T cell tolerance induced by therapeutic antibodies

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Ever since the discovery of Medawar, over 50 years ago, that immunological tolerance was an acquired phenomenon that could be manipulated in neonatal mice, the ability to induce therapeutic tolerance against autoantigens, allergens and organ grafts has been a major driving force in immunology. Within the last 20 years we have found that a brief treatment with monoclonal antibodies that block certain functional molecules on the surface of the T cell is able to reprogramme the established immune repertoire of the adult mouse, allowing indefinite acceptance of allografts or effective curing of autoimmune diseases. We are only now just beginning to define many of the regulatory mechanisms that induce and maintain the tolerant state with the aim of being able to safely and reliably apply these technologies to human clinical situations.

Keywords: monoclonal antibody; tolerance; regulatory T cell; transplantation; CD4 antibodies

1. INTRODUCTION

The immune system develops tolerance to self-antigens early in life, primarily through the process of deleting self-reactive T cell clones in the thymus. This means that in order to impose tolerance in the adult to new antigens, such as those on an allograft, it is necessary either to ablate the entire immune system and attempt to recapitulate development with presentation of the new antigens in the thymus with a fresh source of haemopoietic stem cells, or to find a means to reprogramme the peripheral T cell repertoire *in situ*. The development of monoclonal antibodies that can deplete or modulate T cell function *in vivo* have made both of these routes to tolerance a practical possibility. Monoclonal antibodies that could deplete either CD4⁺ or CD8⁺ T cells in mice became available in the 1980s (Cobbold *et al.* 1984) and were found to be able to suppress the rejection of allogeneic skin or bone marrow grafts (Cobbold *et al.* 1986). While T cell depletion strategies of immunosuppression are still practically useful in clinical bone marrow (Hale *et al.* 2001) and organ transplantation to this day (Calne *et al.* 2000), it was the discovery that a brief treatment with non-depleting CD4 antibodies could induce a permanent state of antigen specific tolerance in mice (Qin *et al.* 1990) that has provided a potential route to true therapeutic reprogramming of the adult immune system.

The discovery that induction of tolerance was possible in mice given a foreign protein, such as human IgG, under the cover of monoclonal anti-CD4 antibody (Benjamin *et al.* 1986), led to many attempts to use such antibodies clinically as potential immunosuppressive agents in organ transplantation and autoimmunity. Most of these early attempts failed, mainly

because we did not understand enough about the mechanisms of action and pharmacokinetics of the anti-CD4 antibodies used. For example, we now know that it is important that the antibodies should be used at sufficient doses to maintain saturation of the CD4 expressed on T cells for at least three weeks (in mice) without eliciting any depletion of the CD4⁺ T cells (Scully *et al.* 1994) and without the induction of any neutralizing antiglobulin response. We also now know that successful reprogramming for tolerance depends on the sufficient generation of antigen specific regulatory T cells (Chen *et al.* 1996a) that are able to control any residual effector cells, and that concomitant treatment with many conventional immunosuppressive drugs can inhibit regulatory T cell development (Kirk *et al.* 1999; Smiley *et al.* 2000). More recently, it has been shown that a humanized, non-depleting aglycosyl anti-CD4 antibody can be used, at doses equivalent to those used in mice, to induce tolerance to equine IgG in baboons (Winsor-Hines *et al.* 2004), demonstrating that the general principle of immune reprogramming by coreceptor blockade is not restricted to rodent models.

Although antibodies against CD4 were the first to be found capable of inducing tolerance to protein antigens, it has become clear that many other antibody specificities are capable, either when used alone or in combinations, of reprogramming the immune system (Waldmann & Cobbold 2001), as shown in table 1. While non-depleting CD4 antibody used alone is sufficient to achieve tolerance to long-lived protein antigens, such as foreign IgG, it was found to be essential to combine this with anti-CD8 antibodies to achieve reliable tolerance to skin grafts (Qin *et al.* 1990). Other specificities that have been shown to induce tolerance to organ or tissue grafts include CD154 (CD40L) that may be used in combination with CTLA4-Ig (Zheng *et al.* 1999), CD11a plus ICAM-1 (Ohta *et al.* 1998), CD2 (Sido *et al.* 1996), CD45RB (Sido *et al.* 1996) and non-mitogenic CD3

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Table 1. Monoclonal antibodies that can induce operational immune tolerance.

