of IL-12Rβ1 and IL-12Rβ2 (122), which when activated by IL-12p70 induces STAT4 and T-bet to stabilize the Th1 phenotype and IFN-γ production (123, 124). Resting T cells do not express high affinity IL-12Rβ2 (117), but both chains are
up-regulated by TCR and CD28 stimulation, as well as by IL-2 and IFN-γ. IL-4 and IL-10 decrease expression of IL-12Rβ2.

Because IL-12p70 promotes induction of Th1 and cytotoxic T cell responses, it was predicted to amplify rejection and GVHD (125). Paradoxically, treatment with one dose of IL-12p70 at the time of bone marrow transfer inhibits fully allogeneic GVHD (126). Prevention of GVHD by IL-12p70 is dependent on donor IFN-γ (127) acting via Fas to inhibit donor T cell expansion (128). IL-12p70 treatment delays allograft rejection (98) and inhibits autoimmune including uveitis (129) and EAE (130). The protective effects of IL-12p70 are associated with induction of IFN-γ and iNOS (129). Blocking IFN-γ or iNOS with L-NIL prevents IL-12p70 prolonging graft rejection (98). In other models IL-12 promotes autoimmunity (131–133).

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12p35−/− (134), IL-12Rβ2−/− (135), IFN-γ−/− (136), and IFNRF−/− (94) mice are more prone to type 1 diabetes and have reduced numbers of CD4+CD25+FoxP3+ Treg that are less suppressive in vitro (137). Some Treg express the IL-12Rβ2 (137). In a situation of an uncontrolled Th1 response, IL-12p70 induces Treg to express T-bet and with high IL-2p70 levels these Treg produce IFN-γ (138). These changes only occur when there is limited IL-2 (138).

In our studies, nTreg cultured with IL-2 and alloantigen (Ts1) expressed IL-12Rβ2 and proliferated with IL-12p70. Ts1 cells activated by specific antigen and IL-12p70 in the absence of IL-2 had greater capacity to suppress alloimmune responses in vitro at 1:1000 and in vivo at 1:100 (Verma et al., unpublished data). Further, these Ts1 cells cultured with IL-12p70 in the absence of IL-2, expressed mRNA for T-bet and IFN-γ. They continued to express CD25, FoxP3, and mRNA for IFNFR and IL-12Rβ2. Ts1 cultured with IL-2 and IL-12p70 did not express mRNA for T-bet or IFN-γ. The concept of how Th1 cytokines induce Ts1 cells that are activated to a specific antigen to express IFNFR and IL-12Rβ2, and the effects of IFN-γ and IL-12p70 on their further expansion of Ts1 to Th1-like Treg is illustrated in Figure 1.

Many of the anti-inflammatory effects of IL-12p70 are attributed to increased production of IFN-γ that in turn induces iNOS to produce NO (98) but this was not required for Th1-like Treg development in vitro. Our results suggested that Ts1 cells, express IL-12Rβ2, and that IL-12p70 directly promotes Treg proliferation and maturation of Ts1 to more potent Th1-like Treg similar to that described by others (72, 138).

**IL-27 PROMOTES Th1-LIKE Treg**

IL-27 is a member of the IL-12 family of hetero-dimers, that was thought to promote Th1 responses (139). A subset of CD4+CD25+ Treg express IL-27Ra (140) a receptor required to control excess inflammation during infection (141). IL-27 inhibits Th1, Th2, and Th17 by direct inhibition of cells and induction of T effectors to produce IL-10 (142, 143). IL-27 promotes T-bet and CXCR3 expression in Treg at mucosa sites (116). IL-27 produces specialized Treg that control immunity at sites of inflammation and these Treg appear to express IL-27 as well as IL-27Ra (116). For IL-27 iTreg to function, they must express IFNFR1 and IL-10 (116). The IL-27 induced Th1-like Treg express different genes to Th1-like Treg induced by IFN-γ (116).

IL-27 via the STAT1 pathway, promotes FoxP3 expression by STAT1 binding to the FoxP3 promoter region in iTreg (144).

