The peripheral generation of CD4$^+$ CD25$^+$ regulatory T cells

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INTRODUCTION

One of the crucial unanswered questions in the field of T-cell regulation is the origin of CD4$^+$ CD25$^+$ regulatory T cells. Does the thymus produce regulatory T cells as a functionally mature separate lineage, or can some T cells acquire regulatory function in the periphery? While mice live for 2·5 years, humans can live for eight decades and beyond. The constraints on the persistence of human CD4$^+$ CD25$^+$ regulatory T cells will therefore be more extreme as a result of thymic involution early in the human lifespan. A central question is whether the full complement of CD4$^+$ CD25$^+$ regulatory T cells are generated early in life and if so, how do they manage to persist? A consistent observation in both mice and humans is that CD4$^+$ CD25$^+$ T cells in adults have the phenotypic characteristics of a highly differentiated T-cell population. This review seeks to reconcile the observations that CD4$^+$ CD25$^+$ T cells are anergic but highly differentiated. We suggest that it is possible that a subpopulation of CD4$^+$ CD25$^+$ regulatory T cells may arise in the periphery as a consequence of anergy induction in antigen-specific CD4$^+$ T cells that are approaching end-stage differentiation.

CD4$^+$ CD25$^+$ REGULATORY T CELLS HAVE A HIGHLY DIFFERENTIATED PHENOTYPE

Upon repeated stimulation by antigen over time, T cells proliferate and acquire effector functions. There is also a change in expression of surface receptors after stimulation and dynamic alterations of cytokine, homing, adhesion and signalling receptors occur.[1–3] Naïve, recently primed and highly differentiated T cells have been distinguished from each other on the differential expression of these molecules.[4] The expression of one molecule in particular has been used to discriminate between cells at early and late stages of differentiation. While naïve or recently activated T cells express high levels of CD45RB, highly differentiated CD4$^+$ T cells express low levels of this molecule.[5] A consistent observation that has been made by many groups is that CD4$^+$ CD25$^+$ regulatory T cells have a CD45RB^{low}

phenotype in mice and a CD45RO$^+$ CD45RB^{low} phenotype in humans, indicating that they have experienced many rounds of cell division.[6–11]

FUNCTIONAL EVIDENCE FOR THE ADVANCED DIFFERENTIATION STATE OF CD4$^+$ CD25$^+$ T CELLS

The expansion of T cells through re-activation cannot occur indefinitely through life and continuous re-activation induces a state of growth arrest known as replicative senescence after a finite number of cell divisions.[12–14] This is considered to be a strategy for preventing potentially malignant expansions of cells.[12] The reason why replicative senescence occurs is that telomeres, repeating hexameric nucleotide units at the ends of chromosomes, shorten by around 50 base pairs with each cell division.[15–17] Telomeres protect against end-to-end fusion of chromosomes and promote their stability.[18,19] Although the enzyme telomerase can compensate initially for telomere loss, this enzyme is not induced upon repeated activation of T cells.[20–22] Repeated T-cell stimulation therefore leads to a critical point where telomeres are sufficiently reduced and growth arrest as a result of chromosomal aberrations occurs. The key point here is that highly differentiated T cells will have shorter telomeres than those that are naïve or have not experienced extensive cell division.[20,23]

Human CD4$^+$ CD25$^+$ T cells have short telomeres, which supports the possibility that they are highly differentiated T cells.[24] An intriguing possibility is that CD4$^+$ T cells do not differentiate into an end-stage effete population, rather they may give rise to regulatory cells that are very antigen-experienced. This raises the important possibility that old T cells may be useful after all and offers a plausible mechanism by which high concentrations of antigen may actually direct the development of specific regulatory cells. This is a reworking of the venerated concept of high-zone tolerance, but with a new twist.