antibody specificity	successful applications	limitations	mechanism of action	references
CD4 (depleting)	proteins; MHC mismatched hearts (with DST)	ineffective for skin grafts (minors or MHC)	depletion of CD4 ⁺ T cells: tolerance to mAb	Benjamin <i>et al.</i> (1986) and Bushell <i>et al.</i> (1994)
CD4 (non-depleting)	heart grafts plus DST	ineffective for most skin grafts	induction of CD4 ⁺ CD25 ⁺ Treg	Saitovitch <i>et al.</i> (1995)
CD4 + CD8 (non-depleting)	minors or MHC mismatched skin, heart, marrow	less effective in black background mice strains	induction of regulatory T cells (foxP3 ⁺ and Tr1)	Cobbold <i>et al.</i> (1996)
CD40L	MHC mismatched heart grafts in rodents and primates	ineffective after priming or homeostatic expansion: thromboembolic toxicity	depletion of activated T cells and costimulation blockade	Blair <i>et al.</i> (2000)
CD40L + hCTLA4-Ig	MHC mismatched heart grafts in rodents and primates	ineffective after priming or homeostatic expansion: thromboembolic toxicity	depletion of activated T cells and costimulation blockade	Kirk <i>et al.</i> (1997)
hCTLA4-Ig	autoimmune disease models and human trials	ineffective after priming or homeostatic expansion: thromboembolic toxicity	costimulation blockade and induction of IDO	Goronzy & Weyand (2004)
CD3 (OKT3)	prophylaxis and treatment of acute renal rejection in humans	cytokine release syndrome, immunosuppression not tolerance	T cell depletion, TCR modulation	Smith (1996)
CD3 (non-mitogenic)	treatment of diabetes in NOD mouse and human trials	risk of EBV lymphoproliferation, some mild cytokine release	induction of TGF- β dependent CD4 ⁺ CD25 ⁺ Treg	Herold <i>et al.</i> (2002)
CD52 (CAMPATH)	prophylaxis of acute renal rejection in humans (prope tolerance)	long term T cell depletion and immunosuppression	depletion of lymphocytes	Calne <i>et al.</i> (2000)
CD80/CD86	proteins, SRBC, heart grafts in primates	ineffective in minors skin grafts	costimulation blockade (and IDO?)	Kirk <i>et al.</i> (2001)
CD2 (CD2R)	neonatal/xeno heart grafts	ineffective in minors skin grafts	immune suppression	Sido <i>et al.</i> (1996)
CD25	prophylaxis and treatment of acute renal rejection in humans	no evidence for tolerance induction, may theoretically deplete Treg cells	depletion or proliferation block of activated T cells	Sollinger <i>et al.</i> (2001)
CD45RB	heart allografts, islet xenografts	not known	depletion of effectors but not Treg cells	Luke <i>et al.</i> (2003)
CD28	chronic renal rejection in rats; GVHD in mouse	ineffective in minors skin grafts	costimulation blockade	Laskowski <i>et al.</i> (2002)
CD200R	induction of tolerogenic DC <i>in vitro</i> ; collagen induce arthritis <i>in vivo</i>	not known	modulation of APC function	Gorczyński <i>et al.</i> (2004)

antibodies (Chatenoud 2003). The fact that so many different antibody specificities are able to promote tolerance induction suggests that the mechanism may not be related to the individual functions of the target molecules on the T cell or antigen presenting cell surface, but more to a predetermined default response of T cells when they are subjected to non-optimal signalling through the immune synapse (Dustin 2004).

2. TOLERANCE DEPENDENT ON REGULATORY T CELLS

There are three types of experiment that have aimed to demonstrate that non-optimal or chronic stimulation with antigen induces tolerance and regulatory T cells.

The first example makes use of our knowledge of how the T cell receptor interacts with the specific antigen peptide being presented by the MHC-II molecule to design variant antigen peptides with conservative amino acid substitutions at T cell receptor contact sites (Chen *et al.* 1996b). These altered peptide ligands (APL) can modify the response of antigen specific T cells and selected variants have been found to act as either weak agonists or even antagonists when compared to the original agonist peptide. Altered peptide ligands of autoantigens have been demonstrated to modulate autoimmune disease induction in mouse models (Nicholson *et al.* 1995; Paas-Rozner *et al.* 2003), and variants of the male transplantation

antigen DBY peptide presented by H-2E^k can induce tolerance to male skin grafts in TCR transgenic recipients (Chen *et al.* 2004b). In the latter case, the APL was found to induce some deletion of antigen reactive T cells, but also both foxP3 positive CD4⁺CD25⁺ regulatory T cells and Tr1-like cells producing IL-10.

