Axotrophin and leukaemia inhibitory factor (LIF) in transplantation tolerance

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Immune self-tolerance is controlled by a subset of T lymphocytes that are regulatory (Treg) and epigenetically programmed to suppress auto-reactive immune effector cells *in vivo*. By extrapolation, donor-specific transplantation tolerance might be controlled by donor-specific Treg that have acquired the appropriate epigenetic program for tolerance. Although such tolerance has yet to be achieved in man, proof of concept comes from mouse models where regulatory transplantation tolerance can be induced within the complex micro-environment of the spleen or draining lymph node. By studying whole spleen cell populations in a murine model of transplantation tolerance we have incorporated a complexity of environmental factors when looking for specific features that characterize tolerance versus aggression. This approach has revealed unexpected patterns of gene activity in tolerance and most notably that a novel stem cell gene, *axotrophin*, regulates T lymphocyte responsiveness both in terms of proliferation and in release of leukaemia inhibitory factor (LIF). Since LIF is a regulator of stem cells in addition to being a key neuropoietic cytokine, these preliminary results linking both axotrophin and LIF to transplantation tolerance lead us to propose that regulatory pathways encoded during the epigenetic development of Treg cells are related to pathways that regulate fate determination of stem cells.

Keywords: axotrophin; leukaemia inhibitory factor; transplantation tolerance; stem cells

1. INTRODUCTION

The immune response is subject to fate determination signals that ensure protective tolerance towards self-tissues while simultaneously being capable of aggressive attack towards foreign pathogens. Specificity of a given response is driven by antigen while the phenotypic fate of the responding lymphocyte is guided by micro-environmental cues to result in either aggression or tolerance. Self-tolerance is regulated at several levels, including apoptotic deletion of high-affinity self-reactive T cells upon engagement of antigen presented by thymic stromal cells (Venanzi *et al.* 2004), a process that requires induction of the pro-apoptotic gene Bim (Liston *et al.* 2004b) and includes promiscuous thymic expression of organ specific antigens such as insulin or thyroglobulin (Liston *et al.* 2003). Those self-reactive cells that escape thymic deletion may become anergized when they first encounter antigen in the periphery (Macian *et al.* 2004), or be suppressed by regulatory T cells (Treg) that arise within the thymus and impose anergy within their immediate environment in a non-specific manner by various mechanisms that include release of IL-10 and transforming growth factor β (TGF- β). Treg in turn are regulated by the expression profile of receptors on their surface, including TCR/CD4, CD25, cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumour necrosis factor receptor family related gene product (GITR) and CD134 (also known

as OX40; Sakagushi 2000; Levings *et al.* 2001, 2002; Read & Powrie 2001; Shevach 2001; Valzasina *et al.* 2004; Fehérvári & Sakaguchi 2005).

Foreign organ grafts are recognized as foreign 'pathogens' by the immune system and become subject to full immune attack leading to their rejection. At the molecular level, full activation of donor-specific T cells occurs as the result of donor-antigen-induced clustering of T cell receptors (TCR) to form the immune synapse. Although a threshold of some 8000 clustered TCR molecules are required for activation, co-stimulatory molecules lower this threshold by amplifying intracellular signalling and in the presence of CD28, 1500 TCRs suffice (Viola & Lanzavecchia 1996, 1999). The synaptic signalling threshold may also be altered by lectins and defective glycosylation at the T cell surface has been shown to result in T cell hypersensitivity (Demetriou *et al.* 2001). Despite the predetermined programme for graft rejection, signalling through the immune synapse may be guided to produce donor-specific Treg-type cells by manipulations that reduce the strength of TCR-driven signalling upon first engagement with donor antigen. Such attenuation can be achieved by several routes, including (i) a low affinity TCR-peptide interaction (Chen *et al.* 2004); (ii) presentation of higher affinity peptide by immature or regulatory dendritic cells together with any associated vitamin D activity (Adorini 2003; Cobbold *et al.* 2003; Jeudes & Von Herrath 2003); (iii) reduced T cell responsiveness due to blockade of the TCR co-receptors CD4 and CD8 (Qin *et al.* 1993; Chen *et al.* 1996); and (iv) reduced T cell responsiveness due to blockade of CD28 co-stimulation (Perez *et al.* 1997; Tang *et al.* 2003).

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The one common denominator in tolerance induction in peripheral graft-reactive T cells appears to be a net reduction in signal strength from the TCR to the genome of the responding T cell. The discovery that expression of a single gene, *foxp3*,¹ is able to orchestrate the differentiation of naive CD4⁺ T cells into Treg (Fontenot *et al.* 2003; Hori *et al.* 2003; Khattri *et al.* 2003; Ramsdell 2003; Ramsdell & Ziegler 2003; Cobbold *et al.* 2004; Fontenot & Rudensky 2004) implies the existence of 'master' epigenetic switches where hierarchical positioning of pivotal gene activity will trigger pathways leading to a given phenotype, with *foxp3* being a nodal gene for tolerance. It might be argued that a low level TCR signal at the immune synapse induces at least one round of T cell division wherein epigenetic remodelling occurs for tolerogenic gene expression patterns. Below, I outline some links between TCR engagement and transcriptosome patterning and then present novel data on axotrophin, a stem cell gene product that has been discovered to have profound effects on T cell responsiveness.

