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Selection of $CD4^+CD25^+$ Regulatory T Cells by Self-Peptides

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Abstract Regulatory T cells have been shown to prevent the development of autoimmune disease, and can modulate immune responses during infections or following tissue transplantation. Recently, the processes by which $CD4^+CD25^+$ regulatory T cells are produced during immune repertoire formation have begun to be elucidated. This review focuses on the role of self-peptides in mediating $CD4^+CD25^+$ regulatory T cell selection in the thymus. How self-peptides continue to have an important influence on the accumulation of $CD4^+CD25^+$ regulatory T cells in the periphery is also discussed.

1 Introduction

A singular characteristic of the immune system is its ability to identify and eradicate a multitude of pathogens, while at the same time existing in and remaining tolerant of an environment that possesses a comparable diversity of self-antigens. The healthy organism will maintain this characteristic, or will otherwise become susceptible to infection or autoimmunity. For cells of the adaptive immune system, the capacity to distinguish between self and foreign antigens is acquired during B and T lymphocyte development and maintained in the periphery. Autoreactive T and B cells can undergo deletion if they encounter their antigen during development (Sprent and Kishimoto 2002; Starr et al. 2003). Yet there is evidence that potentially self-reactive clones of T and B cells are present in healthy, non-autoimmune individuals (Wekerle et al. 1996). Thus, the immune system has developed additional mechanisms to establish self-tolerance. One of these is the production of regulatory cells, which can suppress the activity of potentially autoreactive cells (Shevach 2000).

Although several types of regulatory cells are likely to exist, a well-characterized population comprises approximately 5%–10% of the peripheral $CD4^+$ T cell repertoire in mice and humans and is identified by the constitutive expression of the IL-2Rα chain (CD25) (Maloy and Powrie 2001; Shevach 2000). $CD4^+CD25^+$ regulatory T cells have been shown to prevent the development of several autoimmune diseases, and can also modulate immune responses to infections and in transplantation settings (Maloy and Powrie 2001; Sakaguchi 2004). $CD4^+CD25^+$ regulatory T cells are hypoproliferative in response to TCR stimulation in vitro; however, once stimulated via the TCR, they can suppress the function of responder cells (Piccirillo and Shevach 2001; Takahashi et al. 1998; Thornton and Shevach 1998). This review will describe studies aimed at determining how $CD4^+CD25^+$ regulatory T cells are generated during $CD4^+$ T cell repertoire formation in the thymus based on their interactions with self-peptides. How ongoing interactions with self-peptides in the periphery contribute to the development of a repertoire that effectively prevents autoimmune disease, while not compromising the ability to respond to infectious agents, will also be described.

2 $CD4^+CD25^+$ Regulatory T Cell Selection in the Thymus

T cell development begins in the thymus, where developing thymocytes rearrange their TCR genes. Positive selection rescues thymocytes from programmed cell death based on the ability of the TCR to react with host MHC

molecules, which are mostly occupied by self-peptides (Starr et al. 2003). This ensures that only thymocytes expressing TCRs that have the capacity to recognize the host's MHC molecules when they are displaying foreign peptides will be exported to the periphery. However, since an additional outcome of gene rearrangement is the production of autoreactive TCRs, thymocytes must also survive negative selection. Negative selection can eliminate or functionally inactivate those thymocytes with autoreactive TCR specificities, and, like positive selection, must also be guided by the reactivity of TCRs toward MHC molecules expressing self-peptides (Sprent and Kishimoto 2002). How do interactions between the TCR of developing thymocytes and the repertoire of self-peptides that are presented by MHC molecules influence positive and/or negative selection? Perhaps the most popular model is one in which the probability of selection is based on the strength of the signal received through the TCR; thymocytes survive to maturity if they receive a signal that is strong enough to indicate MHC restriction, yet weak enough to ensure non-self-specificity. This avidity model proposes that a window of signal strength from peptide:MHC-TCR interactions exists in which DP thymocytes must fall in order to be positively selected and avoid negative selection (Sprent and Kishimoto 2002; Starr et al. 2003). However, it is also possible that different thymic cell types may specialize in promoting these different outcomes; for example, cortical epithelium appears to be efficient at inducing positive selection (Laufer et al. 1999; Lo and Sprent 1986; Vukmanovic et al. 1992). More recently, the formation of $CD4^+CD25^+$ regulatory T cells has been found to represent another thymic selection event that is based on the reactivity that thymocytes exhibit toward self-peptides presented by MHC molecules (Jordan et al. 2001), although the cues that cause these processes to differ from positive or negative selection remain to be defined.