HUMAN CD4$^+$ CD25$^+$ T CELLS ARE SUSCEPTIBLE TO APOPTOSIS

The anti-apoptotic molecule Bcl-2 plays a central role in the decision of expanded populations of T cells to live or die.[3] This molecule is involved in the mitochondrial pathway leading to death and it is down-regulated after T-cell expansion. Highly differentiated T cells are therefore extremely susceptible to apoptosis.[5,25,26] However, down-regulation of Bcl-2 is not an

irreversible event and certain groups of cytokines can prevent T-cell apoptosis and do so by re-inducing Bcl-2 expression.[26,27] A second group of cell surface molecules that are related to the tumour necrosis factor receptor can also trigger T-cell apoptosis if ligated at the time of T-cell activation in the absence of co-stimulation. This phenomenon is known as activation-induced cell death.[28] The expression of one of these molecules, CD95 increases as a T cell progressively differentiates.[5] Induction of T-cell apoptosis by CD95 ligation can also be prevented by certain cytokines, e.g. interferon-α/β (IFN-α/β)[29–31] Highly differentiated T cells, that express low levels of CD45RB also have decreased levels of Bcl-2 but increased levels of CD95.[5] However, this does not indicate their imminent death rather that these cells are more dependent on exogenous factors to maintain their survival.[26]

In humans, freshly isolated $CD4^+ CD25^+$ T cells are susceptible to cytokine starvation-induced apoptosis and this is related to low expression of Bcl-2.[10] The apoptosis of these cells can be prevented by the addition of interleukin-2 receptor (IL-2R) γ-chain signalling cytokines, such as IL-2 and IL-15, and also IFN-α/β.[10] An important observation was that the regulatory activity of these cells was maintained when these cytokines were added.[8,10] $CD4^+ CD25^+$ T cells express elevated levels of CD95 but are not particularly sensitive to activation-induced cell death.[10] These observations suggest that $CD4^+ CD25^+$ T cells may depend on the continual presence of exogenous factors to prevent their apoptosis.

EVIDENCE FOR THYMIC GENERATION OF $CD4^+ CD25^+$ T CELLS

Many studies have shown that $CD4^+ CD25^+$ T cells can develop in the thymus. As these have been extensively reviewed elsewhere[32–34] we will briefly mention some of the evidence supporting this concept.

$CD4^+ CD25^+$ represent 5–10% of $CD4^+ CD8^-$ thymocytes, in humans, mice and rats.[6,35–37] A number of early studies showed that mature $CD4^+ CD8^-$ thymocytes have the capacity to prevent autoimmunity.[38–40] Adoptive transfer experiments showed that transfer of single-positive CD4 thymocytes depleted of $CD4^+ CD25^+$ cells produced similar autoimmune diseases in syngeneic T-cell-deficient mice, as do peripheral CD4 T cells. These autoimmune diseases can be prevented by the adoptive transfer of $CD25^+$ thymocytes.[6,36,41]

Mice thymectomized 3 days after birth develop spontaneous autoimmunity (such as gastritis) which correlates with reduced numbers of immunoregulatory $CD25^+$ T cells present in the peripheral lymphoid pool. Performing the thymectomy earlier (day 0) decreased the likelihood of autoimmunity, probably because of a decreased number of self-reactive T cells. On the other hand, delaying thymectomy until day 7 was also less efficient in inducing autoimmunity, presumably because a sufficient number of regulatory T cells have already emerged into the periphery.[42]

A number of experiments exclude the possibility that regulatory T cells from the periphery migrate to or recirculate through the thymus, and confirm the thymus as the source of regulatory T cells. $CD25^+ CD4^+ CD8^-$ thymocytes can develop in the fetal thymic organ culture *in vitro* and emerge *in vivo*

following direct injection of CD4-CD8-thymic precursors into the thymus.[6] Furthermore, intrathymic fluorochrome labelling demonstrates that $CD4^+ CD25^+$ regulatory T cells leave the thymus to populate the periphery.[37]

IS THYMIC PRODUCTION THE ONLY WAY TO GENERATE $CD4^+ CD25^+$ T REGULATORY CELLS?