2. EPIGENETIC PLASTICITY AND THE PERIPHERAL NAIVE T CELL

Epigenetic factors determine cell fate, acting as key regulators of genomic function by the control of dynamic changes in the local and global organization of chromatin in a heritable manner. The expression pattern of inducible genes becomes established as the result of environmental cues during development down a particular cell lineage, and these patterns are controlled in a tissue-specific manner to allow expression of relevant tissue specific genes while silencing those that are irrelevant. Silencing results from DNA methylation of cytosine residues in CpG-rich sequences of the promoter region, so blocking transcription of the gene, and silencing is central to the establishment and maintenance of lymphocyte identity (Smale 2003). The relevant tissue-specific genes for a given cell type remain unmethylated and available for expression in response to stimulation, while house-keeping genes that are required in all cell types remain open because any CpG-rich islands in their promoter regions also remain unmethylated. Methylation of DNA is not the only means of epigenetic control and other biochemical modifications of chromatin include histone phosphorylation, methylation, ubiquitination and deacetylation (Smale 2003; Gill 2004; Wang *et al.* 2004a; Khan & Krishnamurthy 2005).

Since different fate determination pathways are open to the peripheral naive T cell, a level of epigenetic plasticity is indicated wherein heritable chromatin structure and gene expression patterns can be influenced at the time of antigen engagement, so allowing further epigenetic modelling for full differentiation. It is the pattern of receptor engagement at the time of first engagement between the TCR and presented antigenic peptide that will establish development of the Th-1, or Th-2, phenotypes (Dong & Flavell 2000; O'Shea & Paul 2002; Madrenas 2003; Maldonado *et al.* 2004; Smale 2003). Alternatively, donor-specific regulatory T cells will develop a distinct set of expressed genes that will usually include *foxp3*.

Exactly how this epigenetic patterning becomes established is unknown. It is possible to unmask epigenetically silenced genes by modifying chromatin structure, as occurs for *STAT3* and leukaemia inhibitory factor (*LIF*)- $R\alpha$ in mouse spleen cells treated with FR901228, a histone deacetylase inhibitor (Blanchard *et al.* 2002). The apparent flexibility in expression of *STAT3* and *LIF-R* is of interest since LIF signals through *STAT3* and we have found that both LIF and *STAT3*, in addition to axotrophin, are candidate regulatory mediators in transplantation tolerance (Metcalfe *et al.* 2005a,b).

3. THE IMMUNE SYNAPSE AND CYTOSKELETON IN T CELL FATE DETERMINATION

The immune synapse is central to fate determination of the naive T cell (Penninger & Crabtree 1999; Jun & Goodnow 2003; Davis & Dustin 2004; Finkelstein & Schwartzberg 2004; Huang & Wange 2004; Le Bras *et al.* 2004; Meiri 2004, 2005; Sumen *et al.* 2004). Analytic studies show the synapse as a micro-domain structure comprising central active zones of exocytosis and endocytosis encircled by adhesion domains. Here molecular information is integrated according to TCR affinity for the presented antigen together with concurrent engagement of inhibitory and co-stimulatory receptors. In addition, formation of the synapse is qualified by changes in the nature and frequency of cytokine and chemokine interactions while cytoskeletal changes at the synapse in turn qualify the strength and duration of the synaptic structure, so tuning the immune response between tolerance and full activation. Reversible polymerization of actin is linked to the rapid inactivation of ezrin–radixin–moesin proteins to result in disanchoring of the cortical actin cytoskeleton from the plasma membrane, so increasing plasma membrane fluidity and permitting more efficient synaptic formation (Faure *et al.* 2004). Thus the cytoskeleton itself is recognized as an active modulator of specific synaptic signalling. Importantly, cytoskeletal changes may be linked to generation of second messengers in the form of phosphoinositides; phosphoinositides also influence chromatin remodelling, providing a direct interface between strength of synaptic signalling and chromatin regulation (Rando *et al.* 2003). Overall, immune synaptic activity acts in concert with receptor-mediated reversible phosphorylation of the JAK/STAT signalling pathways (e.g. O'Shea & Paul 2002) to result in the developmental fate of the newly activated T cell.

4. AXOTROPHIN IS LINKED TO EXPRESSION OF LIF IN T LYMPHOCYTES

We first discovered a link between axotrophin and immune tolerance when searching for genes that showed increased expression in tolerance (Metcalfe & Muthukumarana 2005). Using subtractive gene array of Affymetrix chips (36 000 genes per chip), we compared spleen cells from CBA mice that were tolerant to a BALB/c heart graft, versus spleen cells from a CBA mouse that had rejected a BALB/c heart graft (figure 1). Compound analyses at progressive time points following stimulation with donor antigen

revealed differential expression of 129 genes, a few of which showed a progressive increase in expression that only occurred in the tolerant cell population. Two such genes were *axotrophin* and *Par-1*. It has been recently found that *Par-1* null mice develop autoimmune disease, revealing a role for *Par-1* in regulatory immune self-tolerance (Hurov *et al.* 2001).

