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Human CD4+CD25+ regulatory T cells

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Abstract

In this report, we review studies of human CD4+CD25+ regulatory T cells (T-reg). Although lagging a few years behind the discovery of these cells in the mouse, the equivalent population of CD4+CD25+ regulatory T cells has also been isolated from human peripheral blood, thymus, lymph nodes and cord blood. In general, the characteristics of this T cell subset are strikingly similar between mouse and man. In the recent explosion of research reports on human CD4+CD25+ cells, although the majority of the characteristics ascribed to these cells appear to be consistent, contrasting results have been found primarily in regards to potential involvement of TGFβ and production of IL-10. One explanation for this variability may reside in the fact that markedly different techniques are used to isolate human CD4+CD25+ T-reg cells and thus may result in the comparison of T-reg populations that differ in cellular composition and/or activation state. Another potential explanation for differences in human T-reg function may rest on the extreme variability of the culture conditions and TCR stimuli that have been used to test the functional properties of these cells in vitro. The strength of the TCR signal provided to the culture greatly affects the functional outcome of the co-culture and can result in the difference between suppression and full activation. Surprisingly, it appears that stronger stimulation has a greater and more rapid effect on the T-resp cell than on the T-reg cell as it causes T-resp cells to quickly become resistant to suppression. Thus, the details of in vitro culture conditions may at least partially account for disparate findings in regard to the functional characterization of human CD4+CD25+ cells. Here we review the evidence regarding the identification of human CD4+CD25+ regulatory T cells and their possible mechanism(s) of function.

Keywords: Regulatory; Suppression; CD25

1. Physical characteristics of human CD4+CD25+ T-reg cells

A number of investigators have recently demonstrated the presence in humans of CD4+CD25+ T cells that exhibited similar features to the homologous mouse CD4+CD25+ regulatory T cell subset. These regulatory CD4+ T cells were identified by their expression of the IL-2Rα chain (CD25) [1–6]. The *in vitro* characteristics of the human CD4+CD25+ T cells were similar to the murine population as they were anergic to stimulation by T cell receptor cross-linking (TCR) in the absence of exogenous IL-2 and in their ability to suppress the activation of other T cells in a cell-contact dependent manner that could not be inhibited by blocking IL-10. The T-regs in both humans and mice could suppress both proliferation and cytokine production by CD4+CD25− responder (T-resp) T cells in response to a number of different polyclonal stimuli as measured by *in vitro* assays. Moreover, the T-reg cells down modulated the responses of CD8+ T cells and NK cells and the antigen-specific responses of CD4 T cells to specific antigens [2,7].

Mechanistic studies have demonstrated that regulation by CD4+CD25+ T cells can be abrogated by the addition of IL-2 to the culture, by providing strong co-stimulatory signals, or by increasing the TCR signal strength to target CD4 cells [2,5,6,8]. Furthermore, as these assays could be performed in the absence of irradiated "feeder cells", CD4+CD25+ cells act directly on the target T cells and do not suppress in vitro by altering accessory cell function. Thus, a model has been proposed by which T-reg cells suppress the full activation of responder T cells receiving low strength TCR signals, as likely occurs during self-antigen activation, while they do not suppress T cells receiving strong TCR signals such as those induced by pathogens during inflammatory responses.

1.1. Surface phenotype of human CD4+CD25+ T-reg cells

There are significant differences in the expression of CD25 on human as compared to mouse T cells that in-

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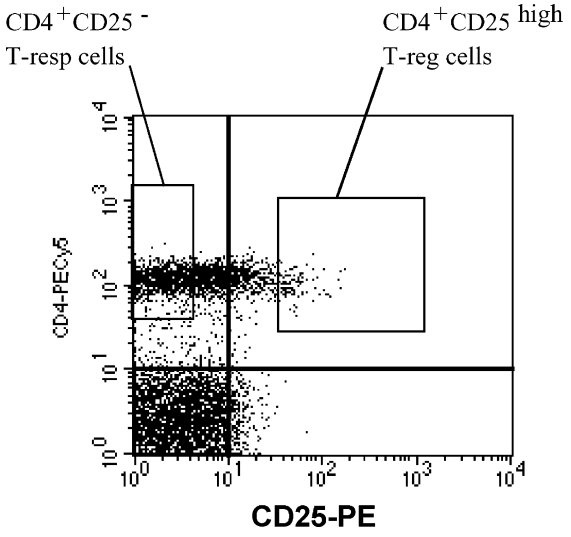

Fig. 1. CD4+CD25^{high} represent a 2–4% of peripheral blood CD4 T cells. This histogram is the typical staining pattern seen when PBMCs are gated to include only small lymphocytes by forward and side scatter, and the monocytes have been excluded by virtue of staining with a cocktail of antibodies including anti-CD14, anti-CD32, and anti-CD116. The low percent of cells that express high levels of CD25 are found only in the CD4+ population as the CD4− T cells (that includes CD8 and NK T cells) do not express similar endogenous high levels of CD25.