**ACTIVATION OF Treg IN ASSOCIATION WITH Th2 RESPONSES**

Dominance of Th2 responses (145–148) and Th2 cytokines IL-4 (148–150), IL-10 (151), and IL-13 (152), can protect against autoimmunity, but their effects are variable. Th2 cytokine expression is associated with prolongation of allograft survival in some models (153–158), including neonatal (159–161), and irradiation (162, 163) induced tolerance, but not in all models (164). Th2 cells transfer protection against chronic rejection (165) but do not directly mediate tolerance (166).

**IL-4 EFFECTS ON nTreg AND iTreg**

IL-4 is key to the induction of Th2 responses by binding to the IL-4Rα and common gamma chain and inducing STAT6 signaling (167) which is required for GATA3 and Th2 cell induction (168). IL-4 makes Th2 cells resistant to Treg (169).

IL-4 also induces STAT6 in Treg and stabilizes expression of FoxP3 (169). GATA3 is essential for full expression of FoxP3 by nTreg and binds to a conserved element of the FoxP3 locus to enhance transcription of FoxP3 (170). GATA3 expression is required to maintain FoxP3 expression in nTreg (77). GATA3 binds to the CNS2 site of the Foxp3 promoter site as well as the Th2 locus, whereas in Th2 cells it only binds to the Th2 locus (77). This induction of GATA3 in nTreg is not via the IL-4/STAT6 pathway (171), whereas induction of GATA3 via the IL-4/STAT6 pathway in nTreg and iTreg (172) suppresses FoxP3 expression by binding to the Foxp3 promoter region (172).

GATA3 is induced in nTreg during inflammation, and sustains FoxP3 expression (171) especially in Treg at sites of low grade inflammation such as mucosa and skin. Absence of GATA3 in Treg results in a spontaneous inflammatory disorder and defective nTreg that gain a Th17 phenotype (77). Th1 polarizing conditions down regulate GATA3 in Th2 and Treg cells (77). GATA3 induced in nTreg in early inflammation inhibits induction of polarizing factors and generation of effector T cells from nTreg (171). This early induction of GATA3, is dependent upon IL-2 as it is enhanced by IL-2/anti-IL-2 mAb complexes and is absent in IL-2 deficient mice (171).

TGF-β inhibits T-bet expression (173) and GATA3 expression (174) in CD4+ T cells reducing Th1 and Th2 cell expansion, thereby favoring Foxp3 expression and iTreg development. On the other hand GATA3 inhibits Foxp3 expression in iTreg activated from CD4+ T cells by TGF-β (77) and diverts the cells to an IL-9 producing effector CD4+ T cell (175, 176). Thus IL-4 may promote nTreg, but inhibit induction of iTreg by promoting GATA3 induction, that down regulates Foxp3 expression. GATA3 is not expressed by RORγt or T-bet expressing Treg, nor by Th17 and Th1 cells (171).

IL-4 in culture prevents apoptosis of mice nTreg (177), but IL-4 does not induce proliferation of nTreg only inducing proliferation of CD4+CD5RBII+CD25− T cells (177). IL-4 enhances the capacity of nTreg to suppress IFN-γ induction in CD4+CD25− T cells (177). Others found IL-4 induces nTreg proliferation (178) and expression of CD25, Foxp3, and IL-4Ra (169, 177). In cultures, IL-4 induces proliferation of both CD4+CD25+ and CD4+CD25−
T cells but promotes survival of CD4+CD25− T cells countering inhibition by nTreg (179).

IL-5 and antigen activation of nTreg

We found IL-5 and antigen in culture induced nTreg to antigen specific Treg (46, 56). This activation induces expression of the specific receptor for IL-5 (IL-5Rα) as well as for IL-4 (IL-4Rα) but not IFNGR or IL-12Rβ2, that we observe in cultures with IL-2 and an antigen (46). We call these antigen and IL-4 activated Treg, Ts2 cells (46). They continue to express FoxP3, but do not express GATA3, T-bet, or IL-2 (46). Ts2 cells features are summarized in Table 2. Ts2 cells have less expression of IL-5, enhanced expression of IFN-γ, and no change in expression of IL-4, IL-13, TGF-β, or IL-10 (46) (Table 2). These changes are not observed when nTreg were cultured with IL-4 and self antigen, suggesting they are due to activation of antigen specific Treg (see Figure 2).