The second example of chronic antigen stimulation is one in which TCR transgenic anti-male (DBY) T cells were adoptively transferred into male RAG knockout recipients (Chen *et al.* 2004a). In this example the T cells in the recipient were unable to avoid their specific antigen and one would have expected graft versus host disease (GVHD) as the outcome. Surprisingly, none of the male recipients of male specific T cells developed any long term signs of GVHD and it was found that all the T cells had become profoundly anergic when tested for proliferation *in vitro*. Further to this, the T cells were able to completely suppress the proliferation and IL-2 secretion of naïve T cells in the conventional *in vitro* suppression assay. The phenotype of these anergic and regulatory T cells was found to be CD4⁺CTLA4⁺ but they were negative for foxP3, CD25 and IL-10 production.

The third example of chronic stimulation leading to tolerance and regulatory T cells is by administering antigen peptide continuously to either TCR transgenic or normal mice subcutaneously via an osmotic pump (Apostolou & von Boehmer 2004). Dominant tolerance could be induced in normal mice to the intact antigen by administration of a single peptide and this was dependent on the generation of CD4⁺CD25⁺ regulatory T cells that appeared indistinguishable from the natural CD4⁺CD25⁺ Treg cells.

The examples above demonstrate that tolerance can be generated by chronic or non-optimal antigen stimulation and that the outcome is dependent on the generation of regulatory T cells, but that, surprisingly, different phenotypes of regulatory T cells seem to be involved, even when the same transgenic TCR is considered. It also raises the question of how anergic or apparently unresponsive T cells can have suppressive properties, at least in the *in vitro* assays. This phenomenon was originally observed in the tolerance of Vβ6 T cells to the MLS-1a antigen after transplantation of spleen or bone marrow (Qin *et al.* 1989) and the 'civil service model' of passive competition for antigen presentation and cytokines was proposed (Waldmann & Cobbold 2001). A number of experiments have been published to suggest that anergic T cells can both compete for antigen presentation and IL-2 (Lombardi *et al.* 1994; Barthlott *et al.* 2003), and that they also can down-modulate antigen presenting cell function (Taams & Wauben 2000; Lechler *et al.* 2001) in common with other types of regulatory T cells.

It now seems that there are many different types of T cells with regulatory capacity (figure 1) and it is not yet clear how or whether these are related to each other in terms of their origin, the signals that generate them, and their mechanisms of action. The so called 'natural' Treg cells that are generally identified by the co-expression of CD4 and CD25 (Sakaguchi *et al.* 1995; Thornton & Shevach 1998) are thought to be

generated primarily in the thymus (Sakaguchi *et al.* 2001) and seem to be biased towards recognition of agonistic, high avidity self-peptides (Jordan *et al.* 2001), although it is not yet clear if this is as a result of positive selection or as a resistance to clonal deletion (Lerman *et al.* 2004; van Santen *et al.* 2004). These natural Treg cells remain CD62L high (Chatenoud *et al.* 2001), populate the peripheral lymphoid system, and are thought to be involved in controlling lymphocyte homeostasis (Annacker *et al.* 2001) and innate immunity (Maloy *et al.* 2003). It has been demonstrated that the transcription factor foxP3 is essential for the generation of these Treg and that human or mice that lack foxP3 function develop an autoimmune pathology involving uncontrolled T cell proliferation in the gut and endocrine tissues (Bennett *et al.* 2001; Brunkow *et al.* 2001). The evidence that foxP3 is a master gene determining Treg function is that viral transduction of the gene into naïve T cells converts them both phenotypically and functionally into regulatory T cells (Fontenot *et al.* 2003; Hori *et al.* 2003; Khattry *et al.* 2003).

While it is still not clear what the natural mechanism is in the thymus for specifying the differentiation of thymocytes into Treg cells, it now seems that exposing naïve, peripheral T cells to TGF-β during their activation by antigen can also induce foxP3 and regulatory function (Chen *et al.* 2003; Fantini *et al.* 2004; Fu *et al.* 2004; Park *et al.* 2004). This is intriguing because in, for example, the mouse models of autoimmune colitis, the tolerance induced by regulatory T cells can be broken by the administration of neutralizing antibodies to TGF-β, suggesting this cytokine plays a role in the activity of Treg cells as well as their induction (Powrie *et al.* 1996). Most recently, it has also been shown that non-depleting CD4 antibodies that induce transplantation tolerance are also able to induce foxP3 expression during exposure to graft antigens, and this is also TGF-β dependent (Cobbold *et al.* 2004). While the source of active TGF-β in these systems remains to be defined, it is of interest to note that a subset of differentiated CD4⁺ memory T cells, termed Th3 cells (Fukaura *et al.* 1996; Weiner 2001), have been shown to act as regulatory T cells in, for example, tolerance to alloantigens presented to the immune system via the anterior chamber of the eye (Kosiewicz *et al.* 1998). While there is considerable evidence that natural Treg cells can play a role in transplantation tolerance, particularly in adoptive transfer models (Wood & Sakaguchi 2003; Graca *et al.* 2004), it is therefore becoming clear that other regulatory T cells, in some cases lacking either CD25 or foxP3, are also involved (Graca *et al.* 2002; Chen *et al.* 2004a,b).