fluence techniques in isolating T-reg cells from human peripheral blood. FACS profiles of T cells from mouse spleen and human peripheral blood stained with antibodies against CD25 and CD4 are not equivalent. In the mouse, CD4+CD25+ cells are seen as a distinct population of cells that is easily distinguished from the CD4+CD25− cells and comprise approximately 10% of splenic CD4+ T cells. Thus, the isolation of mouse T-reg cells is rather straightforward. In contrast, human CD4+ T cells exhibit a continuous and primarily low expression of CD25 in which 2–4% express high levels of CD25, while up to 30% express low levels of CD25 (see Fig. 1). This staining continuum makes it more difficult to determine whether all or only a subset of the CD25+ cells should be included into the CD25 T-reg population. The analysis of other cell surface proteins expressed on the surface of CD4+CD25^{high} or CD4+CD25^{low} subsets isolated from peripheral blood indicates that the CD25^{high} subset is homogeneous as over 95% of the cells express CD45RO, CD62L, and CD122 and includes the majority of cells that express HLA-DR and the transferring receptor (CD71) [6,9]. In contrast, the CD25^{low} subset contains a more heterogeneous mixture of cells as demonstrated by expression of CD45RO (80%), CD62L (80%) and CD122 (28%). Analyses of these cells from thymus, cord blood, and synovial fluid similarly demonstrated increased heterogeneity by CD4+CD25^{low} cells as compared to cells restricted to the CD4+CD25^{high} subset [7,9]. Thus, surface phenotype analysis indicates that restricting CD4 cells to those that are CD25^{high} cells may allow the isolation of a more homogeneous T-reg population.

It has long been known that there are major differences in the proteins expressed by human as compared to mouse T cells, most strikingly in respect to HLA-DR and CD45RA/CD45RO. In contrast to the studies in the mouse demonstrating high levels of expression of CD62L and CD38 by CD4+CD25+ T-reg cells [10,11], these same surface markers have not been useful for isolation of human regulatory T cells [1,2]. Peripheral blood T-reg and T-resp cells also show no difference in the levels of expression of CD3 or α/β TCR when analyzed directly ex vivo [2,3]. On the other hand, the human CD4+CD25+ T-reg population expresses HLA-DR directly ex vivo, while mouse T cell do not express class II. Furthermore, whether human CD4+CD25+ T-reg cells can always be restricted to cells expressing CD45RO appears to depend upon the source of the cells, as discussed later. Ultimately, it appears that for now, the constitutive expression of CD25 is the primary characteristic of human T-reg cells that allows consistent isolation of functionally suppressive cells from different human sources. Although the expression of certain surface markers such as CD62L have been useful to enhance the isolation of CD4+CD25+ T-reg cells from mice, the same surface proteins do not necessarily identify human T-reg cells.

CD4+CD25+ T-reg cells appear to express a highly differentiated phenotype characteristic of chronically activated CD4 T cells. It is a constant concern that this regulatory population may contain a number of “contaminating” responder-type T cells that express CD25 due to recent in vivo activation. Although the CD4+CD25+ cells isolated from blood lack expression of CD69, as this is primarily a recent activation antigen, it may not address concerns raised about the isolation of cells in a state of later activation [1]. Analysis of T-reg expression of different CD45 isoforms has resulted in their classification as primarily CD45RO+, CD45RA− and CD45RB^{low} when isolated from adult peripheral blood or tonsil [1,2]. Expression of CD45RO is usually associated with cells that have experienced antigen and are often referred to as memory cells. When adult peripheral blood CD4+CD25+ T cells were separated into CD45RO− and CD45RO+ subsets, the CD45RO− subset exhibited five-fold more proliferation and less than 25% of the suppressive ability of the CD45RO+ subset [3]. In contrast, the CD4+CD25+ cells isolated from cord blood were both suppressive and expressed CD45RA+ [7]. This suggests that differences in the T-reg surface expression of CD45 isoforms may be related to antigen experience or maturation. However, the majority of T-reg activity does appear to be restricted to cells expressing CD45RO if the cells are isolated from adults.

1.2. Methods of isolation of human CD4+CD25+ T-reg cells

At the present time, CD25 expression appears to be the only reproducible marker that can be used to isolate hu-

Table 1
In those reports showing dose–response analysis of T-reg function, there is marked variability both in the method of T-reg isolation and resulting yield, and in the in vitro conditions for T-reg analysis

Reference	T-reg as percentage of cells from PBL	Co-culture stimuli	APC[a]	Serum	Percentage suppression at (T-resp:T-reg) ratios	Isolation method[b]
Ng et al. [13]	15–30% CD4	PHA	Yes	10% HuS (pooled AB)	50% at 1:1 5–10% at 1:1/2 None at >1:1/2	Biotinylated-αCD25; and streptavidin microbeads
Jonuleit et al. [3]	0.7–5.5% total PBMCs	allo-DC	No	1% HuS (autologous)	50% at 1:1 40% at 1:1/2 30% at 1:1/4 10% at 1:1/8	αCD25 microbeads or FITC-αCD25 and αFITC multisort microbeads
Levings et al. [14]	13% CD4	Allogeneic APCs	Yes	1% HuS (pooled AB) and 10% FBS	85% at 1:1 80% at 1:1/2 45% at 1:1/3.3 45% at 1:1/10	PE-αCD25 and anti-PE microbeads
Stephens et al. [5]	10% CD4	PHA	Yes	10% FBS	60% at 1:1 40% at 1:1/2 20% at 1:14	FITC-αCD25 and αFITC microbeads
Taams et al. [1]	7–10% CD4	OKT3 (pb)	Yes	5% FBS	75% at 1:1 50% at 1:1/2 None at >1:2	αCD25 and αmouseIg beads
Dieckmann et al. [2]	6% CD4	Allogeneic DC	No	None	>90% at 1:1 65% at 1:1/5 45% at 1:1/10	αCD25 microbeads
Baecher-Allan et al. [6]	1–3% CD4	Anti-CD3 (Hit3a, sol), anti-CD28 (sol)	Yes	5% HuS (pooled AB)	>90% at 1:1 80% at 1:1/3 40% at 1:1/10	FACS sorted $CD25^{high}$