2.1 $CD4^+CD25^+$ Regulatory T Cells Develop Intrathymically

Early studies showing that $CD4^+CD25^+$ T cells possess important regulatory activities pointed to thymic processes in their formation. In these studies, thymectomy of 3-day-old neonatal mice (d3Tx) led to the development of organ-specific autoimmune diseases unless mice were given unfractionated $CD4^+$ T cells, or just the $CD4^+CD25^+$ subset of T cells, within 2 weeks of thymectomy (Shevach 2000). Sakaguchi's group went on to show that approximately 3%–5% of CD4SP thymocytes also express CD25, and that these CD4SP $CD25^+$ thymocytes are as suppressive as peripheral $CD4^+CD25^+$ regulatory T cells in in vitro suppression assays (Itoh et al. 1999). Furthermore, 1 week

after Thy1.2 CD4CD8 DP BALB/c thymocytes were injected intrathymically into Thy1.1 BALB/c recipients, a significant fraction of CD4SP $CD25^+$ cells were $Thy1.2^+$, providing evidence that $CD4^+CD25^+$ T cells could develop intrathymically from CD4CD8 DP thymocytes (Itoh et al. 1999). $CD4^+CD25^+$ cells are also present in the thymus when no $CD4^+$ T cells are detectable in the spleen, and BrdU-labeling studies showed that $CD4^+CD25^+$ thymocytes acquire label before $CD4^+$ T cells in the periphery (Asano et al. 1996; Jordan et al. 2001). Collectively, these studies indicated that $CD4^+CD25^+$ regulatory T cells could be formed intrathymically, diminishing the possibility that the detection of $CD4^+CD25^+$ thymocytes was due to the formation of these cells in the periphery and their subsequent recirculation to the thymus.

2.2 Self-Peptides Can Direct $CD4^+CD25^+$ Thymocyte Selection

Studies aimed at defining how TCR specificity guides T cell development in many cases rely on the use of TCR transgenic mice. TCR transgenic mice were used to develop the avidity model of thymocyte development, for example, and have the practical advantage of simplifying the enormously diverse repertoire of TCRs that would otherwise be expressed by thymocytes and mature T cells. Of course, this approach has limitations; it creates mice containing unusually high proportions of T cells with a particular specificity, and the TCR transgenes may be expressed at unusual stages of thymocyte development that may affect selection events. In addition, co-expression of endogenous non-transgene-encoded TCRs (particularly TCR α-chains, which are not subjected to efficient allelic exclusion even in non-transgenic T cells (Heath and Miller 1993; Heath et al. 1995; Zal et al. 1996)) can exert a significant impact on the specificity of the T cells under study.

Indeed, early clues that regulatory T cells were important in preventing autoimmune disease came in studies in which mice expressing an encephalatogenic $CD4^+$ TCR as a transgene were protected against the development of autoimmune encephalitis when maintained on a background that permitted endogenous TCR gene rearrangement (termed T/R^+ mice). However, T/R^- mice (generated by mating with RAG–/– mice to ensure exclusive expression of the transgenic TCR) developed severe encephalitis (Olivares-Villagomez et al. 1998; Van de Keere and Tonegawa 1998). Transfer of unfractionated $CD4^+$ T cells from non-transgenic mice into T/R^- mice was sufficient to prevent disease, and an interpretation of these data was that $CD4^+$ regulatory T cells were playing a role in disease prevention (Olivares-Villagomez et al. 1998; Van de Keere and Tonegawa 1998). Subsequent studies showed that in T/R^+ mice, $CD4^+CD25^+$ regulatory T cells only develop among T cells that co-express

endogenous TCR chains in addition to the transgenic TCR; $CD4^+CD25^+$ regulatory T cells do not develop in RAG-deficient T/R^- mice (which could not express endogenous TCR chains) (Hori et al. 2002).

In this model, restricting $CD4^+$ T cells to expression of the transgenic TCR prevented the formation of an effective regulatory T cell repertoire, and allowed $CD4^+$ T cells expressing the transgenic TCR to induce disease. Indeed, the development of $CD4^+CD25^+$ regulatory T cells depended on the co-expression of endogenous TCR chains, and the transgenic TCR appeared incapable of undergoing $CD4^+CD25^+$ T cell selection. These studies suggested that TCR specificity could play a role in directing $CD4^+CD25^+$ regulatory T cell formation, although the exact mechanism was not discernable.

We developed a transgenic mouse system in which specific interactions between a TCR and a single self-peptide could be shown to provide the basis for $CD4^+CD25^+$ regulatory T cell selection. TS1 mice express a transgenic TCR from a $CD4^+$ T cell clone that had been isolated from an influenza virus PR8-infected BALB/c mouse. The TS1 TCR recognizes the S1 determinant of the PR8 hemagglutinin (HA) presented in the context of the MHCII I-E^d, and can be detected with the anti-clonotypic monoclonal antibody 6.5 (Kirberg et al. 1994). The 6.5^+CD4^+ T cells that develop in TS1 mice are largely $CD25^-$ T cells; however, approximately 5%–10% are $CD25^+$ regulatory T cells (Jordan et al. 2000, 2001; Thornton and Shevach 2000). In contrast, $6.5^+CD4^+CD25^+$ T cells are undetectable in $TS1.RAG^{-/-}$ mice (which are incapable of endogenous TCR gene rearrangement) (Jordan et al. 2001). Thus, as was observed in T/R^+ mice, the development of $CD4^+CD25^+$ regulatory T cells in TS1 mice depends on the expression of endogenous TCR α-chains.