The strong evidence from animal models showing that $CD4^+ CD25^+$ T cells can develop in the thymus does not rule out alternative mechanisms for the generation of regulatory T cells. Mice have a much shorter lifespan than humans and a central question is whether the thymus can replenish the peripheral pool of $CD4^+ CD25^+$ T regulatory cells throughout the human lifespan. There are a number of caveats that restrict the possibility of thymic generation as the sole source of $CD4^+ CD25^+$ T cells in humans.

(1) As a result of thymic involution the number of $CD4^+ CD25^+$ Tregs should decrease over time and yet evidence suggests that this is not the case: therefore thymically derived Tregs must be efficiently maintained in the periphery.

(2) Tregs are anergic and prone to apoptosis and it is not clear how they can be efficiently maintained despite their poor proliferative capacity.

(3) How would regulatory T cells to antigens that are encountered in the periphery develop after thymic involution occurs?

EVIDENCE FOR THE PERIPHERAL GENERATION OF $CD4^+ CD25^+$ T CELLS *IN VIVO*

All the studies that support the peripheral generation of $CD4^+ CD25^+$ T cells have been performed in animals. The most convincing data suggesting the peripheral generation of $CD4^+ CD25^+$ T cells *in vivo* comes from the work of Thorstenson and Khoruts[43] who adoptively transferred CD4 cells, from OVA-specific DO11.10 T-cell receptor (TCR) transgenic mice on a RAG$^{-/-}$ background, into recipient BALB/c mice. Following low dose tolerance protocols *in vivo*, intravenous or oral administration of antigen led to the generation of TCR-transgenic $CD4^+ CD25^+$ T cells. Since RAG-2-deficient mice do not contain $CD4^+ CD25^+$ T cells, this population had to be generated from the $CD4^+ CD25^-$ cells. These regulatory T cells therefore arise from naïve $CD4^+$ precursors but share many characteristics with naturally occurring $CD4^+ CD25^+$ T cells and are able to suppress the responsiveness of naïve T cells in an antigen-specific manner. Zhang and colleagues[44] reached the same conclusion. They observed an increase in the number of clonotype-specific $CD4^+ CD25^+$ T cells following oral administration of OVA to OVA-TCR transgenic mice. Regulatory T cells expanded in this way are activated and more potent than Tregs present in controls.

As discussed earlier in a classic study Asano and colleagues[42] observed that neonatally thymectomized mice lack $CD4^+ CD25^+$ T cells. However, although absent at first, $CD4^+ CD25^+$ T cells in these mice slowly reappear and reach normal numbers by 3 months. These newly emerging regulatory T cells must arise from the $CD4^+ CD25^-$ population.

Additional evidence for peripheral induction of Tregs comes from studies with male mice that have been orchiectomized at birth and thymectomized as adults. *De novo* development of a mature prostate in these mice can be induced by treatment with dihydrotestosterone. Interestingly this newly developed organ does not become the subject of autoimmune attack. In fact, these mice concomitantly develop regulatory T cells that have the capacity to prevent autoimmune prostatitis in the d3Tx models.[45] In addition, various tolerance-inducing protocols using monoclonal antibodies against CD4, CD8, or co-stimulatory molecules can also induce regulatory T cells *in vivo*.[46–48]