[a] If the in vitro assay included irradiated PBLs as accessory cells.
[b] All microbeads were actually paramagnetic beads from Miltenyli Biotec.

man CD4+CD25+ regulatory T cells. Most published reports have utilized anti-CD25 magnetic beads to isolate these cells from CD4+ T cells, while others have utilized sorting by FACS to isolate a CD4+CD25+ regulatory cell population that specifically expresses only high levels of CD25 ($CD25^{high}$) (see Table 1). As a result of differences in the method of cell isolation, and the previously described increased heterogeneity of cells expressing low levels of CD25, it is likely that regulatory cell populations isolated with different degrees of purity would result in variability of in vitro suppression or more complex variation in apparent mechanism(s) of action.

Our data suggests that it is not only the expression of CD25 on the surface of CD4 T cells, but the actual level of CD25 expression on the CD4 cell that is critical in distinguishing between regulatory and non-regulatory CD4 T cells. When CD4 T cells expressing either low or high levels of CD25 were isolated from the circulation of normal healthy individuals and tested for regulatory function, suppressive function segregated with the minor subset of cells exhibiting the $CD25^{high}$ phenotype [6]. This $CD25^{high}$ subset represents approximately 2–4% of human PBL CD4+ T cells. In contrast, we find that the CD4+$CD25^{low}$ cells usually proliferate quite well to polyclonal T cell stimuli and may or may not exhibit minor suppressive ability in comparison to the CD4+$CD25^{high}$ population. Thus, it is our working hypothesis that the CD4+$CD25^{low}$ cells found in human peripheral blood are primarily in vivo activated T-resp cells that may contain an unknown number of CD25+ regulatory T cells, possibly depending upon the donor and their state of health. Others have also demonstrated that suppressive function is most consistently restricted to the cells expressing the highest levels of CD25 as opposed to the CD4+$CD25^{low}$ population when cells were isolated from synovial fluid or again from peripheral blood [9,12].

Isolation by magnetic beads likely selects the $CD25^{high}$ population accompanied by varying numbers of the $CD25^{low}$ subset. It seems that this technique is very sensitive to small differences in procedures since the number of cells reported to be isolated by the same magnetic bead reagents varies from 6 to 30% of the CD4+ cells from adult peripheral blood (Table 1). Since most reports using similar paramagnetic beads can produce strikingly different yields, it is unfortunate that the ratio of bead volume to cell number used to isolate the CD25+ cells from the CD4+ population is usually not provided. Regardless, it is reasonable to propose that the relative ratio of the $CD25^{high}$ to the $CD25^{low}$ cells in the isolated T-reg population would inversely correlate with cell yield. If the small $CD25^{high}$ population contains the most regulatory activity, then, in theory, increased yield would correlate with increased numbers of $CD25^{low}$ cells and decreased T-reg purity. As in vitro suppression usually

cannot occur if the target (T-resp) to effector (T-reg) ratio is greater than 10:1, the suppressive activity should dilute out more quickly from a T-reg population that is already a co-culture of regulatory and non-regulatory cells than from a more homogeneous T-reg population. Only a general trend might be seen, at best, in studies comparing extreme differences in T-reg cell yield. For example, Ng et al. report a quick loss of regulatory function at greater than a 2:1 ratio of T-resp to T-reg cells using a T-reg population that represented a high percentage (30%) of the total number of CD4+ cells [13]. In contrast, others report sustainable suppression at ratios approaching 10:1 using CD25 populations that were isolated by the similar paramagnetic beads but only represented 6–7% of peripheral blood CD4+ T cells [1,2]. In fact, published FACS analysis of low yield T-reg populations after bead-based positive isolation do contain an apparent prevalence of $CD25^{high}$ cells, while the non-selected T-resp population actually contains a fair number of $CD25^{low}$ cells [14]. Thus, these data indicate that lower yield magnetic bead isolation may allow a preferential isolation of the $CD4+CD25^{high}$ cells possibly as a result of performing the isolation under CD25 limiting conditions such as reducing the bead ratio or incubation time.