Despite extensive searches nothing was found on axotrophin until Google revealed that *axot* null mice had been generated by Dr Gary Lyons, University of Wisconsin, USA, and that these mice showed neuronal defects with failure of axonal migration to cross the midline of the brain at the corpus callosum. This led us to speculate that axotrophin might be similar to the semaphorins, since semaphorins play a role in axonal migration by influencing the degree of actin polymerization at the neuronal growth cone, thus enabling responsiveness to extracellular guidance cues (Huber *et al.* 2003; Kantor *et al.* 2004). If axotrophin was somehow similar to the semaphorins, then the role in immune tolerance that was suggested by our gene array data might fit in with the recent realization that certain semaphorins influence lymphocyte developmental fate (Kumanogoh *et al.* 2002; Kuchroo *et al.* 2003; Kumanogoh & Kikutani 2003; Takegahara *et al.* 2005).

To our surprise, direct studies on *axotrophin* null spleen cells revealed profound effects on mitogen-activated T lymphocytes, with marked hyper-proliferation and over production of LIF; this proved to be gene-dose-dependent and was in striking contrast to a lack of effect on B lymphocytes (Metcalf *et al.* 2005a). Comparison of *axotrophin* null with wild-type spleen cells also suggested that LIF release is directly regulated by axotrophin (Metcalf *et al.* 2005a). At the mRNA level, comparison of *axotrophin* null versus wild-type thymocytes has revealed that loss of *axotrophin* results in increased LIF expression and decreased Foxp3 expression (Lyons & Metcalfe, in preparation) while in regulatory Tr1 cells *axotrophin* and *LIF* gene expression is relatively high when compared to isogenic clones of TH1 and TH2 phenotypes (Steve Cobbold, personal communication).

Since LIF is a critical growth factor in fate determination versus self-renewal of stem cells (Zandstra *et al.* 2000; Murray & Edgar 2001), this implies an interactive role for axotrophin and LIF not only in the regulation of immune tolerance but also in the regulation of stem cells and of neuronal stem cells in particular. The link between axotrophin and LIF is of special interest since we had previously found that LIF is produced in high amounts both by tolerant spleen cell populations and by cloned Tr1 cells, in marked contrast to cloned Th1 or Th2 cells (Metcalf *et al.* 2005b). Thus we believe that axotrophin plays a critical and previously unrecognized role in immune tolerance *in vivo*, and that this is linked to Foxp3, LIF and T cell replication.

Having shown that axotrophin may act as a negative regulator of LIF, at least in activated T cells, we suggest that LIF expression is functionally coupled to axotrophin expression, with axotrophin playing a role in co-ordinating the positive and negative regulation of LIF release. This would place axotrophin as a nodal regulator of fate determination via LIF. The molecular

function of axotrophin has yet to be determined and how axotrophin might influence LIF release is unknown. The one clue to date is a RING domain encoded by the axotrophin gene, endowing axotrophin with E3-ligase potential. The E3 ligases guide specificity of ubiquitin-mediated degradation of target proteins by the proteasome and play a critical role in regulating protein half life, a function that operates in anergic T cells where specific signalling molecules are rapidly degraded to prevent full activation (Heissmeyer *et al.* 2004; Macian *et al.* 2004). E3 ligases are also involved in peptide generation for antigen presentation by the MHC and the extent of their role in immunity is an expanding field of study (Liu 2004). In self-tolerance, autoimmune regulator (AIRE) regulates tolerance to organ specific gene products including insulin and thyroglobulin and the AIRE protein has been reported to function in the thymic medulla both as a transcription factor and as an E3 ligase (Liston *et al.* 2004; Uchida *et al.* 2004). This raises the intriguing concept of AIRE-mediated induction of target genes (transcription) and degradation of target gene products (E3-guided ubiquitination) for thymic presentation, the outcome being deletion of the respective self-reactive thymocytes. The potential role of axotrophin as an E3 ligase able to regulate T cell responsiveness is currently being investigated.

5. LIF

It is notable that the phenotype of the *LIF* null mouse (Bugga *et al.* 1999) shows similarities with the *axotrophin* null mouse phenotype: both show normal development with exception of specific hippocampal structures. The viability of the LIF null mouse, despite LIF having wide ranging functions, relates to promiscuity of the LIF receptor and closely related cytokines, for example IL6, IL11, ciliary neutrophin factor, and oncostatin M, may substitute for LIF due to their common receptor subunit, gp130 (Heinrich *et al.* 2003; Metcalf 2003). Thus, although LIF specificity is endowed by the LIF-R α chain in the gp130/LIF-R α heterodimer, this specificity is not exclusive and other family members can substitute for LIF. A small number of exceptions show an absolute requirement for LIF–LIF-R signalling: blastocyst implantation (Cheng *et al.* 2001) and, apparently, limited sites in the hippocampus. The *LIF-R* null mouse is embryo lethal (Metcalf 2003), demonstrating an absolute requirement for LIF-R mediated signalling *in vivo*. The correlation between LIF and tolerance (Metcalf *et al.* 2005a,b) may also be LIF specific, since IL6 failed to show a similar correlation in these experiments (Metcalf, unpublished observations) while others have found an association between IL6 and breakdown of regulatory tolerance (Pasare & Medzhitov 2003).