EVIDENCE FOR PERIPHERAL GENERATION OF CD4⁺ CD25⁺ REGULATORY T CELLS FROM EXPERIMENTS *IN VITRO*

The ability to generate CD4⁺ CD25⁺ regulatory T cells from CD4⁺ CD25⁻ T cells *in vitro* would lend credence to the fact that they may not be a unique lineage that is derived from the thymus. The repeated stimulation of naïve cord blood cells with immature DC leads to the presence of a population that is suppressive, hyporesponsive and has a similar mechanism of suppression as CD4⁺ CD25⁺ cells (contact-dependent, antigen non-specific, antigen-presenting cell-independent, partially overcome by IL-2).[49] In addition, treatment with immunosuppressive drugs such as vitamin D3 in combination with mycophelate mofetil leads to the development of CD4⁺ CD25⁺ T cells *in vivo*.[50] On the other hand treatment with vitamin D3 and dexamethasone leads to the generation of IL-10-secreting regulatory T cells,[50,51] The relationship between these and CD4⁺ CD25⁺ T cells is not clear. This brings up a very important point that not all cells that exhibit regulatory function are CD4⁺ CD25⁺.[32]

In humans, it has been shown that clones of antigen-specific CD4⁺ T cells can be anergised by TCR stimulation without co-stimulation or by peptide presented by T cells to each other (T : T presentation).[52–54] This T : T presentation of specific peptide occurs because activated human T cells express major histocompatibility complex (MHC) class II.[55,56] Rat T cells also express MHC class II and anergy can be induced in T-cell clones from these animals by T : T presentation.[57,58] A fundamentally important observation is that once anergy is induced in human or rat T cells, they acquire the ability to suppress the proliferation of non-anergised cells.[24,52,57,59] Furthermore, the phenotype of anergised antigen-specific T cells is virtually identical to that of naturally occurring CD4⁺ CD25⁺ regulatory T cells (Table 1). The main point here is that CD4⁺ CD25⁺ T cells that are both anergic and suppressive can be generated from responsive, highly differentiated antigen-specific CD4⁺ CD25⁻ T cells. This indirectly supports the possibility that these cells may be generated in the periphery. It is of note that mouse T cells do not express MHC class II after activation and that peripheral generation of regulatory T cells by this route may not play an important role in this species.

WHERE DO HIGHLY DIFFERENTIATED HUMAN CD4⁺ CD25⁺ T CELLS COME FROM?

The TCR repertoire of CD4⁺ CD25⁺ regulatory T cells in adult humans is as diverse as that of CD4⁺ CD25⁻ T cells, indicating that they can recognize and suppress many different antigens.[24] This, together with their highly differentiated phenotype that is virtually identical to that of memory T cells (CD45RO⁺, CD45RB^low, short telomeres) suggests that they may be derived from a population that has encountered antigen extensively, not a unique lineage from the thymus. However, CD4⁺ CD25⁺ T cells that are anergic and suppressive are also found in human neonates in cord blood but these cells do not have a highly differentiated phenotype.[60,61] It is currently not clear if the CD4⁺ CD25⁺ cells that are found in adulthood are derived from the neonatal population. If this were the case, these regulatory cells would have to be stable over time and have the capacity to proliferate extensively, to acquire the characteristically short telomeres of this population. Two observations argue against this. First, these cells are susceptible to apoptosis induced by cytokine starvation. Even if their death is prevented by cytokines *in vivo*, they will be at a continuous disadvantage because of competition with activated responsive T cells that utilize and sequester the same cytokines for their own survival after immune responses.[62] Second, CD4⁺ CD25⁺ T cells are anergic and even if cytokines like IL-2 can temporarily break anergy, their relatively poor ability to proliferate will impede their persistence through competition with activated and responsive T cells for the same mediators.

Further work is essential to determine the relationship between neonatal and adult CD4⁺ CD25⁺ regulatory T cells. The relative expression of unique markers for regulatory T cells, such as FoxP3, may provide important clues to similarities or differences between them.[63,64] In view of the multitude of many new types of regulatory T cell that continue to be described,[65,66] the possibility that CD4⁺ CD25⁺ regulatory T cells in neonates and adults are generated by different mechanisms should not be ignored.

Table 1. Similarities between T-cell clones and CD4⁺ CD25⁺ T cells

CD4⁺ CD25⁺ T cells	CD4⁺ clones
Low CD45RB[24]	Low CD45RB[5,24]
Short telomeres[24]	Short telomeres[24]
Anergic[24]	Easily anergised[52,59]
Apoptosis prone[10]	Apoptosis prone[5]
High CTLA-4[10]	High CTLA-4*
CD25⁺	CD25⁺

*G. Lombardi, personal communication.