Another type of differentiated, or elicited, CD4⁺ regulatory T cell is the Tr1 cell that is defined primarily because it is dependent both for its generation and much of its suppressive function on the cytokine IL-10 (Groux *et al.* 1997; Roncarolo *et al.* 2001). Tr1 cells do not express the foxP3 gene (Cobbold *et al.* 2003a,b), although they may express some of the other surface markers associated with regulatory T cells such as CD25, CTLA4, GITR and CD103 (Zelenika *et al.* 2002; Cobbold *et al.* 2003a,b). The overall pattern of

Figure 1. Different types of regulatory T cells. The four panels depict the four main types of regulatory T cells that have been characterized so far on the left of each panel, together with an indication of their interaction with antigen presenting cells (APC) on the right. (a) Natural regulatory T cells (Treg) are $CD4^+CD25^+foxP3^+$ cells that are associated with TGF- β for their generation and some of their functions. They can down-modulate APC by CTLA4 cross-linking of CD80/86 molecules, the induction of indoleamine dioxygenase (IDO) and the catabolism of tryptophan. Th3 cells that make predominantly TGF- β may be similar to these Treg. (b) Anergic T cells are associated with T cell activation in the absence of costimulation and are hyporesponsive to antigen due to the induction of the E3-ubiquitin ligases GRAIL, Itch and cbl-b that cause degradation of the T cell receptor signalling pathway. Anergic cells may suppress by competing for IL-2 and costimulation. (c) Tr1 cells are dependent on IL-10 for their generation, and some of their suppressive functions. They can also modulate the APC via CTLA4, CD80/86 and IDO. (d) The $CD8^+CD28^-foxP3^+$ suppressor T cell is also associated with an IL-10 rich environment and is able, at least in the human system, to modulate the dendritic antigen presenting cell to express the molecules ILT3 and ILT4 that present to further T cells to induce anergy and $CD4^+CD25^+$ Treg cells.

gene expression of Tr1 cells seems to be similar to Th2 cells, although resting Tr1 cells seem to lack a number of Th2 associated genes such as GATA-3, Egr-1, ST2L and IL-4, and only very few genes such as the repressor or GATA (ROG; Zelenika *et al.* 2002) and leukaemia inhibitory factor (LIF; Metcalfe *et al.* 2005) seem to be overexpressed specifically in Tr1 cells. After strong activation with either mature dendritic cells (DC) or CD3 cross-linking, Tr1 cells can express IL-4 and IL-5 cytokines, and appear genetically even more similar to Th2 cells (Cobbold *et al.* 2003a,b), suggesting that the Tr1 cell is perhaps an anergic or partially differentiated Th2. The main functional difference is that while Th2 cells are perfectly capable of rejecting skin grafts rapidly after adoptive transfer to T cell depleted or RAG deficient recipients, Tr1 cells fail to reject such grafts and will furthermore suppress rejection by either Th2 or Th1 cells (Zelenika *et al.* 2002; Cobbold *et al.* 2003a,b).

There are also other types of regulatory T cells besides the natural Treg, Th3 and Tr1 populations. These include subpopulations of Th1, Th2 (Stock *et al.* 2004) and $CD8^+$ T cells that express the foxP3 gene (Cosmi *et al.* 2003; Manavalan *et al.* 2004), as well as more distantly related cells such as NKT cells. The latter population has been particularly implicated in the regulation of autoimmunity in the NOD mouse model of type I diabetes (Ikehara *et al.* 2000; Sharif *et al.* 2002) and is thought to act early in the response by producing cytokines such as IL-4 that deviate the later T cell response from Th1 to Th2. The role, if any, of these, as yet, less well studied regulatory populations in tolerance induced by monoclonal antibodies remains to be determined.