Although restricting the T-reg populations to those CD4 T cells that express high levels of CD25 does increase T-reg homogeneity, it is difficult to assess whether all activated T-resp cells have been removed from the isolated T-reg population. Most surface proteins that are expressed on T-reg cells such as CD25, CTLA4, GITR, PD-L1, CD45RO, and HLA-DR, can also be found on activated T-resp cells. However, it has been shown that 1–2 weeks after polyclonal activation in the presence of exogenous IL-2, bulk populations of T-reg cells demonstrate sustained high expression of CD25 and CTLA4 on their surface while activated CD4+CD25− T-resp cells are low or negative for CD25 and CTLA4 as they are only transiently expressed [2]. Thus, it is possible that this feature of maintenance of certain surface protein expression could be used to indicate the purity of an isolated T-reg population in a retrospective manner. Sustained surface expression of CD25, CTLA4 and GITR was also found to be a feature of suppressive clones generated from the $CD4+CD25^{high}$ population while non-suppressive clones did not show this phenotype [15].

While it has not been reported whether T-reg clones can be generated in the mouse, functionally suppressive T-reg clones can be generated from human $CD4+CD25^{high}$ cells [15]. The ability to study T-reg clones will likely prove to be a significant advance in the future study of these cells even though the technique is extremely inefficient as only 10% of seeded wells actually produce clones. It is possible that either the optimal procedures to grow these cells have not yet been identified or that limited cell expansion is an intrinsic unavoidable feature of $CD4+CD25^{high}$ cells. Regardless, $CD4+CD25^{high}$ clones grown by stimulation with PHA/IL-2 and allogeneic feeder cells, could ultimately be categorized as either suppressive (45%), anergic (35%), or responder (20%) (i.e. proliferative) when tested for ability to suppress fresh CD4+ T-resp cells. Furthermore, there was a direct correlation between sustained high expression of CD25 on the clones and their suppressive ability ([15] and our unpublished results). Specifically, we have reproduced the results of Levings et. al. in our laboratory using identical culture conditions. Of those wells plated with $CD4+CD25^{high}$ cells demonstrating growth, between 10 and 30% contained clones that grew vigorously and both proliferated to a high degree and were non-suppressive when tested for function. In contrast, the clones that grew poorly usually exhibited sustained expression of CD25 but were either too low in number to be tested for function or did expand sufficiently to be tested and were found to be either functionally anergic or suppressive (50–80% of testable clones). Two caveats of this assay are that it is unknown whether the regulatory function of expanded CD4+CD25+ T-reg clones changes as a result of in vitro culture and that as it is based on the need to generate clones, it is biased against CD4+CD25+ T-reg cells which grow poorly. However, as non-suppressive clones are generated from the $CD4+CD25^{high}$ population, it is likely that the $CD4+CD25^{high}$ subset still contains a low number of non-suppressive responder T cells.

1.3. In vitro measures of suppression by human CD4+CD25+ cells

The best evidence for the regulatory classification of CD4+CD25+ cells isolated from humans should ultimately come from in vivo demonstration of their inhibitory properties, which of course is not possible. Thus, in human studies, assessment of the regulatory function of the isolated CD4+CD25+ population depends upon in vitro assays that reflect parallel in vivo and in vitro observations in mice. The widely utilized method to examine regulatory cell function is the in vitro co-culture suppression assay in which the potential T-reg cells are added, often in decreasing numbers, to target cell CD4+CD25−, responder T cells (T-resp) in the presence of polyclonal stimulation as initially published by Thornton and Shevach [16]. As these human and mouse assays are almost identical and the mouse in vitro experiments have been shown to correspond to in vivo T-reg function, it is likely that human in vitro experiments may also be reflective of in vivo events. The most informative in vitro assay is a dose–response co-culture in which the regulatory population is added in decreasing numbers to a constant number of responder T cells. This assay addresses either the purity of the isolated T-reg population or the efficiency of the suppression by the isolated cells when co-cultured under different conditions. In order to classify CD25+ subset of CD4 cells as CD4+CD25+ T-reg as opposed to Tr1 or Tr3 T-reg cells,

the suppressive ability of the isolated population must be shown to depend on cell contact. This is achieved by culturing target and effector cells either in the same or in separate transwell compartments that do or do not allow direct cell contact. Additional studies involving the addition of antibodies to block specific cytokines, indicate that suppression by CD4+CD25+ T-reg cells does not require IL-10 and may or may not involve TGF-β as there are conflicting results [2,17].