However, a crucial observation was made when TS1 mice were mated with HA28 mice, which express HA as a neo-self-antigen under the control of the SV40 early region promoter/enhancer. In TS1xHA28 mice, 6.5^+CD4^+ T cells develop in similar numbers to TS1 mice (that lack the HA transgene), but in TS1xHA28 mice approximately half of these 6.5^+CD4^+ T cells are $CD25^+$ regulatory T cells (Jordan et al. 2000; Jordan et al. 2001). These studies showed that interactions with a single self-peptide (S1) induced thymocytes expressing the 6.5 TCR to undergo selection to become $CD4^+CD25^+$ regulatory T cells. Moreover, when $TS1.RAG^{-/-}$ bone marrow, which could not rearrange endogenous TCR genes, was given to HA28 recipients, $6.5^+CD4^+CD25^+$ regulatory T cells developed as efficiently as in TS1xHA28 mice (Jordan et al. 2001). Thus, thymocytes that can only express the 6.5 TCR cannot undergo $CD4^+CD25^+$ T cell selection in response to the self-peptides that are presented by thymic MHC molecules in TS1 mice. However, they do so abundantly when a single additional peptide (S1) is presented in the diverse milieu of self-peptides in TS1xHA28 mice.

Similar processes guiding $CD4^+CD25^+$ regulatory T cell development have also been described using another transgenic system. In DO11.10 TCR transgenic mice, a small fraction (5%–10%) of the $CD4^+$ T cells expressing the clonotypic KJ-126 TCR are $CD25^+$ regulatory T cells when the mice are on a RAG-sufficient background, but KJ-$126^+CD4^+CD25^+$ T cells are undetectable in DO11.10 $RAG^{-/-}$ mice (Itoh et al. 1999). As was observed in TS1 (and T/R^+) mice, the KJ-126 TCR lacks a ligand in BALB/c mice that can induce selection of $CD4^+CD25^+$ regulatory T cells; the $CD4^+CD25^+$ regulatory T cell selection that occurs in a RAG-sufficient background is most likely mediated by endogenous TCR chains interacting with self-peptide:MHC complexes (Itoh et al. 1999; Suto et al. 2002). However, when DO11.1 TCR transgenic mice were mated with mice expressing ovalbumin either as a nuclear antigen or under the control of a rat insulin promoter, KJ-$126^+CD4^+CD25^+$ regulatory T cells were formed in increased numbers compared to DO11.10 mice (Kawahata et al. 2002; Walker et al. 2003). KJ-$126^+CD4^+CD25^+$ regulatory T cells were also formed in mice lacking RAG expression and co-expressing the OVA peptide, again showing that introduction of a single peptide into the milieu of thymic self-peptides can provide a ligand that promotes $CD4^+CD25^+$ regulatory T cell formation (in this case of the KJ-126 TCR) (Kawahata et al. 2002; Walker et al. 2003). It seems likely, based on these transgenic models, that $CD4^+CD25^+$ regulatory T cell formation in non-transgenic mice (and humans) similarly involves thymic selection events driven by TCR recognition of self-peptide:MHC complexes.

2.3 Thymocytes Can Undergo Both Deletion and $CD4^+CD25^+$ Regulatory T Cell Selection in Response to an Agonist Peptide

One of the most prominent features of the findings in TS1xHA28 mice was the high frequency of 6.5^+CD4^+ T cells (both $CD25^+$ and $CD25^-$) that were present. Indeed, 6.5^+CD4^+ T cells were as abundant in the LNs and spleens of TS1xHA28 mice as they were in TS1 mice (that lack S1 peptide); however, in TS1xHA28 mice, approximately half of the 6.5^+CD4^+ T cells were $CD25^+$ (Jordan et al. 2000). The minimal deletion of 6.5^+CD4^+ T cells in TS1xHA28 mice was also in sharp contrast to findings in other lineages of mice we had examined (termed TS1xHA12 and TS1xHA104), in which HA expression is also driven by the SV40 early region promoter/enhancer (Riley et al. 2000). Thymocytes expressing the 6.5 TCR are subject to much more extensive deletion in TS1xHA12 and TS1xHA104 mice than in TS1xHA28 mice (Jordan et al. 2001; Riley et al. 2000). The findings in these lineages showed that thymocytes bearing TCRs with identical specificities for a self-peptide could

undergo either overt deletion or abundant $CD4^+CD25^+$ regulatory T cell formation as processes of tolerance induction. Moreover, differences in the expression of S1 peptide between these lineages (induced by differences in their transgene integration sites) must play a decisive role in directing these different outcomes, since the same TCR could be subjected to these differing fates.

To extend these findings, we have generated additional lineages of HA transgenic mice, in part to better understand the relationship between $CD4^+CD25^+$ regulatory T selection and deletion of autoreactive thymocytes. We were also interested in determining how idiosyncratic TS1xHA28 mice might be, and whether the development of such large numbers of $6.5^+CD4^+CD25^+$ T cells might be dependent on some aspect of the presentation of the S1 peptide that could be unique to this lineage. We used the β-globin locus control region to target transgene expression to erythroid lineage cells in PevHA mice (Antoniou and Grosveld 1990; Yeoman and Mellor 1992), and the β-myosin heavy chain promoter to target HA expression to cardiac and skeletal muscle in β-myoHA mice (Rindt et al. 1993). We found that HA mRNA could be detected in the thymus of each lineage; similar findings have been made with other transgenic mice, using ostensibly tissue-specific promoters, that promiscuous expression by thymic epithelial cells may make a significant contribution to establishing $CD4^+$ T cell tolerance to tissue-specific self-antigens (Derbinski et al. 2001; Klein et al. 1998).