3. CORECEPTOR BLOCKADE FOR TRANSPLANTATION TOLERANCE

Although the ability to induce tolerance was initially associated with the use of single monoclonal antibodies against CD4 this was soon found to be limited to certain protein antigens and the induction phase of certain autoimmune disease models. Most attempts to use CD4 antibodies alone to induce tolerance to skin or heart allografts gave enhanced graft survival, with gradual graft loss once the antibody was cleared. Experiments with combinations of depleting CD4 and CD8 antibodies soon showed that either subset could rapidly reject bone marrow or skin allografts (Cobbold & Waldmann 1986), and that many of the autoimmune diseases also involved both $CD4^+$ and $CD8^+$ T cells once they were established (Kantwerk *et al.* 1987; Hayward *et al.* 1988). Therefore, a number of ways to additionally target the CD8 component of the response have evolved, including giving donor specific transfusions (Pearson *et al.* 1990) or bone marrow to enhance the deletion of $CD8^+$ T cells while they are blocked from $CD4^+$ help (Seung *et al.* 2003), using initial T cell or lymphocyte depletion, or by adding CD8 antibodies to deplete or block the CD8 response (Honey *et al.* 1999). Such approaches were found to allow the induction of indefinite graft survival and tolerance to skin, heart or bone marrow even across fully MHC mismatched donors (as defined by the acceptance of a second donor type graft but rejection of a third party graft indicating the absence of non-specific immunosuppression). It is worth noting that tolerance could even be induced in primed recipients by giving initial CD4 plus CD8 depleting antibodies and then continuing with non-depleting CD4 plus

CD8 blockade for a period of three further weeks (Cobbold *et al.* 1990). It is interesting to speculate whether this allowed co-receptor independent expansion of the regulatory T cell population while the CD4 and CD8 antibodies blocked the homeostatic expansion of residual naïve and memory T cells that would normally be a barrier to tolerance induction in other protocols (Wu *et al.* 2004).

The first really powerful evidence of immune regulation was observed in the mouse models of skin graft tolerance induced by non-depleting CD4 and CD8 antibodies as two related phenomena. The first was that of linked suppression (Davies *et al.* 1996), where A-type mice tolerant of B-type skin grafts would reject C-type third party grafts, demonstrating specificity, but would often accept (B×C)_{F1} skin as an indication that regulatory processes targeted to B antigens could override rejection of C antigens if they were expressed on the same tissue or antigen presenting cell. Furthermore, the individuals that failed to reject the (B×C)_{F1} skin were later fully tolerant of pure C-type grafts, suggesting the tolerance had spread to the third party specific T cells. It was further demonstrated that the regulatory and tolerant phenotype could be transferred from one population of CD4⁺ T cells to another via the process termed infectious tolerance (Qin *et al.* 1993). The tolerant and regulatory state of the immune system was therefore self-sustaining in the continued presence of antigen, with any new T cells generated by the thymus (or artificially introduced from outside) also becoming tolerant. This whole process has generally been called dominant tolerance. Much of the recent work in this area has been to determine which regulatory T cell and antigen presenting cell populations play an important role in generating and maintaining dominant tolerance and how different therapeutic manipulations can generate or break the tolerant state.

Previous experiments had suggested that such CD4 treatment *in vitro* could make T cells anergic to restimulation (Vincent *et al.* 1995; Woods *et al.* 1998), but there had been no clear indication of any mechanism or direct link to regulation. A major step forward has been the discovery that CD4 blockade during T cell activation by cognate antigen can induce the expression of the regulatory master gene foxP3 (Cobbold *et al.* 2004), both *in vitro* and *in vivo*, and that in both cases this is dependent on the presence of active TGF-β. As indicated previously, TGF-β has been strongly implicated in both the differentiation and suppressive function of natural CD4⁺CD25⁺ Treg cells (Nakamura *et al.* 2001; Yamagiwa *et al.* 2001; Chen & Wahl 2003), although some of the details, such as whether Treg themselves are required to express TGF-β, remain controversial (Piccirillo *et al.* 2002; Gregg *et al.* 2004; Nakamura *et al.* 2004; Tang *et al.* 2004; Wahl *et al.* 2004). Furthermore, the addition of active TGF-β1 or TGF-β2 can induce foxP3 during activation by antigen or CD3 plus CD28 stimulation *in vitro* of naïve CD4⁺CD25⁻ T cells and such cultures generate regulatory T cells that are active both in standard suppression of proliferation assays and after adoptive transfer in autoimmune models *in vivo* (Chen *et al.* 2003; Fu *et al.* 2004).