There is such variation in the in vitro assay conditions used to study the regulatory activity of human CD4+CD25+ cells that the experimental conditions themselves may produce different mechanistic conclusions. These in vitro assays of suppression most often involve polyclonal stimulation methods that range from undefined allogeneic responses, to mitogenic lectins (PHA, Con A), to the use of different monoclonal antibodies against CD3 in the presence or absence of anti-CD28 co-stimulation (see Table 1). However, even in studies where the same anti-CD3 or anti-CD28 monoclonal antibodies are used as the stimulus, the antibodies are often provided in different physical formats (immobilized or soluble) or at different concentrations. The assays are further varied by the presence or absence of accessory cells (autologous or allogeneic) and the presence or type of serum ranging from pooled human AB serum at 0–10%, autologous human serum at 1–5% or even FBS. While at a very broad level different reports appear to use the same stimulus such as through CD3 and CD28, it is highly possible that seemingly small variations in the details of the culture conditions could produce opposing results of co-culture suppression or co-culture proliferation. For example, we find that stimulation of co-cultures with platebound anti-CD3 (clone UCHT1 at 2.5 μg/ml) and soluble anti-CD28 (clone 28.2 at 2.5 μg/ml) in the presence of irradiated PBMC feeders and 5% pooled human AB serum results in strong co-culture proliferation, even though CD4+CD25^{high} T-reg cells are added at a 1:1 ratio [6]. In contrast, Dieckmann et al. use these same stimulatory antibodies (platebound UCHT1 at 10 μg/ml, and soluble 28.2 at 10 μg/ml) in the absence of irradiated PBMC feeders or serum to produce strong co-culture suppression using bead isolated CD4+CD25+ T-reg cells [2]. Since the strength of the signals given to the different T cells determines whether suppression occurs, it is possible that a strong signal delivered in the presence of serum and accessory cells that does not typically result in in vitro suppression may become a much weaker stimulus in the absence of serum and accessory cells which then allows suppression to occur. Or alternatively, the addition of serum and feeders may enhance the baseline activation state within the T cell so that the signals generated by anti-CD3/anti-CD28 stimulation rise above a theoretical threshold that is no longer sensitive to suppression. It is most likely a result of the highly suppressive nature of human CD4+CD25+ T-reg cells that they are able to suppress T cell proliferation under so many different conditions in vitro.

2. Functional features of human CD4+CD25+ T-reg cells

2.1. Growth potential of CD4+CD25+ cells

Although CD4+CD25+ T-reg cells have been described to be hypo-responsive in vitro, the mechanism by which they maintain their anergic state is largely unknown. It has been suggested that these T-reg cells represent the accumulation of highly antigen-experienced CD4 T cells that have reached the end stage of differentiation [18]. This provides a unifying explanation for their characteristics of limited growth potential, activated surface phenotype, and long-term presence in vivo in the face of thymic involution. The poor ability to propagate T-reg cells in vitro would further support this hypothesis. Yet, it may simply be that we do not yet know the correct requirements to grow these cells as it is not clear whether T-reg cells should respond with proliferation to culture conditions originally identified to support T-resp cell growth. Furthermore, although the population of human T-reg cells do exhibit features similar to those of repeatedly activated T cells in that they have short telomeres, contain low levels of Bcl-2, and express CD95 on their surface, they are not particularly sensitive to activation induced cell death (AICD) and are sensitive to death induced by cytokine deprivation [1,19]. Studies in mice suggest that CD4+CD25+ T-reg cells may arise from a separate lineage of CD4 T cells that leave the thymus after neonatal day 3 since adult mice thymectomized by neonatal day 3 lack T-reg cells but still contain CD4+CD25− T-resp cells [20,21]. In this regard, it is quite interesting that functional CD4+CD25+ T-reg cells can be isolated from human cord blood at birth which would basically be equivalent to neonatal day 0. Thus, whether human CD4+CD25+ T-reg cells are a separate lineage of cells or arise from a highly specialized process of differentiation remains to be determined.

Certain features of T-reg cells indicate the obstacles that will need to be overcome in order to achieve reasonable levels of T-reg cell expansion, assuming it is possible. Many reports have shown that bulk populations of T-reg cells can undergo limited expansion in vitro by stimulation through the TCR in the presence of cytokines that signal through the IL-2R-γ chain, and still retain their suppressive ability when tested in co-culture with fresh CD4+ T-resp cells [1,3,14]. However, it is important to note that even in the presence of exogenous cytokines, T-reg cells exhibit lower proliferative capacity as compared to similarly activated T-resp cells [2,6]. We typically find that the CD4+CD25^{high} cells expand 10- or 40-fold less than similarly stimulated CD4+CD45RA+CD25− or CD4+CD45RA−CD25− T-resp cells, even when exogenous cytokines are provided. Thus, these cells require exogenous sources of cytokine since they die in response to cytokine deprivation but can be rescued by the addition of IL-2 or IFN-β and possibly IL-15 [1–3]. Interestingly, as the addition of these cytokines also inhibits their suppressive function, it is possible that stim-

ulated T-reg cells may actually suppress their own growth. When stimulated in the absence of IL-2, it appears that T-reg cells do not undergo increased apoptosis, but rather arrest at the G1/G0 phase as the percent of cells in S phase from CD4+CD25+ or CD4+CD25− cultures were 3 and 11% while the percent of cells in G1/G0 were 95 and 80%, respectively in response to allogeneic dendritic cells [3]. However, as the cells are not apoptotic, it may be that there are certain conditions that would allow them to re-enter the cell cycle. Yet there may be an unavoidable limit to T-reg expansion since human, bead isolated, CD4+CD25+ T-reg cells were shown to have slightly shorter telomeres than CD4+CD25− T-resp cells, as indicated by decreased hybridization with a telomere probe giving the respective mean fluorescent intensities of 25 and 29 [19]. However, if this small difference in fluorescence intensity and thus telomere length is the reason for the observed limitation in T-reg expansion is currently unknown. Furthermore, whether a small subset of T-reg cells has longer telomeres and/or increased expansion potential and may be represented by the ability to generate a low number of suppressive clones remains to be determined. In fact, even with the low efficiency of generating T-reg clones and the possibility that the most highly suppressive T-reg cells may not be clonable, the ever-present concern on the purity of bulk T-reg populations makes it attractive to study the growth conditions and proliferative potential of T-reg cells on a clonal basis. Thus, if human CD4+CD25+ T-reg cells can be efficiently expanded in vitro, it would be useful for the future study of these cells and the development of potential therapeutics.