Ts2 cells have increased potency of suppression in vitro as they fully suppressed naive CD4+ T cells proliferation in MLC at 1:32 (46), whereas nTreg only fully suppress MLC at 1:1 or greater (59). Evidence that Ts2 cells are antigen specific Treg is that Ts2 cells suppress specific donor allograft rejection mediated by naive CD4+ T cells at a ratio of 1:10 (46), whereas naive nTreg only suppress rejection at 1:1 (57). Ts2 cells do not suppress third party rejection at 1:10 demonstrating the Ts2 cells are antigen specific (46). The animals restored with Ts2 cells to suppress rejection develop tolerance to the allograft and after 150 days have CD4+CD25+FoxP3+ T cells that expressed IL-5Rα and IFN-γ. These tolerant Treg proliferate in culture to specific donor, but not to self or third party alloantigen, if IL-5 is present (46). This is consistent with these alloantigen specific Treg retaining their phenotype over a long period and IL-5 being key to the maintenance of tolerance mediated by antigen specific CD4+CD25+FoxP3+ Treg.

In other hosts with transplant tolerance, we have identified CD4+CD25+FoxP3+ Ts2 cells that expressed IL-5Rα and IFN-γ, that in vitro responded to specific donor and not third party when IL-5 was present (unpublished). Alloantigen with IL-5, but not IL-4, promoted in vitro survival of transplant tolerance transferring alloantigen specific CD4+ T cells (Plain et al., unpublished data). We suggest that these Ts2 cells maintain alloantigen specific tolerance, albeit animals with tolerance can have both antigen specific Ts1 and Ts2 cells.

In an autoimmune model, we have also generated antigen specific Ts2 cells in vitro by culture of nTreg with IL-4 and autoantigen. These Ts2 cells are induced to express IL-5Rα and IFN-γ, not IFNGR, and IL-12Rβ2 (56).

Human CD4+CD25+CD127loFoxP3+ Treg cultured with antigen and IL-4, but not IL-2, express IL-5Rα, suggesting IL-5 may promote these antigen specific Treg (56).

Th2-like Treg

Th2-like Treg express the transcription factor Interferon regulatory factor-4 (IRF4) to control Th2 responses (73). IRF4 also promotes Th2 and Th17 (181) responses. IRF4 binds to the promoter region of FoxP3 and induces Treg to express IL-4 and IL-5 (73). Thus induction of IRF4 results in a Th2-like Treg. Antigen specific Th2-like Treg are induced in Th2 responses by IL-10 and ICOS/ICOS ligand interaction and secrete IL-10 and some IL-4 but not IL-13 (182). ICOS expressed on Treg promotes their expansion in sites of inflammation during parasitic infestation, whereas in lymphoid tissues ICOS promotes Th2 responses not Treg expansion (183).

During parasitic infestations, CD4+CD25+ Treg develop in parallel with the Th2 polarization and regulate the size of the immune response (184). These Th2 iTreg inhibit Th1 responses, thereby facilitating Th2 polarization (185, 186). The early immune response to parasites is markedly controlled by Treg (187). Persistence of parasitic infestation is due to CD4+CD25+ Treg (188, 189) and these hosts have expanded CD4+CD25+FoxP3+ Treg populations (190).

Chronic infestation with parasites is associated with dominance of Treg, which suppress Th1 and Th2 responses against the parasite (191, 192). Animals who fail to eliminate parasites have protective CCR8+CD4+CD25+ Treg producing IL-10 that regulates Th2 response (193). Transfer of CD4+CD25− T cells confer some protection against infestation, while transfer of activated CD4+CD25+FoxP3+CD103+ Treg impairs parasite clearance with greater effect than nTreg (194).

Animals with parasitic infections and an active Th2 response are resistant to the induction of autoimmunity (195, 196) through the
effects of TGF-β (197) and have delayed allograft rejection (198–200). This suggests the Th2 milieu and possibly Th2 activated Treg protect these animal from Th1 and Th17 responses (201).

Multiple sclerosis patients with eosinophilia from parasitic infestation have markedly reduced episodes of relapses and new MRI lesions in brain associated with increased CD4+CD25+ Treg (202). Treatment of parasitic infestations leads to increased relapses and progression of multiple sclerosis with a reduction in Treg (203). Trials of therapeutic parasitic infestation are underway in inflammatory bowel disease (204) and MS (205). As parasitic infestation is associated with Th2 responses and production of IL-5, that induces eosinophilia, one possibility is that this IL-5 promotes antigen specific Ts2 cells to control autoimmunity.