There was a striking similarity in 6.5^+CD4^+ T cell development between TS1xHA28 and TS1xPevHA mice. Similar total numbers of 6.5^+ CD4SP thymocytes and 6.5^+CD4^+ LN cells are generated in each lineage, and among these the percentages that were $CD25^+$ were also very similar (Lerman et al. 2004). Smaller percentages of CD4SP and $CD4^+$ cells were 6.5^+ in TS1xβ-myoHA mice than in TS1xHA28 and TS1xPevHA mice, indicating that more extensive deletion of 6.5^+ cells occurs. Nevertheless, large fractions of the 6.5^+CD4SP thymocytes and 6.5^+CD4^+ T cells in TS1xβ-myoHA mice were $CD25^+$ regulatory cells, as is the case in TS1xHA28 and TS1xPevHA mice (Lerman et al. 2004). Similar studies have been carried out in mice expressing HA under the control of an Igκ promoter; in this case too, substantially fewer $6.5^+CD4^+CD25^+$ T cells were present in the periphery than in TS1xHA28 mice, although they again existed as mixtures of $CD25^+$ and $CD25^-$ T cells (Apostolou et al. 2002). These studies show that the 6.5 TCR can be subjected to substantial deletion by the S1 peptide; however, even under these conditions of extreme deletion some $CD4^+CD25^+$ regulatory T cell formation can occur. However, in other cases, the peptide is presented in a way that imposes much less deletion; instead, it induces the selection of the 6.5 TCR into $CD4^+CD25^+$

regulatory T cells at frequencies near those directing positive selection of the 6.5 TCR into the $CD4^+$ T cell repertoire of BALB/c mice.

2.4 Role of TCR Specificity in Thymic $CD4^+CD25^+$ Regulatory T Cell Selection

These studies using TCR transgenic mice have provided evidence that the generation of $CD4^+CD25^+$ T cells can occur through a thymic selection process that has characteristics of positive selection (e.g., upregulation of CD69 and CD5 among 6.5^+ DP thymocytes [Azzam et al. 1998; Dutz et al. 1995; Jordan et al. 2001; Merkenschlager et al. 1997]). However, $CD4^+CD25^+$ regulatory T cell selection is different from conventional positive selection in that it is associated with increased CD25 expression and the acquisition of unique phenotypic and functional characteristics (i.e., regulatory activity). Thymic selection of $CD4^+CD25^+$ regulatory T cells also appears to differ from positive selection with respect to the specificity requirements for recognition of self-peptide(s). An elegant series of studies compared the peptides that could promote the positive selection of thymocytes expressing a MHC class I-restricted TCR in FTOC with the agonist peptide that was known to promote full activation of mature $CD8^+$ T cells expressing this TCR (Ashton-Rickardt et al. 1994; Hogquist et al. 1994; Sebzda et al. 1994). Peptides bearing minimal sequence identity with and reactivity relative to the agonist peptide could promote positive selection of the transgenic TCR (Hogquist et al. 1994, 1997). Positive selection appears then to be based on low-level reactivity with self-peptides that are presented by thymic MHC molecules. By contrast, the studies in both the HA and OVA systems showed that introducing a peptide that is a known agonist for the transgenic TCR could promote thymic $CD4^+CD25^+$ regulatory T cell selection, and as outlined above, the endogenous pool of self-peptides is incapable of promoting this selection.

To begin to examine the specificity with which thymocytes and $CD4^+$ T cells must react with self-peptides to undergo $CD4^+CD25^+$ regulatory T cell selection, we generated an additional TCR transgenic mouse, termed TS1(SW), using TCR genes from a $CD4^+$ T cell hybridoma that recognizes a homologue of the S1 peptide, termed S1(SW), which differs from the S1 determinant by two amino acid substitutions. The TS1(SW) TCR is roughly 100-fold less reactive toward the S1 peptide than is the 6.5 TCR (Jordan et al. 2001). $CD4^+CD25^+$ T cells expressing the TS1(SW) TCR were no more abundant in TS1(SW)xHA28 mice than in TS1(SW) mice, unlike the findings in TS1xHA28 mice where $6.5^+CD4^+CD25^+$ T cells were increased relative to $6.5^+CD4^+CD25^+$ T cells in TS1 mice. In addition, TS1(SW) mice were mated with the HA12 and HA104 lineages, which induce overt deletion of the 6.5 TCR, as well as with

an additional lineage (termed HACII mice), which expresses PR8 HA under control of a MHC class II-promoter and induces extreme deletion of 6.5^+ thymocytes. Although the TS1(SW) TCR could undergo deletion in response to the S1 peptide (particularly in TS1(SW)xHACII mice), in none of the mice did it undergo increased selection to become $CD25^+$ (Jordan et al. 2001). These findings provide evidence that $CD4^+CD25^+$ regulatory T cell selection may require a high intrinsic affinity of an autoreactive TCR for a selecting peptide, although it remains possible that some unknown properties of the TS1(SW) TCR contribute to an inability to undergo $CD4^+CD25^+$ selection. This issue can be examined more closely by generating additional transgenic mouse lineages that express the S1(SW) peptide and by determining whether S1(SW) expression induces the TS1(SW) TCR to undergo $CD4^+CD25^+$ regulatory T cell selection. The evidence to date suggests that the thymic selection of $CD4^+CD25^+$ regulatory T cells is exquisitely sensitive to, and dependent upon, interactions between thymocytes and individual self-peptides against which the TCR is highly reactive.