Induced expression of the *LIF* gene requires *ets* transcription factors (Bamberger *et al.* 2004) and LIF-induced signal transduction is mediated by STAT3 (Moon *et al.* 2002). In responding cells, LIF-induced signalling is tightly regulated by negative feedback and LIF-response genes include suppressor of cytokine signalling-3 (SOCS-3), encoding a dominant suppressor of LIF-induced STAT3 signalling (Bartoe &

Figure 1. The model used to study transplantation tolerance *in vivo* and *ex vivo*. Briefly, tolerance to vascularized heart grafts is generated under cover of CD4 and CD8 blockade. Spleen cells from tolerant recipients, and from recipients that have rejected a heart graft, are cultured *ex vivo* and challenged with donor antigen in the form of irradiated donor-type spleen cells (panel a). Donor-specificity controls include recipient spleen cells challenged with recipient-type (self-self-controls) or with third-party-type spleen cells. The inserts to (a) illustrate the donor-responsive recipient spleen cells at 7d (top) and 14d. Panel (b) illustrates the molecular analyses applied to the spleen cell populations harvested from the *ex vivo* cultures, where identical conditions are maintained between the 'tolerant' and 'rejecting' cultures. Panel (c) details the sequential samples taken, each representing an individual flask to avoid disturbance of responding cells, and the molecules assayed at each time point. Very few showed a selective increase in tolerance: these were the proteins LIF, STAT3, and Foxp3, and c-Kit, together with six genes, one of which was *axotrophin*. These results led to the discovery that axotrophin regulates T cell proliferation and T cell-derived LIF.

Nathanson 2002; Takahashi *et al.* 2003). Such feedback regulation of a critical cytokine ensures balanced physiological responsiveness and permits changes in intensity of signal and we speculate on the possibility that axotrophin plays a role in feedback regulation of LIF, for example by co-operating with SOCS-3 activity. Accordingly, loss of *axotrophin* would enhance LIF-induced signalling by reducing negative feedback control of potential autocrine activity in T cells.

Considering the effect of axotrophin on T cell proliferation and an observed tight relationship between proliferation and endogenous LIF release in the *axot*^{+/+} cells, together with our finding that exogenous LIF does not alter T cell proliferation (Metcalfe *et al.* 2005a), we also speculate that a coupled feedback regulation exists that incorporates an intrinsic link between LIF and T cell division. Uncoupled, hyper-proliferation and increased LIF release would result from loss of endogenous regulation, as observed for the *axotrophin* null T cells (Metcalfe *et al.* 2005a).

6. ALLO-TOLERANCE ASSOCIATED WITH AXOTROPHIN AND LIF IS SELF-SUSTAINING

Our discovery of axotrophin and LIF in tolerance came from an *ex vivo* model where the complexity of the micro-environment of responding T cells was preserved during their allo-activation so as to mimic the *in vivo* situation (figure 1). The *ex vivo* model was directly derived from an *in vivo* mouse model where we have already shown that established regulatory tolerance is donor-specific and permanent (Chen *et al.* 1996), lacks B cell allo-reactivity (Chen 1997), and shows good vascular preservation within the graft (Moffatt 1999). Tolerance was induced by 21 days therapy with blocking monoclonal antibodies to CD4 and CD8 and, although this induction could be prevented by high dose interleukin 2 (IL2), once established, donor-specific tolerance was very robust and could not be broken by IL2 nor by removal of IL4 using anti-IL4 monoclonal antibody (Chen *et al.* 1996). Crucially, tolerance became self-perpetuating and adoptive transfer of tolerant spleen cells into

Figure 2. Stemness theory of development and maintenance of regulatory T cells. Cartoons (a) and (b) show the proposed alternative outcomes following peripheral CD4⁺ T lymphocyte activation, as qualified by strength of signal delivered via the T cell receptor (TCR) at the time of first antigen engagement. In (a), signalling from the TCR is sufficient to trigger differentiation of aggressive effector functions, these being further qualified by environmental cues to guide Th1-type, or Th2-type, development. In (b), the signalling threshold at the immune synapse is too weak to generate differentiation towards effector function, but is sufficient to induce epigenetic changes for regulatory tolerance (step 1). This is associated with a checkpoint that arrests the responding T cell in a relatively undifferentiated state. This stage is then maintained in the presence of presented antigen due to proliferation and release of LIF from the responding T cell (step 2), LIF in turn being regulated by axotrophin. The LIF–axotrophin link appears to be endogenously controlled in the epigenetically stable Treg cells. The term ‘stemness’ is suggested since the relatively undifferentiated state of the regulatory cell population is characterized by molecular pathways that are in common with the regulation of stem cells.

fully immune competent recipients revealed not only the spread of tolerance to whole (un-manipulated) naive recipients of a donor-type graft but also spread of tolerance at the cellular level to naive donor-reactive T cells (*infectious tolerance*). In contrast, all third party heart grafts were rejected, while 50% of [donor×third party] (F1) heart grafts were accepted in the presence of adoptively transferred donor-tolerant spleen cells, demonstrating spread of specific tolerance to linked third party antigen. Two absolute requirements for maintenance of dominant transplantation tolerance were found: allo-specific CD4⁺ T cells, and continuous presence of allo-antigen (Chen *et al.* 1996). In our *ex vivo* analyses of tolerance we sought to parallel the *in vivo* state, preserving the full cellular environment of the alloreactive T cells. Having identified novel molecules selectively expressed in the un-manipulated tolerant cell population, whole spleen cell population studies at a single cell level by flow cytometry will identify those cells expressing axotrophin and LIF together with their co-expressed markers and proliferation profile.