THE RELATIONSHIP BETWEEN ANERGY, APOPTOSIS AND SUPPRESSION IN CD4⁺ CD25⁺ REGULATORY T CELLS

The induction of anergy by various means in responsive antigen-specific T-cell clones *in vitro* is associated with extensive activation-induced apoptosis in a large proportion of these cells.[59,67] The cells that survive the apoptotic onslaught are anergic to a second round of stimulation with peptide presented by professional antigen-presenting cells.[68] The dose of antigen is crucial in the induction of either anergy, suppression, or death of the T cells.[69] However, anergy and apoptosis can be

dissociated because the addition of anti-CD95 antibody[67] and IFN-α/β prevents death but leads to the presence of increased numbers of anergic cells in culture.[59] This rescue by IFN-α/β is the only natural means by which CD95-mediated death can be prevented and has been suggested as a potential mechanism that promotes the persistence of anergic and suppressive T cells *in vivo*.[59] Mechanisms that prevent apoptosis may therefore be important both during the induction of anergy in potential T regulatory cells and also for the maintenance of these cells in the periphery.[59] It is of note that T cells that have been rendered anergic *in vitro* can act as suppressor cells both *in vitro* and *in vivo*.[70] The speculation about differences in how neonatal and adult CD4$^+$ CD25$^+$ T cells arise could be rephrased to how anergy may be induced in immature cells in the thymus and highly differentiated T cells in the periphery. The new focus of the question would then be what prevents deletion of the cells during anergy induction and whether the surviving cells are suppressive.