There are variable reports as to whether human CD4+CD25+ T-reg cells secrete IL-10. In general, it appears that FACS isolated CD4+CD25^{high} T-reg cells do not secrete IL-10, while bead isolated CD4+CD25+ T-reg cells do. It has also been shown that suppressive clones generated from CD4+CD25^{high} cells do not secrete IL-10 ([15] and our unpublished results). In fact, in contrast to what is usually reported on IL-10 secretion by T-reg cells, we find that IL-10 is produced primarily by T-resp cells by stimulation with strong TCR signals that usually do not permit suppression of proliferation [6,8]. Furthermore, as IL-10 is found at higher levels in supernatant from the T-resp cultures as compared to the corresponding T-resp–T-reg co-cultures, and is not in cultures of T-reg cells, it appears that the T-reg cells must either inhibit IL-10 production by T-resp cells or utilize the IL-10 released into the supernatant. We have observed that while only a subset of stimuli actually induce IL-10, it is found at higher amounts in the T-resp cultures than in the corresponding T-reg co-cultures [8].

2.2. Mechanisms of suppression by human CD4+CD25+ cells

The mechanism of suppression by CD4+CD25+ cells is poorly understood. The most basic feature indicated by our in vitro models is that suppression requires direct cell contact between the CD4+CD25+ T-reg cell and the target T-resp cell. Furthermore, it appears that human CD4+CD25+ T-reg cells must be activated through their TCR in order to be operationally suppressive. This was shown by two separate studies in which fixed, bead isolated T-reg cells could suppress T-resp cell proliferation, but only if the T-reg cells had been pre-activated before the fixation [22,23]. These studies also indicate that while the T-reg cells need to be activated to exhibit suppressive function, they do not need to be viable during the actual co-culture. Furthermore, the changes that occur during this pre-activation were shown to require protein synthesis and surface expression during a 20 h incubation with anti-CD3 (2% serum) [23] or a 10 h incubation with anti-CD3/anti-CD28 (no serum) [22]. No role has been shown for the surface molecules GITR, CTLA4, or PD-L1 using currently available blocking antibodies as the addition of increasing numbers of CD4+CD25+ cells cause suppression even in the presence of these reagents [3,6,8,14]. In contrast to the consistent finding that CD4+CD25+ T-reg cell function is independent of IL-10, it is difficult to say whether TGF-β is involved as most reports studying T-reg cells isolated from adult blood show no effect of blocking TGFβ while others find that neutralizing TGF-β reduces suppression [15,17].

The type of stimulus provided to the co-culture of T-reg and T-resp cells has a strong influence on whether suppression or proliferation will be the end result of the interaction. Suppression by CD4+CD25+ cells is lost if the co-cultures are supplemented with growth promoting cytokines or strong co-stimulation. Furthermore, as co-cultures stimulated with increasing amounts of platebound anti-CD3 exhibit less suppression, it appears that the degree of suppression is inversely related to the strength of the TCR stimulation [6,8]. Whether strong TCR stimulation abrogates suppression by increasing the resistance of T-resp cells or by decreasing the functional ability of T-reg cells was addressed in a two step co-culture system in which T-reg or T-resp cells were pre-activated with different strength stimuli for different lengths of time before they were combined in subsequent co-culture. Upon combining differentially stimulated T-reg or T-resp cells, it became apparent that increasing the strength of the TCR signal primarily increased the resistance of T-resp cells to regulation. Thus, the degree of TCR stimulation significantly alters the outcome of T-reg and T-resp interactions.

Two groups have reported data suggesting that contact with CD4+CD25+ T-reg cells causes CD25− responder type T cells to become suppressive themselves by the production of the inhibitory cytokines TGF-β or IL-10. This mechanism is referred to as “infectious tolerance”. In a complex multiple donor system, Jonuleit et al. found that if CD4+CD25− HLA-A2^{-} cells were cultured for 6 days with anti-CD3/anti-CD28 in the presence of allogeneic CD4+CD25+ HLA-A2^{+} T-reg cells, then when re-isolated, the T-resp cells could suppress freshly stimulated CD4 cells in secondary cultures [23]. However, unlike

suppression mediated by CD4+CD25+ T-reg cells, suppression mediated by these re-isolated T-resp cells did not require cell contact and could be inhibited by anti-TGF-β, but not anti-IL-10. In contrast, Dieckmann et al. published a similar study indicating that T-resp cells that had been co-cultured with T-reg cells, also became subsequently suppressive but through the production of IL-10 and not TGF-β [22]. In this second study, CFSE labeled CD4+CD25− T-resp cells were re-isolated after a 2 day co-culture with T-reg cells stimulated with anti-CD3/anti-CD28 in 1% autologous serum. Although these two studies reach opposite mechanistic conclusions, it is possible that differences in the stimuli and the length of T-reg contact could cause the T-resp cells to differentiate into cells that selectively produce one or the other of these inhibitory cytokines. This mechanism of infectious tolerance could explain how such a small population of cells can regulate a much larger population of responder T cells in vivo.