7. IS STEMNESS A CHARACTERISTIC OF PERIPHERAL TOLERANCE?

We have previously found selective expression of both STAT3 and c-kit in tolerance (Metcalfe & Watson 2002); c-kit is the receptor for stem cell factor (SCF) and both SCF/c-kit and LIF/LIF-R signal through STAT3. (Ning *et al.* 2001; Heinrich *et al.* 2003). Others have shown that STAT3 activity indirectly inhibits dendritic cell maturation (Wang *et al.* 2004b) opening up the possibility that LIF and/or SCF in the micro-environment arrests differentiation of antigen presenting cells.

As a working model we propose that LIF activity, regulated by axotrophin, is associated with immune tolerance. LIF may guide naive T cells towards a relatively undifferentiated, non-aggressive phenotype in response to presented antigen where the circumstances of presentation initiate the tolerogenic LIF activity, either directly or indirectly. Thereafter, epigenetic changes, including expression of *foxp3* and *ROG*, and induction of Id (*Inhibitors of differentiation*) transcription factors (Ying *et al.* 2003) would stabilize the tolerant phenotype for inheritable Treg activity.

Axotrophin, LIF and STAT3 would continue to play a role in the self-perpetuation of tolerance, actively driven by presented antigen/TCR interactions to maintain both specificity and self-renewal (figure 2). Removal of antigen would result in loss of tolerance, while co-expressed F1 antigen may induce linked tolerance, as described previously (Chen *et al.* 1996). Overwhelming expansion of the lymphocyte repertoire might reduce the option of regulated epigenetic programming towards the tolerant phenotype, and homeostatic proliferation has been described as a barrier to transplantation tolerance (Wu *et al.* 2003).

Having discovered that certain features of immune tolerance are common to regulation of stem cell fate, the question arises: do 'stemness' signals play a role in peripheral tolerance by suppressing terminal differentiation of immune effector cells? Secondly, we consider the possibility that allogeneic stem cells might bias the allo-immune response towards allo-tolerance by signalling for stemness, so favouring successful therapeutic engraftment. Thus, in addition to tolerance in transplant recipients, any links between regulatory immune tolerance and stem cell biology should benefit regenerative medicine and the successful outcome of implanted stem cell allografts, in particular of neuronal stem cells where LIF release might both suppress aggressive immunity and act as a neural growth factor.

ENDNOTE

¹Foxp3 belongs to the family of Forkhead (Fox) transcription factors that are represented by a wide range of members including the Foxp and Foxo sub-groups and recent evidence suggests a generalized role for the forkhead proteins in T cell tolerance (Coffer & Burgering 2004; Lin *et al.* 2004).

REFERENCES

- Adorini, L. 2003 Tolerogenic dendritic cells induced by vitamin D receptor ligands enhance regulatory T cells inhibiting autoimmune diabetes. *Ann. NY Acad. Sci.* **987**, 258–261.
- Bamberger, A. M. *et al.* 2004 Regulation of the human leukemia inhibitory factor gene by ETS transcription factors. *Neuroimmunomodulation* **11**, 10–19. (doi:10.1159/000072964.)
- Bartoe, J. L. & Nathanson, N. M. 2002 Independent roles of SOCS-3 and SHP-2 in the regulation of neuronal gene expression by leukemia inhibitory factor. *Brain Res. Mol. Brain Res.* **107**, 109–119.
- Blanchard, F., Kinzie, E., Wang, Y., Duplomb, L., Godard, A., Held, W. A., Asch, B. B. & Baumann, H. 2002 FR901228, an inhibitor of histone deacetylases, increases the cellular responsiveness to IL-6 type cytokines by enhancing the expression of receptor proteins. *Oncogene* **21**, 6264–6277. (doi:10.1038/sj.onc.1205777.)
- Bugga, L., Gadiant, R. A., Kwan, K., Stewart, C. L. & Patterson, P. H. 1999 Analysis of neuronal and glial phenotypes in brains of mice deficient in leukaemia inhibitory factor. *J. Neurobiol.* **36**, 509–524. (doi:10.1002/(SICI)1097-4695(19980915)36:4<509::AID-NEU5>3.0.CO;2-#.)
- Chen, Z. 1997 Induction and maintenance of tolerance in murine allo- and xeno-grafts. Ph.D. thesis, University of Cambridge.
- Chen, Z. K., Cobbold, S. P., Waldmann, H. & Metcalfe, S. M. 1996 Amplification of natural regulatory immune mechanisms for transplantation tolerance. *Transplantation* **62**, 1200–1206. (doi:10.1097/00007890-199611150-00002.)
- Chen, T. C., Cobbold, S. P., Fairchild, P. J. & Waldmann, H. 2004 Generation of anergic and regulatory T cells following prolonged exposure to a harmless antigen. *J. Immunol.* **172**, 5900–5907.
- Cheng, J. G., Chen, J. R., Hernandez, L., Alvord, W. G. & Stewart, C. L. 2001 Dual control of LIF expression and LIF receptor function regulate Stat3 activation at the onset of uterine receptivity and embryo implantation. *Proc. Natl Acad. Sci. USA* **98**, 8680–8685. (doi:10.1073/pnas.151180898.)
- Cobbold, S. P. *et al.* 2003 Regulatory T cells and dendritic cells in transplantation tolerance: molecular markers and mechanisms. *Immunol. Rev.* **196**, 109–124. (doi:10.1046/j.1600-065X.2003.00078.x.)
- Cobbold, S. P., Castejon, R., Adams, E., Zelenika, D., Graca, L., Humm, S. & Waldmann, H. 2004 Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J. Immunol.* **172**, 6003–6010.
- Coffer, P. J. & Burgering, B. M. 2004 Forkhead-box transcription factors and their role in the immune system. *Nat. Rev. Immunol.* **4**, 889–899. (doi:10.1038/nri1488.)
- Davis, D. M. & Dustin, M. L. 2004 What is the importance of the immunological synapse? *Trends Immunol.* **25**, 323–327. (doi:10.1016/j.it.2004.03.007.)
- Demetriou, M., Granovsky, M., Quaggin, S. & Dennis, J. W. 2001 Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* **409**, 733–739. (doi:10.1038/35055582.)
- Dong, C. & Flavell, R. A. 2000 Control of T helper cell differentiation—in search of master genes. *Sci. STKE* **49**, PE1.
- Faure, S., Salazar-Fontana, L. I., Semichon, M., Tybulewicz, V. L., Bismuth, G., Trautmann, A., Germain, R. N. & Delon, J. 2004 ERM proteins regulate cytoskeleton relaxation promoting T cell–APC conjugation. *Nat. Immunol.* **5**, 272–279. (doi:10.1038/ni1039.)
- Fehérvári, Z. & Sakaguchi, S. 2005 CD4⁺ regulatory cells as a potential immunotherapy. *Phil. Trans. R. Soc. B* **360**, 1647–1661. (doi:10.1098/rstb.2005.1695.)
- Finkelstein, L. D. & Schwartzberg, P. L. 2004 Tec kinases: shaping T-cell activation through actin. *Trends Cell Biol.* **14**, 443–451. (doi:10.1016/j.tcb.2003.07.001.)
- Fontenot, J. D. & Rudensky, A. Y. 2004 Molecular aspects of regulatory T cell development. *Semin. Immunol.* **16**, 73–80. (doi:10.1016/j.smim.2003.12.002.)
- Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. 2003 Foxp3 programs the development and function of CD4⁺ CD25⁺ regulatory T cells. *Nat. Immunol.* **4**, 330–336. (doi:10.1038/ni904.)
- Gill, G. 2004 SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev.* **18**, 2046–2059. (doi:10.1101/gad.1214604.)
- Heinrich, P. C., Behrmann, I., Haan, S., Hermanns, H. M., Muller-Newen, G. & Schaper, F. 2003 Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem. J.* **374**, 1–20. (doi:10.1042/BJ20030407.)
- Heissmeyer, V. *et al.* 2004 Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat. Immunol.* **5**, 255–265. (doi:10.1038/ni1047.)
- Hori, S., Nomura, T. & Sakaguchi, S. 2003 Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057–1061. (doi:10.1126/science.1079490.)
- Huang, Y. & Wange, R. L. 2004 T cell receptor signaling: beyond complex complexes. *J. Biol. Chem.* **279**, 28 827–28 830. (doi:10.1074/jbc.R400012200.)