2.5 Role of Thymic Epithelium in $CD4^+CD25^+$ Regulatory T Cell Selection

The production of regulatory T cells by thymic epithelium was first suggested by studies in which allogeneic thymic epithelium from strain A mice was engrafted into athymic strain B mice. Low numbers of $CD4^+$ cells from these engrafted animals would induce autoimmune disease when transferred into additional athymic strain A mice, but this did not occur when larger numbers of cells were transferred (Modigliani et al. 1996). These results were interpreted to indicate that thymic epithelium normally generates mixed populations of autoreactive and regulatory T cells with overlapping specificities, and that insufficient regulatory T cells had been introduced to prevent autoimmunity when low doses of cells were transferred. In bone marrow chimera studies, $6.5^+CD4^+CD25^+$ regulatory T cells only developed when HA was expressed on radioresistant cell types, and in this setting $6.5^+CD4^+CD25^+$ T cell selection closely resembled that of intact TS1xHA28, TS1xPevHA, and TS1xβ-myoHA mice (Jordan et al. 2001; Lerman et al. 2004). Radioresistant thymic epithelial cells (TECs) were also shown to direct $6.5^+CD4^+CD25^+$ regulatory T cell selection in Igκ-HA mice (Apostolou et al. 2002). Moreover, in a different system, $CD4^+CD25^+$ regulatory T cells were able to develop in transgenic mice in which expression of MHCII is largely restricted to cortical TEC (cTEC) (Bensinger et al. 2001).

Work from several groups has demonstrated that TECs express mRNA transcripts for proteins that are otherwise generally restricted to differenti-

ated peripheral tissues (Anderson et al. 2002; Derbinski et al. 2001). More recently, the transcription factor AIRE was found to direct expression of mRNA transcripts of "peripheral" antigens in thymic epithelial cells (principally medullary TECs [mTECs]) (Anderson et al. 2002; Liston et al. 2003). However, it appears that disruption of the AIRE gene may affect deletion of autoreactive thymocytes to a greater degree than it affects $CD4^+CD25^+$ regulatory T cell formation (Anderson et al. 2002; Liston et al. 2003). Nevertheless, "promiscuous" expression of peripheral antigens, perhaps selectively by cTECs and perhaps under the control of some other as yet unidentified transcription factor, may play an important role in directing formation of $CD4^+CD25^+$ regulatory T cells for tissue-specific antigens. In this respect, however, it is worth noting that the studies in T/R^+ and T/R^- mice suggest that peptides from some tissue-specific antigens might not be expressed in the thymus in a way that can induce either $CD4^+CD25^+$ regulatory T cell formation or substantial deletion of an encephalotogenic TCR. Yet, encephalotogenic T cell activity can nonetheless be prevented by $CD4^+CD25^+$ regulatory T cells either with distinct TCRs specific for the same self-peptide, or that more likely underwent selection in response to different self-peptides.

3 Role of the Periphery in $CD4^+CD25^+$ Regulatory T Cell Repertoire Formation

Although thymic development plays a major role, peripheral processes also significantly influence the composition of the $CD4^+$ T cell repertoire. Homeostatic mechanisms that affect lifespan and proliferation control the size and composition of the $CD4^+$ T cell compartment and are mediated by cytokine and TCR signals (Jameson 2002). Cytokines (particularly IL-7) are important in promoting the proliferation and survival of naïve $CD4^+$ T cells (Fry and Mackall 2001). In addition, TCR-derived signals can induce homeostatic proliferation of naïve $CD4^+$ T cells and may be important for their survival (Jameson 2002). In contrast to cytokine-mediated signals, those that result from $CD4^+$ T cells interacting with MHC may promote expansion or survival based on specificity for self-peptides. When naïve TCR transgenic T cells are adoptively transferred into lymphopenic recipients that do not express their cognate antigen, they can undergo homeostatic division, showing that in a lymphopenic environment they can divide in response to weak interactions with self-peptides:MHC complexes (Ernst et al. 1999; Goldrath and Bevan 1999). Moreover, there is evidence that the peptides mediating positive selection in the thymus can also be responsible for directing the homeostatic

proliferation and survival of naïve T cells in the periphery (Ernst et al. 1999; Goldrath and Bevan 1999).