Figure 2. Blocking CD4 induces foxP3 and dominant tolerance to grafted tissue. Under normal circumstances a foreign tissue graft will stimulate alloantigen reactive CD4⁺ T cells to generate activated and effector T cells that lead to graft destruction. In the presence of non-depleting, but blocking, monoclonal antibodies to CD4, a proportion of the T cells express the regulatory master gene foxP3 in a TGF-β dependent manner. These foxP3⁺ T cells not only develop the ability to suppress both the activation and differentiation of naïve T cells, but also seem to promote the generation of IL-10 dependent Tr1 cells. The foxP3⁺ and Tr1 cells are both found at the site of the tissue graft and probably act together to maintain robust dominant tolerance of the graft.

Although these observations have now been confirmed by many groups, there is as yet no published evidence for a direct link between TGF-β signalling through the SMAD pathway and the control elements of the foxP3 promoter, although there is data to suggest that down-regulation of the SMAD7 inhibitor of TGF-β signalling may be involved (Fantini *et al.* 2004). In addition, although the induction of tolerance by coreceptor blockade *in vivo* is blocked by neutralizing TGF-β, it has generally not been possible to break established tolerance in this way, indicating that the actions of TGF-β alone are not sufficient to explain the state of dominant and infectious tolerance.

TGF-β has also been implicated in the differentiation of Tr1 regulatory cells (Roncarolo *et al.* 2001) and, as has already been discussed, Tr1 cells have been shown capable of suppressing both Th1 and Th2 mediated skin graft rejection (Cobbold *et al.* 2003a,b). The tolerant state induced by the combination of partial CD4 depletion together with donor specific transfusion (DST) also seems to depend on both IL-10 and CTLA4 (Kingsley *et al.* 2002) that may be indicative of Tr1 activity. Furthermore, in the DBY specific transgenic TCR model of tolerance induction by anti-CD4 there is evidence that RNA transcripts for both foxP3 (as a marker for CD4⁺CD25⁺ Treg) and repressor of GATA (ROG as a marker for Tr1 activity) are highly enriched in tolerated male skin grafts (Cobbold *et al.* 2004). It seems likely that the most robust form of transplantation tolerance induced by coreceptor blockade is maintained by a combination of CD4⁺CD25⁺ Treg and Tr1 cells (figure 2). It remains to be determined whether other types of regulatory populations may also play a role in this situation.

4. COSTIMULATION BLOCKADE FOR TOLERANCE INDUCTION

While coreceptor blockade is, in our hands, the most potent and robust method of inducing transplantation tolerance in mice, it has never been tested in the clinical setting for reasons that are more to do with commercial perceptions rather than scientific validity. Costimulation blockade, using reagents that target either the CD40L (CD154) or CD28 pathways (Pearson *et al.* 1994; Larsen *et al.* 1996; Salomon & Bluestone 2001), however, have for some reason enjoyed a much higher profile, not only in the mouse but also in pre-clinical primate studies (Kirk *et al.* 1997, 2001; Krieger *et al.* 1998; Ossevoort *et al.* 1998; Blair *et al.* 2000) and experimental human therapies (Kalunian *et al.* 2002; Elster *et al.* 2004; Goronzy & Weyand 2004). Trials with CTLA4-Ig (or LEA29Y that has a higher affinity for CD80/CD86) treatment in autoimmune diseases are still ongoing (Moreland *et al.* 2002), but blocking this pathway has often not been effective in rodent models of established disease (Rossini *et al.* 2001). Similarly, it is now recognized that costimulation blockade is not effective in a primed immune system or after T cells have been allowed to expand homeostatically (Wu *et al.* 2004). Preclinical trials in primates demonstrated that costimulation blockade with monoclonal antibodies to CD154 was able to induce indefinite survival of allogeneic renal allografts (Kirk *et al.* 1997). Clinical trials with current generation antibodies to CD154 have, however, found an unexpectedly high rate of thromboembolic complications (Kawai *et al.* 2000), as the target antigen is also expressed on platelets (Sidiropoulos & Boumpas 2004), and this effectively blocks their current use as a practical therapy.