A MODEL FOR THE PERIPHERAL GENERATION OF ADULT CD4$^+$ CD25$^+$ REGULATORY T CELLS IN HUMANS

Based on the observations that are highlighted above, the following model for the peripheral generation of CD4$^+$ CD25$^+$ regulatory T cells in adult humans can be proposed (Fig. 1). After antigenic encounter, naïve T cells replicate to a highly differentiated population with the acquisition of the memory phenotype of such cells. Once these cells reach this highly differentiated state, they synthesize insufficient levels of IL-2 for autocrine proliferation and are easily anergised by high doses of antigen that is presented by non-professional antigen-presenting cells.[71,72] The deletion of these cells can be prevented by the type-Ir IFNs that enable the survival of an anergic and suppressive CD4$^+$ CD25$^+$ T-cell population.[59] This hypothetical mechanism would promote the generation of an anergic/regulatory CD4$^+$ T-cell population that is specific for antigens that are present continuously at high levels *in vivo*. This would include self- and dietary antigens, and also frequently encountered environmental antigens. This model is supported by a number of observations. These include the fact that memory CD4$^+$ T cells are more susceptible to anergy induction than their naïve counterparts.[71,72] Also, highly differentiated antigen-specific CD4$^+$ T cells that are CD45RB^{low} and have short telomeres can be rendered responsive or anergic/suppressive depending on how the antigen is presented.[24,59] In addition, if anergy is induced in these cells, they acquire the phenotype of regulatory CD4$^+$ T cells (CTLA4$^+$, CD25$^+$ G. Lombardi personal communication) and their functional characteristics, including loss of anergy in the presence of IL-2 but reversion to an anergic/suppressive state upon IL-2 removal.[52,57,69,73] This model does not preclude the coexistence of other populations of thymically or cytokine-dependent populations of Tregs.
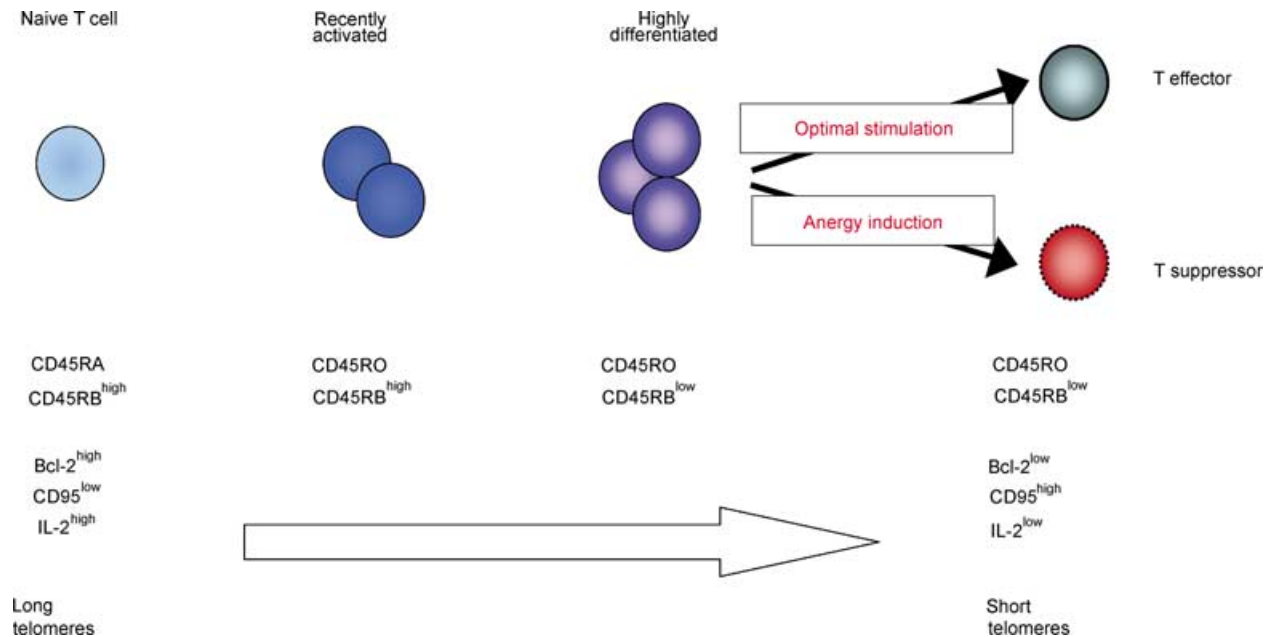

Figure 1. Model for the generation of CD4$^+$ CD25$^+$ regulatory T cells in the periphery. Naïve CD4$^+$ T cells become primed upon encounter with antigen on antigen-presenting cells, lose CD45RA and acquire CD45RO expression. These cells remain CD45RB^{high} during this process and retain the capacity to secrete large amounts of IL-2. They still express Bcl-2 and have long telomeres. Repeated stimulation of the primed cells by frequently encountered antigen leads to generation of highly differentiated CD4$^+$ CD45RO$^+$ CD45RB^{low} memory/effector T cells. These highly differentiated T cells have short telomeres and are prone to apoptosis, as they express low levels of Bcl-2. They also have a profoundly diminished ability to secrete IL-2, which makes them susceptible to anergy induction. These cells can either remain as effector T cells, or they can become anergised and acquire suppressive capacity.

CONCLUSION

The generation of apoptosis-prone regulatory cells makes sense for a number of reasons. First, this limits the capacity of an extensively stimulated population for survival and may protect against the development of malignancy. Second, only cells that have experienced extensive encounters with antigen can be induced to become regulatory. Furthermore, generation of regulatory CD4$^+$ T cells by this route of anergy induction has considerable implications for the maintenance of tolerance to self-antigens. These antigens may be presented by many cell types that do not have specialized antigen-presenting cell function and would thus promote the development of anergy and suppressive activity in self-reactive populations. The available data fit this model. We await further evidence to determine if this may be a way to generate CD4$^+$ CD25$^+$ regulatory T cells in humans *in vivo*.

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