- Huber, A. B., Kolodkin, A. L., Ginty, D. D. & Cloutier, J. F. 2003 Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. *Annu. Rev. Neurosci.* **26**, 509–563. (doi:10.1146/annurev.neuro.26.010302.081139.)
- Hurov, J. B., Stappenbeck, T. S., Zmasek, C. M., White, L. S., Ranganath, S. H., Russell, J. H., Chan, A. C., Murphy, K. M. & Piwnicka-Worms, H. 2001 Immune system dysfunction and autoimmune disease in mice lacking Emk (Par-1) protein kinase. *Mol. Cell Biol.* **21**, 3206–3219. (doi:10.1128/MCB.21.9.3206-3219.2001.)
- Jeudes, A. E. & Von Herrath, M. G. 2003 Using regulatory APCs to induce/maintain tolerance. *Ann. NY Acad. Sci.* **1005**, 128–137. (doi:10.1196/annals.1288.014.)
- Jun, J. E. & Goodnow, C. C. 2003 Scaffolding of antigen receptors for immunogenic versus tolerogenic signaling. *Nat. Immunol.* **4**, 1057–1064. (doi:10.1038/ni1001.)
- Kantor, D. B. *et al.* 2004 Semaphorin 5A is a bifunctional axon guidance cue regulated by heparan and chondroitin sulfate proteoglycans. *Neuron* **44**, 961–975. (doi:10.1016/j.neuron.2004.12.002.)
- Khan, A. U. & Krishnamurthy, S. 2005 Histone modifications as key regulators of transcription. *Front. Biosci.* **10**, 866–872.
- Khattari, R., Cox, T., Yasayko, S. A. & Ramsdell, F. 2003 An essential role for Scurfin in CD4+ CD25+ T regulatory cells. *Nat. Immunol.* **4**, 337–342. (doi:10.1038/ni909.)
- Kuchroo, V. K., Umetsu, D. T., DeKruyff, R. H. & Freeman, G. J. 2003 The TIM gene family: emerging roles in immunity and disease. *Nat. Rev. Immunol.* **3**, 454–462. (doi:10.1038/nri1111.)
- Kumanogoh, A. & Kikutani, H. 2003 Roles of the semaphorin family in immune regulation. *Adv. Immunol.* **81**, 173–198. (doi:full_text.)
- Kumanogoh, A. *et al.* 2002 Class IV semaphorin Sema4A enhances T-cell activation and interacts with TIM-2. *Nature* **419**, 629–633. (doi:10.1038/nature01037.)
- Le Bras, S., Foucalt, I., Foussat, A., Brignone, C. & Acuto, O. 2004 Deckert M Recruitment of the actin-binding protein HIP-55 to the immunological synapse regulates T cell receptor signaling and endocytosis. *J. Biol. Chem.* **279**, 15 550–15 560. (doi:10.1074/jbc.M312659200.)
- Levings, M. K., Sangregorio, R. & Roncarolo, M. G. 2001 Human CD25+ CD4+ T regulatory cells suppress naïve and memory T cell proliferation and can be expanded in vitro without loss of function. *J. Exp. Med.* **193**, 1295–1301. (doi:10.1084/jem.193.11.1295.)
- Levings, M. K., Bachetta, R., Schulz, U. & Roncarolo, M. G. 2002 The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells. *Int. Arch. Allergy Immunol.* **129**, 263–276. (doi:10.1159/000067596.)
- Lin, L., Hron, J. D. & Peng, S. L. 2004 Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. *Immunity* **21**, 203–213. (doi:10.1016/j.immuni.2004.06.016.)
- Liston, A., Lesage, S., Wilson, J., Peltonen, L. & Goodnow, C. C. 2003 Aire regulates negative selection of organ-specific T cells. *Nat. Immunol.* **4**, 350–354. (doi:10.1038/ni906.)
- Liston, A. *et al.* 2004a Gene dosage-limiting role of Aire in thymic expression, clonal deletion, and organ-specific autoimmunity. *J. Exp. Med.* **200**, 1015–1026. (doi:10.1084/jem.20040581.)
- Liston, A. *et al.* 2004b Generalized resistance to thymic deletion in the NOD mouse; a polygenic trait characterized by defective induction of Bim. *Immunity* **21**, 817–830. (doi:10.1016/j.immuni.2004.10.014.)
- Liu, Y. C. 2004 Ubiquitin ligases and the immune response. *Annu. Rev. Immunol.* **22**, 81–127. (doi:10.1146/annurev.immunol.22.012703.104813.)