3. CD4+CD25+ T-reg cells in human disease

One goal of studying human CD4+CD25+ regulatory T cells is to assess whether they play a role in diseases involving aberrant activation of the immune system. Unfortunately, as CD25 expression currently appears to be the best feature for isolating this subset of T-reg cells, potential contamination with activated T-resp cells will always be an unavoidable concern especially when isolating the T-reg populations from patients. Thus, it may not be surprising that some reports have demonstrated increased frequency of CD4+CD25+ cells in the target tissues of specific diseases of autoimmunity, cancer, or chronic infection, while the peripheral blood usually shows little difference in CD4+CD25+ frequency. Surprisingly, in the majority of these studies, these CD25+ cells were shown to be suppressive in vitro [9,12,24,25]. Whether these cells are more or less inhibitory than similar cells isolated from healthy controls cannot be determined since healthy controls will not have T cells in similar target tissues. However, the ability to study ex vivo suppressive function over the course of disease progression or treatments may indicate whether changes in regulatory cell function correlates with the clinical disease state. Thus, if clinical observations of disease relapse and remission can be associated with changes in the in vitro suppressive ability of the CD4+CD25+ cells or the in vitro susceptibility of the CD4+CD25− T-resp cells, it may indicate whether alteration of T-reg suppressive function associates with human inflammatory disease.

In contrast to cancer and those autoimmune diseases that appear to result from a potential accumulation of somatic events, there is also a rare genetically inherited autoimmune disease known as IPEX that often manifests itself soon after birth in which T-reg cells may be absent or impaired due to a genetic mutation in the *foxp3* gene, which is thought to be important in the function of mouse CD4+CD25+ T-reg cells [26–28]. Thus, elucidating the biology of regulation by human CD4+CD25+ T cells may lead to both an increased understanding and the development of potential treatments for specific disease.

Treatments that induce T-reg cell function would be immunosuppressive and might be useful in allo-transplantation by inhibiting allo-reactive T-resp cells. The mechanism by which humanized anti-CD3 mAb treatment produces its favorable results in clinical trials of renal transplantation is unclear. It is possible that it may have a direct effect of killing highly activated, allo-specific T-resp cells by AICD as well as an indirect effect of making T-resp cells more sensitive to T-reg suppression, since the efficacy of anti-CD3 seems to be related to its ability to deliver only partial signals [29]. Thus, humanized anti-CD3 may provide a low strength signal to allo-reactive T cells that makes them sensitive to suppression by CD4+CD25+ T-reg cells.

Our current model for the involvement of CD4+CD25+ T-reg cells in the initiation or progression of autoimmune disease involves the observation that TCR signal strength can change the outcome of T-resp and T-reg cell interaction from suppression to proliferation. Furthermore, time course studies of in vitro suppression show that although weakly stimulated T-resp cells are initially sensitive to T-reg suppression, they do in fact become resistant to suppression after 40 or 60 h of stimulation in a rate that is still dependent upon the relative strengths of the provided stimuli (see Fig. 2). Thus, one potential scenario is that in a given tissue during an inflammatory response, T cells that recognize self-antigens with low strength signals can proliferate as cytokines (i.e. IL-2, IL-15) produced by strongly activated T-resp cells cause T-reg cells to become non-suppressive and possibly even to proliferate. Then, after the initial inflammation resolves, the regulatory ability of the CD4+CD25+ cells would return. However, over time, as a result of multiple interactions with self-antigens and T-reg cells, those auto-reactive cells that recognize self-antigens with greater strength of signal would be preferentially selected from the expanded pool of self-reactive T cells due to their decreased sensitivity to suppression by CD4+CD25+ cells. It is also possible that as a result of chronic inflammation, the T-reg cells may also exhibit decreased activity due to functional exhaustion. This is an attractive explanation for the episodic clinical nature of many autoimmune diseases such as RA or MS. However, this proposed model for developing autoimmunity would still require a predisposition for generating self-reactive T cells that recognize self-antigen more easily or with stronger signals.

3.1. Chronic infection, autoimmunity, and cancer

The definition of chronic infection is the absence of clearance of the offending pathogen. It has been postulated that lack of clearance or immune resolution could be due to undesirable activation of T-reg cells that inhibit the development