A plausible hypothesis is that the evolution of the immune system was with persistent parasitic infestations and Th2 responses that inhibit innate and Th1/Th17 immunity (206). There is an increasing incidence of autoimmunity in the Western World where the parasitic infestation rate has markedly declined (206). Parasites induction of immune responses that promote Treg possibly by production of IL-5, may also explain the reduced incidence of autoimmunity in populations that live closer to the equator and have poorer hygiene (206).

Our hypothesis is that persistent Th2 responses releasing IL-5 may through a by-stander effect promote expansion of activated antigen specific IL-5Rx+ Treg generated to new non-parasite antigens. We demonstrated that IL-5 was an essential growth factor for nTreg activated by IL-4 and these Ts2 cell reduce autoimmune injury (56). We propose that one of the beneficial effects of parasites may be the high IL-5 level produced by a chronic Th2 response, promotes IL-5Rx expressing antigen specific Ts2 cells to control autoimmunity and allograft rejection.

**ACTIVATION OF Treg IN ASSOCIATION WITH Th17 RESPONSES**

Th17-LIKE Treg

T regulatory cells expressing both FoxP3 and IL-17 occur in mice and man (78, 207). IL-17 producing Treg are produced in the periphery not the thymus (78). STAT3, a transcription factor required for Th17 induction, is also required in Treg for induction and maintenance of FoxP3 expression induced by CD28 co-stimulation to produce iTreg (208). Specific deletion of STAT3 in Treg results in a fatal Th17 mediated colitis (209). It is proposed that STAT3 and FoxP3 together coordinate expression of a set of genes that specifically regulate Th17 effector T cells (209). STAT3 induces the receptors for IL-10, and for the pro-inflammatory cytokines IL-6 and IL-23 on Th17 cells and presumably on Treg associated with Th17 responses. IL-27 inhibits Treg via STAT3 (210). IL-10 at the site of inflammation can promote activated FoxP3+ Treg and FoxP3− Treg (211) and can directly inhibit Th17 and Th17/Treg cells at the site of inflammation in colitis (212). This suggests that IL-10R is expressed by Th17, Th1/Th17 cells, as well as Th17-like Treg that suppress Th17.

Human peripheral blood and lymphoid tissue contain CD4+FoxP3+ Treg that express CCR6 and when activated produce IL-17. They express both FoxP3 and RORγt (78). These CD4+CD25+FoxP3+ cells, that produce IL-17, strongly inhibit CD4+ T cell proliferation, and could be cloned (78). Naive CD4+FoxP3+CCR6− Treg that have their TCR stimulated in the presence of IL-1β, IL-2, IL-21, and IL-23 differentiate into IL-17 producing Treg (78). Human Treg that secrete IL-17A express the Th17 transcription factor RORγt (213). Both naïve and memory Treg suppress Th17 cells and inhibit their production of IL-17 and IL-22, as well as their expression of CXCL8 (214).

CD4+CD25+FoxP3+ Treg expressing IL-17, that acquire IL-1R1 can be converted to Th17 cells by IL-1β (215). This group suggested the preferred route of induction of Th17 in man may be via activation of nTreg with lineage differentiating factors, such as activated APC, IL-1β, TGF-β, and IL-23 as well as IL-2 (74). They propose a new role for nTreg as precursors of Th17 effector cells. IL-2 therapy triggers conversion of Th17 producing FoxP3+ Treg to Th17 cells that do not express FoxP3 (216). The Th17 effectors, that no longer suppress, do not express FoxP3 or IL-1R1, but express CCR6; similar to a smaller population of Treg that express FoxP3 and IL-17 (74).

IL-21 synergizes with IL-2 to promote activation of effector CD4+ and CD8+ T cells but inhibits induction of iTreg when combined with IL-2 and TGF-β (217). Thus, there is evidence for activated Treg and iTreg being induced to suppress Th17 responses that use induction pathways, in part, shared with Th17 cells.