- Macian, F., Im, S. H., Garcia-Cozar, F. J. & Rao, A. 2004 T-cell anergy. *Curr. Opin. Immunol.* **16**, 209–216. (doi:10.1016/j.coi.2004.01.013.)
- Madrenas, J. 2003 SLAT in the Th2 signalosome. *Immunity* **18**, 459–461. (doi:10.1016/S1074-7613(03)00089-X.)
- Maldonado, R. A., Irvine, D. J., Schreiber, R. & Glimcher, L. H. 2004 A role for the immunological synapse in lineage commitment of CD4 lymphocytes. *Nature* **431**, 527–532. (doi:10.1038/nature02916.)
- Meiri, K. F. 2004 Membrane/cytoskeleton communication. *Subcell. Biochem.* **37**, 247–282.
- Meiri, K. F. 2005 Lipid rafts and regulation of the cytoskeleton during T-cell activation. *Phil. Trans. R. Soc. B.*
- Metcalfe, D. 2003 Unsolved enigmas of leukaemia inhibitory factor. *Stem Cells* **21**, 5–14. (doi:10.1634/stemcells.21-1-5.)
- Metcalfe, S. M. & Watson, T. J. 2002 *Regulatory tolerance is associated with induction of c-kit, the receptor for stem cell factor.* Abstract 047. London: British Transplantation Society.
- Metcalfe, S. M. & Muthukumarana, P. A. 2005 Transplantation tolerance: gene expression profiles comparing allotolerance vs. allojection. *Int. Immunopharmacol.* **5**, 33–39. (doi:10.1016/j.intimp.2004.09.009.)
- Metcalfe, S. M., Muthukumarana, P. A. D. S., Thompson, H. L., Haendel, M. & Lyons, G. E. 2005a Leukaemia inhibitory factor (LIF) is functionally linked to axotrophin and both LIF and axotrophin are linked to regulatory immune tolerance. *FEBS Lett.* **579**, 609–614. (doi:10.1016/j.febslet.2004.12.027.)
- Metcalfe, S. M., Watson, T. J., Shurey, S., Adams, E. & Green, C. J. 2005b Leukaemia Inhibitory Factor (LIF) is linked to regulatory transplantation tolerance. *Transplantation* **79**. (doi:10.1097/01.TP.0000149324.42994.38.)
- Moffatt, S. D. 1999 Molecular manipulation of lymphocytes leading to allo-tolerance. Ph.D. thesis, University of Cambridge.
- Moon, C. *et al.* 2002 Leukemia inhibitory factor inhibits neuronal terminal differentiation through STAT3 activation. *Proc. Natl Acad. Sci. USA* **99**, 9015–9020. (doi:10.1073/pnas.132131699.)
- Murray, P. & Edgar, D. 2001 The regulation of embryonic stem cell differentiation by leukaemia inhibitory factor (LIF). *Differentiation* **68**, 227–234. (doi:10.1046/j.1432-0436.2001.680410.x.)
- Ning, Z. Q., Li, J. & Arcenci, R. J. 2001 Signal transducer and activator of transcription 3 activation is required for Asp(816) mutant c-Kit-mediated cytokine-independent survival and proliferation in human leukemia cells. *Blood* **97**, 3559–3567. (doi:10.1182/blood.V97.11.3559.)
- O'Shea, J. J. & Paul, W. E. 2002 Regulation of Th1 differentiation—controlling the controllers. *Nat. Immunol.* **3**, 506–508. (doi:10.1038/ni0602-506.)
- Pasare, C. & Medzhitov, R. 2003 Toll pathway-dependent blockade of CD4+ CD25+ T cell-mediated suppression by dendritic cells. *Science* **299**, 1033–1036. (doi:10.1126/science.1078231.)
- Penninger, J. M. & Crabtree, G. R. 1999 The actin cytoskeleton and lymphocyte activation. *Cell* **96**, 9–12. (doi:10.1016/S0092-8674(00)80954-X.)
- Perez, V. L., Van Parijs, L., Biuckians, A., Zheng, X. X., Strom, T. B. & Abbas, A. K. 1997 Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity* **6**, 411–417. (doi:10.1016/S1074-7613(00)80284-8.)
- Qin, S., Cobbold, S. P., Pope, H., Elliott, J., Kioussis, D., Davies, J. & Waldmann, H. 1993 “Infectious” transplantation tolerance. *Science* **259**, 974–977.