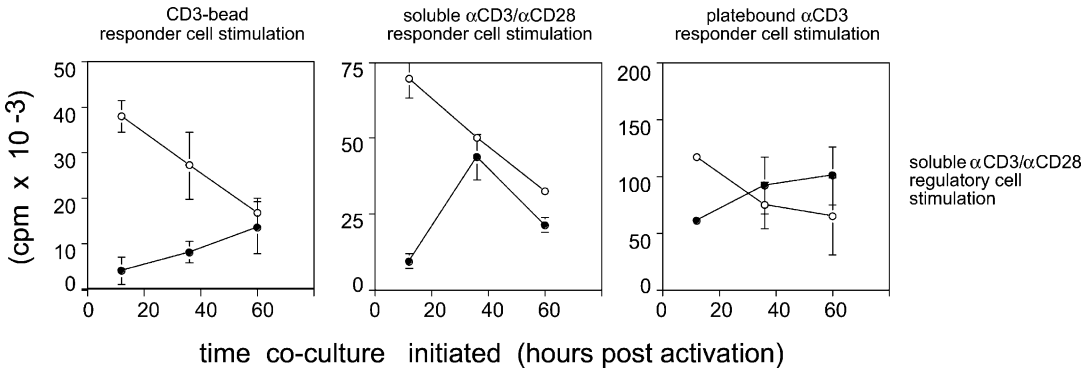

Fig. 2. Increasing the strength of the TCR stimulation provided to the T-resp population decreases the length of time that they remain sensitive to suppression by CD4+CD25^{high} T-reg cells. Cultures of T-resp cells and irradiated feeders (filled symbols) or irradiated feeders only (open symbols) were stimulated with anti-CD3 beads (weak), soluble anti-CD3 and anti-CD28 (stronger), or plate-bound anti-CD3 (strongest) for 12, 38 or 60 h before they were combined in co-culture with T-reg cells that had been stimulated for the same lengths of time with soluble anti-CD3 and CD28, and were analyzed for proliferation at 5 days. The T-resp cells stimulated with the weakest stimulus (anti-CD3 beads) give the lowest level of proliferation and the longest sensitivity to suppression (60 h), while stimulation with increasing strength signals increases the proliferation and decreases the time that these cells remain sensitive to suppression.

and completion of a productive immune response. In the case of *Helicobacter pylori* infection there is no evidence for any immune protection from re-infection after antibiotic clearance in infected individuals. Although it is highly preliminary, it has been reported that in in vitro studies, removing CD25^{high} cells significantly increases the *H. pylori* response by memory T cells isolated from the peripheral blood from infected individuals more so than from uninfected controls [12]. This result suggests that CD4+CD25^{high} cells may play a role in failures of clearance and protective immunity. Furthermore, while this is a situation of chronic inflammation, it is interesting to note that the CD4+CD25^{high} population isolated from the blood, exhibits regulatory as opposed to responder activity.

There are few reports on functional analysis of CD4+CD25^{high} cells isolated from patients with autoimmune disease. In the study of multiple sclerosis we have addressed whether there are differences in this regulatory cell subset isolated from the peripheral blood of patients and healthy controls. While we have found no difference in the frequency of CD4+CD25^{high} cells between patients and healthy controls, T-reg cells derived from patients as compared to healthy controls exhibit significantly less suppressive function (submitted for publication). In the case of rheumatoid arthritis, which has association with HLA-DR4, Cao et al. [9] found that the synovial fluid from patients with active disease often contains an increased number of CD4+CD25^{bright} cells as compared to their levels in peripheral blood. Furthermore, these synovial derived, FACS isolated CD4+CD25^{bright} cells demonstrated “classical” T-reg function in vitro and were able to inhibit the proliferation of T-resp (CD4+CD25−) cells derived from either synovial fluid or peripheral blood. Whether the CD4+CD25^{high} cells from the blood and the CD4+CD25^{bright} cells from the synovial fluid function through different mechanisms and whether they are actually functional in vivo, is unknown.

While CD4+CD25+ T-reg cells isolated from lung cancer biopsies, and from the peripheral blood of patients with pancreatic or breast cancer were shown to be functional, the in vitro conditions used for the analysis were likely less than ideal as the cells isolated from normal controls and peripheral blood were poorly suppressive [24,25]. Regardless, the presence of functional T-reg cell in these states would be deleterious to the ability to mount an anti-tumor response.

3.2. IPEX

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is a very rare human genetic disease in which female heterozygous carriers are asymptomatic. In contrast, the disease in affected male offspring is associated with severe diarrhea, lymphocyte activation, IDDM, thryoiditis and autoimmune deficiency and is usually fatal early in life likely due to overproduction of proinflammatory cytokines [26,30]. A homologous disease occurs in the *scurfy* mouse that has been found to contain a natural mutation in the *foxp3* gene, encoding a member of the forkhead/winged-helix proteins. In humans, the majority of IPEX cases have also been found to correlate with a disruption in the *foxp3* gene product. The wild type foxp3 protein (scurfin) contains a zinc finger domain, a leucine zipper motif, and forkhead domain in the wild type state and is proposed to function as a transcription repressor in T cells. The interest in the *foxp3* gene has arisen mainly from the findings that in the mouse foxp3 expression appears to be restricted to CD4+CD25+ T-reg cells and forced foxp3 expression by viral transduction may confer T-reg activity to normal T cells [27,28]. Although expression of foxp3 has not been shown to be similarly restricted to CD4+CD25+ T-reg cell in man, the findings that many cases of IPEX are associated with truncation or absence of the foxp3 protein, that IPEX is characterized by hyperactivation of T cells, and that

chimerism from donor BMT can treat the disease in a dominant fashion, it is attractive to postulate that the bone marrow gives rise to functional T-reg cells that can then down modulate the recipient's immune system [31–33].

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