An initial indication that the presence of peripheral self-antigen may be required for the sustained persistence of $CD4^+$ regulatory T cells came from work done by Seddon and Mason (Seddon and Mason 1999). In this study, CD4SP thymocytes from athyroid rats did not induce thyroiditis upon transfer into thyroid-bearing recipients, whereas the peripheral $CD4^+$ T cells could induce thyroiditis while remaining protective against diabetes. The interpretation of these data was that thyroid tissue-specific regulatory cells could develop in the thymus of athyroid rats, but in order for those cells to persist in the periphery, the tissue expressing their self-antigen (the thyroid) had to be present. Similarly, studies using mice in which the ovaries had been removed showed that ovary-specific $CD4^+CD25^+$ regulatory T cells, which were present in normal mice, were not detectable (Garza et al. 2000; Sakaguchi et al. 1982; Tung et al. 2001). Thus, interactions with peripheral self-antigen appeared to play a critical role in the persistence of $CD4^+$ regulatory T cells, although how tissue-specific regulatory T cells were being maintained was not known.

3.1 Self-Peptides Drive $CD4^+CD25^+$ Regulatory T Cell Expansion in the Periphery

Even though a defining characteristic of $CD4^+CD25^+$ regulatory T cells is their hyporesponsiveness to TCR stimulation in vitro, adoptive transfer experiments have indicated that these cells can proliferate in vivo (Annacker et al. 2001; Fisson et al. 2003; Gavin et al. 2002; Klein et al. 2003; McHugh and Shevach 2002; Shevach 2000; Walker et al. 2003). Adoptive transfer of polyclonal $CD4^+CD25^+$ regulatory T cells into lymphopenic recipients induced several rounds of homeostatic division, and this division was comparable to the extent of $CD4^+CD25^-$ T cell division (McHugh and Shevach 2002). In this study, however, the specificity of the transferred $CD4^+CD25^+$ regulatory T cells was unknown. Furthermore, when interactions between transferred $CD4^+CD25^+$ regulatory T cells and MHCII were eliminated by transfer into MHCII-deficient hosts, homeostatic proliferation was greatly reduced (Gavin et al. 2002). Proliferation of monoclonal $CD4^+CD25^+$ regulatory T cells in response to cognate self-antigen has also been demonstrated in both the DO11.10/OVA and 6.5/HA transgenic systems. OVA-specific KJ-$126^+CD4^+CD25^+$ T cells proliferated when transferred into OVA-expressing recipients, although their proliferation was reduced compared to that of KJ-$126^+CD4^+CD25^-$ T cells (Walker et al. 2003). Similarly, HA-specific $6.5^+CD4^+CD25^+$ regulatory T cells proliferated in response to stimulation when transferred into mice that had been immunized with S1 peptide (Klein et al. 2003). These studies showed

that $CD4^+CD25^+$ regulatory T cells have the ability to proliferate in response to TCR stimulation in vivo, although how TCR- versus cytokine-derived signals might each contribute to their peripheral expansion under homeostatic conditions was not clear.

To examine this question we analyzed the ability of purified $CD4^+CD25^+$ regulatory T cells, and of $CD4^+CD25^-$ T cells, from TS1xHA28 mice to proliferate following transfer into HA28 or BALB/c mice that either had or had not been made lymphopenic by irradiation (Cozzo et al. 2003). We found that whereas $6.5^+CD4^+CD25^-$ T cells underwent division in response to lymphopenia in irradiated BALB/c mice, $6.5^+CD4^+CD25^+$ regulatory T cells did not divide under these conditions. Significantly, however, the $6.5^+CD4^+CD25^+$ regulatory T cells underwent division when transferred into HA28 mice, even in the absence of lymphopenia. The $6.5^+CD4^+CD25^-$ T cells also divided when transferred into HA28 mice, and the ability of $6.5^+CD4^+CD25^+$ versus $6.5^+CD4^+CD25^-$ T cells to divide in response to S1 peptide in vivo directly correlated with the differing abilities of these populations to proliferate in response to S1 peptide in vitro. The failure of $6.5^+CD4^+CD25^+$ regulatory T cells to proliferate in response to lymphopenia alone correlated with a reduced level of expression of the high affinity receptor for IL-7 (CD127) relative to conventional $CD4^+$ T cells (Cozzo et al. 2003; Gavin et al. 2002; Walker et al. 2003). Thus, the presence of self-antigen drives the expansion of $CD4^+CD25^+$ regulatory T cells, and signals derived from lymphopenia alone are insufficient to promote this proliferation.

It is interesting that $6.5^+CD4^+CD25^+$ regulatory T cells appear to be exquisitely dependent on the presence of S1 peptide for their proliferation in vivo, even under conditions of lymphopenia. As outlined above, thymic selection of $CD4^+CD25^+$ regulatory T cells appears only to occur in the presence of an agonist peptide for the TCR (that is presented in an amount or cell type that can induce selection), whereas positive selection of conventional $CD4^+$ T cells is promoted by interactions with weakly reactive peptide-MHC complexes. The $6.5^+CD4^+CD25^-$ T cells underwent homeostatic proliferation in mice that lack the S1 peptide (Cozzo et al. 2003), and studies in other systems have indicated that interactions with self-peptide MHC complexes are likely to contribute to this expansion (Ernst et al. 1999; Goldrath and Bevan 1999). In this respect, then, low specificity or degenerate recognition events contribute to both positive selection and homeostatic expansion of conventional $CD4^+$ T cells, whereas both selection and peripheral expansion of $CD4^+CD25^+$ regulatory T cells appear to require highly specific interactions with agonist self-peptides. It will be interesting in future experiments to determine whether lower affinity interactions (such as those that might occur between the TS1(SW) TCR and the S1 peptide) can allow for peripheral

expansion of $CD4^+CD25^+$ regulatory T cells, even if they cannot support their thymic selection.