**ACTIVATION OF Treg IN ASSOCIATION WITH Th1 RESPONSES**

Tfh-like Treg are specialized Treg that control germinal center expansion and autoimmune responses that are found in primary B cell follicles. These CD4+CD25+FoxP3+ T cells migrate to the B-T border areas of secondary lymphoid tissues, where they suppress Tfh dependent antibody responses by inhibiting both B cells and T cells (218, 219). These cells are CD4+CD25FoxP3+ T cells that share transcription factors and cell surface phenotype with Tfh cells, including expression of the Tfh chemokine receptor CXCR5 (75, 219) and PD1 which is expressed by Tfh (75). The development of Tfh-like Treg is similar to Tfh cell development as it depends upon expression of the transcription factor Bcl-6 (75). Bcl-6 is a transcription factor that promotes Tfh and represses other Th lineages. They also express Blimp-1, which is repressed in B cells and Tfh that express Bcl-6 (75). Bcl-6 is a transcriptional repressor that promotes Tfh but represses other Th lineages. Bcl-6−/− Treg are selectively impaired at controlling Th2 responses, but not Th1 and Th17 responses, as Bcl-6 suppresses GATA3 and Th2 (220).

Both Tfh and Tfh-like Treg depend upon SAP CD28, and B cells for their activation (75). Similar to Tfh cell induction, the Tfh-like Treg are induced by IL-21 and IL-6 and produce IL-21 with STAT3 expression. Tfh-like Treg are derived from nTreg and are not iTreg (75). Tfh-like Treg prevent over expansion of germinal centers and mediate tolerance in B cell responses.

**CONCLUSION**

This review sets out the evidence that nTreg are activated by cytokines released by the activation of CD4+CD25− T cells in all immune responses. It describes how the responsiveness of antigen activated nTreg changes during the immune response. Initially nTreg are activated by early cytokines such as IL-2 in Th1 and IL-4 in Th2 responses. With persistent active immune responses, the cytokines produced change. In late Th1 responses IFN-γ and
IL-12p70, not IL-2 is produced, and these late Th1 cytokines further expand and activate IL-2 and antigen activated Ts1 cells. In late Th2 responses IL-5 and IL-13 are produced not IL-4. In late Th2 response IL-5 promotes IL-4 and antigen activated Ts2 cells.

Excessive amounts of these cytokines can further induce antigen specific Treg to express the transcription factor of the dominant inflammatory response, so that in Th1 responses T-bet and STAT1 are induced to Th1-like Treg that produce IFN-γ. In Th2 responses Treg express IRF4 and produce IL-5 and IL-4 to become Th2-like Treg. In Th17 responses activated Treg express RORyt and IL-17A to become Th17-like Treg, whereas in Th2 responses, Treg express Bcl-6, and IL-21 to become Th17-like Treg. Each step of activation is associated with an increase in potency to suppress of the activated Treg, so that they can suppress at ratios of 1:10–1:10,000, whereas nTreg only fully suppress at 1:1. These subsets are identifiable by expression of chemokine ligands, CXCR3 in Th1 responses, CCR8 in Th2 responses, CCR6 in Th17 responses, and CXCR5 in Th responses. Highly potent antigen specific Treg, with the potential to migrate to sites of tissue inflammation to control active destructive immune responses, has far reaching potential in therapy for allograft rejection, control of GVHD, and autoimmunity.

These activated Treg include antigen specific Treg and require specific antigenic stimulation and the relevant cytokines to promote their survival. The requirement for specific antigen and a restricted cytokine milieu makes study of these cells in vitro very difficult, unless the correct environment is created to promote their survival. Further, the expansion of enriched nTreg by repeated culture with IL-2 over more than a week, only expands nTreg and probably selects against antigen specific Treg as the cytokines required to sustain antigen specific Treg are absent and IL-2 prevents induction of Th1-like Treg.

It is now appreciated that the number of nTreg to control GVHD, graft rejection, or autoimmunity is impossibly large, as they need to be present at ratios of 1:1 or greater (221). Understanding the pathways for selective activation of antigen specific Treg from nTreg will allow growth of more potent Treg that suppress in a specific manner with smaller numbers of cells. This may be achieved by first culturing nTreg with IL-2 or IL-4, then with other cytokines, respectively IFN-γ or IL-12 and IL-5. The effector mechanisms of each subset or activated Treg also needs resolutions, as there are many effector mechanism other than inhibition of APC with CTLA4 and production of IL-10 and TGF-β, as reviewed (222).

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