- Ramsdell, F. 2003 Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity* **19**, 165–168. (doi:10.1016/S1074-7613(03)00207-3.)
- Ramsdell, F. & Ziegler, S. F. 2003 Transcription factors in autoimmunity. *Curr. Opin. Immunol.* **15**, 718–724. (doi:10.1016/j.coi.2003.09.008.)
- Rando, O. J., Chi, T. H. & Crabtree, G. R. 2003 Second messenger control of chromatin remodeling. *Nat. Struct. Biol.* **10**, 81–83. (doi:10.1038/nsb0203-81.)
- Read, S. & Powrie, F. 2001 CD4(+) regulatory T cells. *Curr. Opin. Immunol.* **13**, 644–649. (doi:10.1016/S0952-7915(01)00273-4.)
- Sakagushi, S. 2000 Regulatory T cells: key controllers of immunological self-tolerance. *Cell* **101**, 455–458. (doi:10.1016/S0092-8674(00)80856-9.)
- Shevach, E. 2001 Certified professionals: CD4+CD25+ suppressor cells. *J. Exp. Med.* **193**, F41–F45. (doi:10.1084/jem.193.11.F41.)
- Smale, S. T. 2003 The establishment and maintenance of lymphocyte identity through gene silencing. *Nat. Immunol.* **4**, 607–615. (doi:10.1038/ni0703-607.)
- Sumen, C., Dustin, M. L. & Davis, M. M. 2004 T cell receptor antagonism interferes with MHC clustering and integrin patterning during immunological synapse formation. *J. Cell Biol.* **166**, 579–590. (doi:10.1083/jcb.200404059.)
- Takahashi, Y. *et al.* 2003 SOCS3: an essential regulator of LIF receptor signaling in trophoblast giant cell differentiation. *EMBO J.* **22**, 372–384. (doi:10.1093/emboj/cdg057.)
- Tang, Q. *et al.* 2003 CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J. Immunol.* **171**, 3348–3352.
- Takegahara, N., Kumanogoh, A. & Kikutani, H. 2005 Semaphorins: a new class of immunoregulatory molecules. *Phil. Trans. R. Soc. B* **360**, 1673–1680. (doi:10.1098/rstb.2005.1696)
- Uchida, D. *et al.* 2004 AIRE functions as an E3 ubiquitin ligase. *J. Exp. Med.* **199**, 167–172. (doi:10.1084/jem.20031291.)
- Valzasina, B., Guiducci, C., Dislich, H., Killeen, N., Weinberg, A. D. & Colombo, M. P. 2004 Triggering of OX40 (CD134) on CD4+CD25+ T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR. *Blood* **105**, 2845–2851. (doi:10.1182/blood-2004-07-2959.)
- Venanzi, E. S., Benoist, C. & Mathis, D. 2004 Good riddance: thymocyte clonal deletion prevents autoimmunity. *Curr. Opin. Immunol.* **16**, 197–202. (doi:10.1016/j.coi.2004.01.008.)
- Viola, A. & Lanzavecchia, A. 1996 T cell activation determined by T cell receptor number and tunable thresholds. *Science* **273**, 104–106.
- Viola, A. & Lanzavecchia, A. 1999 T-cell activation and the dynamic world of rafts. *APMIS* **107**, 615–623. Review.
- Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R. S. & Zhang, Y. 2004a Role of histone H2A ubiquitination in Polycomb silencing. *Nature* **431**, 873–878. (doi:10.1038/nature02985.)
- Wang, T. *et al.* 2004b Regulation of the innate and adaptive immune responses by STAT-3 signaling in tumor cells. *Nat. Med.* **10**, 48–54. (doi:10.1038/nm976.)
- Wu, Z. *et al.* 2003 Homeostatic proliferation is a barrier to transplantation tolerance. *Nat. Med.* **10**, 87–92. (doi:10.1038/nm965.)
- Ying, Q. L., Nichols, J., Chambers, I. & Smith, A. 2003 BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* **115**, 281–292. (doi:10.1016/S0092-8674(03)00847-X.)
- Zandstra, P. W., Le, H. V., Daley, G. Q., Griffith, L. G. & Lauffenburger, D. A. 2000 Leukemia inhibitory factor (LIF) concentration modulates embryonic stem cell self-renewal and differentiation independently of proliferation. *Biotechnol. Bioeng.* **69**, 607–617. (doi:10.1002/1097-0290(20000920)69:6<607::AID-BIT4>3.3.CO;2-6.)