At this stage, the evidence suggests that the expansion of $CD4^+CD25^+$ regulatory T cells in the periphery may be driven by highly specific interactions with peptides that also induced their selection in the thymus. Their differing responsiveness to TCR- vs cytokine-mediated signals provides a mechanism by which the activity and expansion of $CD4^+CD25^+$ T cells specific for tissue-restricted self-antigens may be directed in a manner that promotes tolerance while maintaining immunity. The stringent specificity with which $CD4^+CD25^+$ regulatory T cells must interact with self-peptides during both thymic selection and homeostatic expansion may also play an important role in causing $CD4^+CD25^+$ regulatory T cells to accumulate selectively at sites of antigen expression, even under conditions of lymphopenia.

3.2 $CD4^+CD25^+$ Regulatory T Cell Accumulation and Survival

Studies showing that $CD4^+CD25^+$ regulatory T cells require stimulation with specific peptide:HC complexes to expand in the periphery do not exclude a possible role for cytokine signals in determining their relative survival in the periphery. Little is yet known about the lifespan and turnover of $CD4^+CD25^+$ regulatory T cells compared to that of naïve $CD4^+$ T cells. Signals mediated from IL-2/IL-2R and through CD28/B7 signaling seem to be necessary for the maintenance of $CD4^+CD25^+$ regulatory T cells (Nelson 2004; Salomon et al. 2000; Tang et al. 2003). Still, interactions between the TCR and self-peptide:MHC may also be important for $CD4^+CD25^+$ regulatory T cell survival, as is the case for conventional $CD4^+$ T cells. Transfer into MHCII-deficient recipients inhibited the homeostatic proliferation of $CD4^+CD25^+$ regulatory T cells, but also diminished recovery of these cells (Gavin et al. 2002). An interpretation of these results is that TCR-self-peptide:MHC signals promote $CD4^+CD25^+$ regulatory T cell longevity, although studies addressing the MHC requirement for T cell survival are often complicated by the difficulty in dissecting the contribution of (or lack of) lymphopenia-induced proliferation to the final recovery of cells (Dorfman and Germain 2002). So, given that $CD4^+CD25^+$ regulatory T cells expand in response to and are likely activated by self-antigens, it is likely that contact with cognate self-peptides is important for $CD4^+CD25^+$ regulatory T cell maintenance and survival. But whether this again depends on interactions with a specific peptide–MHC complex, or can be achieved by more degenerate cross-reactive recognition, is not known.

With respect to factors governing their survival in the periphery, it is worth noting that $CD4^+CD25^+$ regulatory T cells express an antigen-experienced

phenotype that may affect their expression of survival factors and their lifespan relative to conventional naïve $CD4^+$ T cells (Read et al. 2000; Schluns et al. 2000; Shimizu et al. 2002; Takahashi et al. 2000; Xue et al. 2002). $CD4^+CD25^+$ regulatory T cells may, for example, have an enhanced ability to respond to small amounts of a trophic cytokine, IL-2, because their constitutive expression of CD25 permits them to respond to low levels that naïve $CD4^+CD25^-$ T cells would not detect. Although both $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ regulatory T cells proliferate in response to interactions with peripheral peptide, whether these two cell types differ in sensitivity to antigen-induced cell death (AICD) as a consequence of peptide-induced proliferation is unknown. Recovery of transferred polyclonal $CD4^+CD25^-$ vs $CD4^+CD25^+$ ($CD62L^{hi}$) regulatory T cells in Thy1.1 congenic recipients demonstrated that although both populations initially increased in number, $CD4^+CD25^-$ T cell numbers quickly decreased, whereas numbers of $CD4^+CD25^+$($CD62L^{hi}$) T cells remained steady for a longer time before decreasing (Fisson et al. 2003). Although the antigen specificity of the $CD4^+CD25^+$ regulatory T cells in these studies was not known, these data suggest that $CD4^+CD25^+$ regulatory T cells have a lower sensitivity to AICD in response to self-antigen than $CD4^+CD25^-$ T cells. In this regard, it is not yet known how the "activated" phenotype expressed by $CD4^+CD25^+$ regulatory T cells affects their patterns of recirculation in the lymphoid tissue, and whether the phenotype of these cells is dependent upon on-going interactions with the self-peptide in vivo. Insight into these processes will be important for a full understanding of the role peptide specificity plays in guiding the regional accumulation and activity of $CD4^+CD25^+$ regulatory T cells.

3.3 Peripheral Generation of $CD4^+CD25^+$ Regulatory T Cells

While there is clear evidence that $CD4^+CD25^+$ regulatory T cells are generated in the thymus and appear to be maintained as a distinct lineage, it remains possible that $CD4^+CD25^+$ regulatory T cells could be generated in the periphery from mature $CD4^+CD25^-$ T cells. In both the OVA and HA systems, the development of clonotype$^+$$CD4^+CD25^+$ regulatory T cells in the presence of an agonist peptide is accompanied by the development of an equivalent number of clonotype$^+$$CD4^+$ T cells that are $CD25^-$ (Apostolou et al. 2002; Jordan et al. 2000; Jordan et al. 2001; Lerman 2004; Walker et al. 2003). These clonotype$^+$$CD4^+CD25^-$ T cells are potentially autoreactive cells that likely also encounter cognate self-antigen. Therefore, one way to keep these cells from becoming pathogenic in response to antigen re-encounter may be to induce $CD4^+CD25^+$ regulatory T cell phenotype and function. Yet whether and how

conversion from $CD4^+CD25^-$ T cells to a $CD25^+$ regulatory T cell phenotype might take place is not yet understood.