In an experimental setting there is still considerable use of costimulation blockade induced tolerance as a means to identify mechanisms of tolerance and immune regulation. While antibodies to CD154 alone can be effective at maintaining long term graft survival in weaker rejection systems, the antibodies are usually combined either with anti-CD8 (Honey *et al.* 1999), CTLA4-Ig or a donor specific cell transfusion (DST; Zheng *et al.* 1999) that seems to provide a more robust tolerance induction and generation of regulatory T cells. Once tolerance has been established by one of these methods it seems that all the phenomena of linked suppression and infectious tolerance as described above are once again observed (Honey *et al.* 1999; Graca *et al.* 2000). The induction of CD4⁺CD25⁺ Treg cells has been described in both transplantation and autoimmunity models (Salomon & Bluestone 2001).

In the case of anti-CD154 plus DST it seems there is an increased activation induced T cell death and effective clonal deletion of CD8⁺ T cells that are otherwise resistant to costimulation blockade alone (Iwakoshi *et al.* 2000; Jones *et al.* 2002). The effects on CD4⁺ T cells are also still somewhat controversial as it seems that the MR1 hamster anti-mouse CD154 generally used is capable of either blocking costimulation (Nagelkerken *et al.* 2004) or depleting activated CD154 expressing T cells (Monk *et al.* 2003), possibly depending on the dose and timing of antibody

administration. If it is working primarily by depleting T cells that are activated by specific antigen (Hargreaves *et al.* 2004) in a process more related to clonal deletion rather than the induction of regulatory T cells, it may still be useful as an adjunct to conventional immunosuppressive agents in the same way that the clinically licensed anti-CD25 antibodies (e.g. daclizumab, basiliximab) are used in human transplantation (Waldmann & O'Shea 1998; Bumgardner *et al.* 2001; Sollinger *et al.* 2001; Adu *et al.* 2003). Monoclonal antibodies that deplete either CD25⁺ or indeed CD4⁺ T cells in mice, however, while immunosuppressive, have generally been found to deplete regulatory T cells (Onizuka *et al.* 1999; Dubois *et al.* 2003) and thereby inhibit the induction of dominant tolerance. It may therefore be important to avoid depletion by anti-CD154 when it is used in tolerogenic protocols by genetically eliminating the function of the Ig-Fc.

The apparent synergy between anti-CD154 treatment and DST may be extended to the classical use of bone marrow transplantation (BMT) as a route to tolerance through mixed chimerism (Tomita *et al.* 1996). The problem with conventional BMT in the context of organ grafting is the toxicity of the recipient conditioning that is required to make 'space' for the donor marrow to engraft. While depletion of the recipients' own immune system with antibodies to CD4, CD8 (Cobbold *et al.* 1986) or CAMPATH-1 (Hale *et al.* 2001) can improve engraftment and reduce the intensity of conditioning required, some reduction in the myeloid compartment is generally still needed. The use of anti-CD154, however, does seem to allow sufficient engraftment of donor stem cells, particularly if they are given in 'megadoses' (Reisner & Martelli 2000), in the absence of myeloablation to induce a state of mixed chimerism and tolerance (Wekerle *et al.* 1999). Recent, as yet unpublished data suggest that the combination of non-depleting CD4, CD8 and CD154 antibodies allow the induction of such chimerism and tolerance with normal, clinically relevant doses of donor marrow (Graca *et al.* submitted). Tolerance via mixed chimerism has generally been associated with clonal deletion (Tomita *et al.* 1996) and an absence of regulatory T cells, most clearly observed as an absence of linked suppression (Graca *et al.* 2004), but experiments using different doses of bone marrow to induce tolerance to the MLS-1a antigen indicate that while high doses of donor cells were associated with deletion of recipient Vβ6 T cells, very low doses given under cover of non-depleting CD4 and CD8 mAbs could induce anergy and regulation (Bemelman *et al.* 1998). It is likely that in a clinical setting, where there may be an issue of tolerance resistance due to heterologous priming (Taylor *et al.* 2004), both clonal deletion and the induction of potent regulation will be required.

5. MONOCLONAL CD3 ANTIBODIES

A major commercial barrier to the clinical exploitation of either coreceptor or costimulation blockade is that both systems require combinations of more than one novel reagent to be truly effective. Such combinations can really only in practice be developed once the

individual components have been proven safe and effective in the clinic, and as we have seen this is not yet the case for any one of CD4, CD8, CD154 or CTLA4-Ig. One monoclonal antibody that has, however, been approved for use as an immunosuppressive agent in transplantation is OKT3 that recognizes the CD3 ϵ chain of the T cell receptor (Smith 1996). While this particular antibody has shown no specific evidence of a tolerogenic activity, and is generally considered to have too toxic side effects due to mitogenic T cell activation for wider application, the ability to target all T cells in a single agent is appealing. In addition, experiments using a diphtheria toxin immunoconjugate of a CD3 antibody to deplete 3 logs of T cells *in vivo* were found to induce long term survival of renal allografts in rhesus monkeys (Armstrong *et al.* 1998). This observation renewed the clinical interest in pan-T cell or lymphocyte depletion (e.g. with CAMPATH1) as an adjunct to reducing conventional immunosuppressive drugs (Knechtle *et al.* 2004) as a route to an operational or 'prope' tolerance (Calne *et al.* 1998).