To date, conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ regulatory T cells has been demonstrated in several systems, both in vivo and in vitro. In one in vitro study, $CD4^+CD25^+$ regulatory T cells were induced from $CD4^+CD25^-$ T cells by combining TCR stimulation with TGF-β treatment (Chen et al. 2003). These TGF-β-induced $CD4^+CD25^+$ regulatory T cells could suppress the development of an induced allergic response. $CD4^+CD25^+$ regulatory T cells could also be induced by alloantigen treatment from a population of polyclonal $CD4^+CD25^-$ T cells in a thymus-independent process (Karim et al. 2004). Antigen-specific $CD4^+CD25^+$ regulatory T cells have been generated from transgenic $CD4^+CD25^-$ T cells via immunization with low doses of antigen, or by orally administered antigen (Thorstenson and Khoruts 2001). In these studies, though, the contribution of recirculation through the thymus to the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ regulatory T cells, and the possibility that peptide immunization was expanding rare populations of $CD4^+CD25^+$ regulatory T cells that had been generated intrathymically (perhaps via co-expression of endogenous TCR chains) could not be assessed. Recently, clear evidence emerged that $6.5^+CD4^+CD25^-$ T cells could undergo conversion to become $CD25^+$ regulatory T cells in the periphery of BALB/c mice into which had been implanted osmotic pumps delivering low doses of S1 peptide (Apostolou and Boehmer 2004). The ability to generate $CD4^+CD25^+$ regulatory T cells with defined specificity in the periphery may have potential therapeutic benefits.

4 Conclusions and Future Directions

This review has described studies aimed at determining how specificity for self-peptides can guide the thymic selection and peripheral expansion of $CD4^+CD25^+$ regulatory T cells. We have presented evidence that both processes are exquisitely sensitive to and dependent on the ability of a TCR undergoing selection to recognize its selecting self-peptide as an agonist ligand. Many questions are raised by these findings and remain to be addressed. What factors determine whether an autoreactive thymocyte undergoes deletion vs $CD4^+CD25^+$ regulatory T cell formation in response to an agonist self-peptide? It is difficult to fit the data outlined here into a simple model in which the avidity with which a TCR reacts with self-peptide:MHC complexes plays a decisive role in directing these outcomes, because thymocytes expressing the 6.5 TCR undergo overt deletion or abundant $CD4^+CD25^+$ regulatory

T cell formation in response to variations in how the S1 peptide is expressed in different lineages. Perhaps expression in different thymic stromal cells (e.g., cTECs vs mTECs) is important, but it may also be that a combination of expression of a self-peptide under conditions of low overall avidity but high specificity, possibly by a particular cell type, provides a signal that promotes $CD4^+CD25^+$ regulatory T cell formation. For example, how might varying signals from peptide:MHC complexes affect the induction of the transcription factor FoxP3 in $CD4^+CD25^+$ regulatory T cell development? As described elsewhere in this volume, expression of FoxP3 is tightly linked with $CD4^+CD25^+$ regulatory T cell formation and activity (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003), and whether particular cues are provided by expression of self-peptides in certain amounts and/or cell types that induce its expression remains to be determined.

Finally, why do $CD4^+CD25^+$ regulatory T cells co-exist with $CD4^+CD25^-$ T cells expressing the same TCR in the transgenic systems that have been studied to date? Even in the context of varying degrees of deletion, $CD4^+CD25^+$ regulatory T cells expressing the transgenic TCR are typically present as mixtures with $CD4^+CD25^-$ T cells. Perhaps stochastic processes governing FoxP3 expression cause a subset of autoreactive thymocytes to develop along the $CD4^+CD25^+$ regulatory T cell pathway, while others do not. But in this model, the selection of $CD4^+CD25^+$ regulatory T cells appears to still depend on the ability of the thymocyte TCR to receive a signal from an agonist peptide ligand, and the processes that protect $CD4^+CD25^-$ thymocytes expressing the same TCR from deletion in these settings are not obvious. An intriguing possibility is that the thymus typically exports mixtures of clonally related $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells; it is clear from the autoimmune diseases that can develop under conditions when $CD4^+CD25^+$ regulatory T cell are selectively eliminated that $CD4^+CD25^-$ T cells with the potential to exert pathologic autoreactivity exist in the normal immune repertoire, and that they can appear to react with the same target organs. The studies to date in TCR transgenic mice raise the notion that these autoreactive and regulatory T cells could possess identical specificities, even if this is difficult to understand on theoretical grounds. A future challenge will be to test these hypotheses in additional transgenic and non-transgenic systems, and doing so may aid in the application of regulatory T cells in therapeutic settings.

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