More recently, second generation, non-mitogenic antibodies to CD3 have been developed (Bolt *et al.* 1993; Plain *et al.* 1999; Meijer *et al.* 2003) and these are less prone to causing the toxic cytokine release syndrome (Vossen *et al.* 1995). A non-Fc binding and humanized variant of OKT3 (OKT3 γ 1Ala-Ala; Herold *et al.* 2003) has been tested in patients with psoriatic arthritis to some effect (Utset *et al.* 2002) and more impressively it was found to reduce the needs for insulin 12 months after a brief treatment of newly diagnosed type I diabetics (Herold *et al.* 2002). Non-mitogenic anti-CD3 antibodies have previously been shown effective at treating diabetes even after the onset of symptoms in the NOD mouse model (Chatenoud 2003) and this has been shown to be due to the TGF- β dependent function of foxP3 positive CD4⁺CD25⁺CD62L⁺ regulatory T cells (Belghith *et al.* 2003; You *et al.* 2004). This suggests that under appropriate conditions non-mitogenic anti-CD3 antibodies may be as effective as, and generate a state of tolerance that is similar to, that obtained by coreceptor or costimulatory blockade.

6. INDUCED IMMUNE PRIVILEGE

While CTLA4-Ig has also been considered as a means to achieve blockade of the CD28 costimulatory pathway by competing for CD80/CD86 ligands, it now turns out that it also may have an alternative mechanism of action through induction of indoleamine dioxygenase (IDO) in the antigen presenting cell (Mellor *et al.* 2003). IDO is an enzyme that catabolizes tryptophan and it has been shown that CD8⁺ T cells in particular absolutely require a source of this amino acid to proliferate and survive (Lee *et al.* 2002). The kynurenine metabolites of tryptophan also seem to be toxic to T cells (Terness *et al.* 2002). This means that IDO activity by either dendritic cells or macrophages generates a local environment that is non-permissive for normal T cell responses to antigen. This mechanism seems to be important in pregnancy to avoid rejection of the semi-allogeneic foetus (Munn *et al.* 1998) and appears to be regulated via the expression of

CTLA4 on the natural CD4⁺CD25⁺ Treg cells (Aluvihare *et al.* 2004) that activates the IDO in antigen presenting cells (APC) through ligation of CD80 and CD86 (Aluvihare *et al.* 2004). Tr1 cells have also been shown to express surface CTLA4 and these can also act to suppress CD8⁺ T cell responses via the IDO pathway (Mellor *et al.* 2004). This mechanism may be one of a number of examples where regulatory T cells are able to modulate the activity of APC and this probably represents an important component of the phenomena of linked suppression and infectious tolerance. Regulatory T cells may similarly induce target tissues to protect themselves from immune attack, in effect generating a local, induced state of immune privilege (Ferguson *et al.* 2002). Examples of gene products that have been implicated in such protection are FasL (Green & Ferguson 2001), PDL-1 (Nishimura & Honjo 2001; Dong & Chen 2003; Gao *et al.* 2003; Aramaki *et al.* 2004), and haemoxygenase (Ke *et al.* 2000), and it is possible that antibodies or other agents that provoke this protection response in tissues may find a role in future tolerogenic therapies.

7. CONCLUSIONS

Monoclonal antibodies have provided us with a powerful tool to manipulate the immune system in general, and have proven to be powerful tolerogenic agents in rodent models. While we have so far failed to successfully translate their use to the clinic for the effective treatment of autoimmune disease or the induction of tolerance to organ grafts, we are now beginning to understand some of the cellular interactions and molecular mechanisms that are involved and hence some of the reasons why we may have so far failed in the clinic. We have learned that we not only have to use monoclonal antibodies that are non-toxic and preferably non-T cell depleting, but that we have to generate sufficient numbers and possibly heterogeneity of regulatory T cells to overcome clinical issues such as heterologous immunity and memory. With this knowledge we are already starting to trial second generation, humanized Fc mutated and higher affinity antibodies or Ig fusion proteins in ways that may allow for true tolerance induction rather than long term immunosuppression.

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