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T cell depletion as a route to transplantation tolerance.

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ABSTRACT.

The aim of this work was that to investigate and possibly establish clinically-feasible therapeutic protocols in order to induce transplantation tolerance in solid organ (kidney) recipients.

The model I adopted was that of murine skin transplantation across the complete MHC barrier, as it is known to be the most stringent model of organ transplantation, and the hardest barrier to overcome with a therapeutic treatment.

My starting point was that T cell antibody depletion, currently utilized in the transplantation clinic as induction therapy, is effective and allows some reduction in maintenance immunosuppression, which is however still required life-long for the prevention of acute rejection. Therefore I sought to establish therapeutic protocols to induce transplantation tolerance building on the benefits of T cell depletion. I used as T cell depleting agent Campath-1, a powerful monoclonal lytic antibody, which is used for lymphocyte depletion in transplantation.

The first strategy I developed was driven by the working hypothesis that the careful guidance of T cell reconstitution towards regulation could induce transplantation tolerance following depletion. Indeed the combination of an anti-IL-7R blocking antibody and Rapamycin could reduce the proliferation of effector T cells and favour the expansion of reconstituting Foxp3+ regulatory T cells, which are essential for tolerance. This combination treatment has succeeded at promoting indefinite survival of fully mismatched transplants, and graft acceptance so obtained proved to be dependent on TGF-ß signalling, as its neutralization limited graft survival.

The second strategy I investigated relies on the knowledge that development of mixed haematopoietic chimerism following bone marrow transplantation can induce tolerance towards donor epithelial graft, but host myelo-ablation, required for bone marrow engraftment, remains a limiting factor for a wider application of this strategy into the clinic. I have developed for the first time a protocol based on T cell depletion, Rapamycin and co-stimulation antibody blockade that enables bone marrow engraftment without any need for myelo-ablation; mixed haematopoietic

chimerism then induces transplantation tolerance towards epithelial graft of donor origin when performed later in time; tolerance so obtained is based on regulation as demonstrated by the phenomenon of linked-suppression.

These results foster the concept that the adult immune system can be reprogrammed with a short-course treatment aimed at inducing tolerance towards not-self graft antigens; the clinical application of such tolerizing treatments will improve long-term transplantation outcomes, by limiting recipients exposure to the side effects inherent in life-long immunosuppression, currently required to prevent rejection.

AKNOWLEDGMENT.

This experimental thesis is the result of the work I have done as a Doctoral Student over the last 3 years, mainly performed in the host laboratory, Therapeutic Immunology Group (TIG), headed by Prof. Herman Waldmann, at the Sir William Dunn School of Pathology, Oxford University.

I am deeply grateful to Prof. Waldmann for the opportunity he gave me to test myself as a medical researcher in an exciting environment such as that of the Dunn School of Pathology.

I sincerely consider Prof. Waldmann an extraordinary and rigorous man of science.

I also want to thank Prof. Steven Cobbold, Doctor Elizabeth Adams and Doctor Christian Peter for the insightful discussions, the generous help, and the useful advices.

I wish these inspiring people and the other members of the TIG lab all the best.

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ABBREVIATIONS.

Ab	Antibody
Ag	Antigen
AIDS	Acquied immunodeficiency syndrome
APC	Antigen Presenting Cell
AR	Acute rejection
ATG	Anti-thymocyte globulins
B6	C57BL/6 mouse
B6.hCD2.KI	B6.Foxp3hCD2 Knock-in mouse
BM	Bone marrow
BrdU	Bromodeoxyuridin
BSA	Bovine serum albumin
CAD	Chronic allograft disease
CBA	CBA/Ca mouse
CFSE	5,6-carboxyfluorescin diacetate succinimyl esterdilution
CN	Calcineurin
CNI	CN Inhibitors
CP1H	hCD52 transgenic mouse
CVD	Cardiovascular disease
DC	Dendritic Cell
dH2O	Distilled water
EAA	Essential amino acids
EDTA	Ethylenediaminetetraacetic disodium salt
EdU	5-ethynyl-2'-deoxyuridine
EP	endogenous proliferation

ESRD	End stage renal disease
FACS	Fluorescence-activated cell sorting
F1	Filial 1
FBS	Fetal bovine serum
FKBP12	FK Binding Protein 12
Foxp3	Forkhead/winged-helix protein
GVHD	Graft versus host disease
HD	Haemodialysis
HINRS	Heat-inactivated rabbit serum
HLA	Human Leukocyte Antigen
HP	homeostatic proliferation
HSC	Haematopoietic Stem Cells
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
IL-R	IL-Receptor
IMD	Inosine monophosphate dehydrogenase
INF	Interferon
IS	Immunosuppression
iTreg	Induced regulatory T cells
mH2O	Millipore water
MHC	Major Compatibility Complex
miH	Minor Leukocyte Antigen
MMF	Mycophenolate mofetil
moAb	Monoclonal antibody
MST	Mean survival time
mTOR	Mechanistic target of Rapamycin

NK	Natural Killer cell
nTreg	Natural regulatory T cells
p-preDC FC	plasmacytoid precursor dendritic facilitating cells
PB	Peripheral blood
PBS	Dulbecco's Phosphate Buffered Saline
PD	Peritoneal Dialysis
PDL1	Programmed Death 1 Ligand 1
PTLD	Post transplant lymphoproliferative disease
RAG	Recombinant Activating genes
Rapa	Rapamycin
RT	Renal transplantation
RT	Room temperature
SCID	Severe combined immunodeficiency syndrome
TBAC	Tris-Buffered Ammonium Chloride
Tconv	Conventional T cells
TCR	T cell Receptor
Tfh	T follicular helper cell
tg	Transgenic
TGF-ß	Transforming growth factor -ß
Th	T helper cell
TMA	Trombotic microangiopathy
TNF	Tumor Necrosis Facotr
Treg	Regulatory T cells
Tx	Transplantation

INTRODUCTION.

1. Organ transplantation in modern medicine.

Since the first successful renal transplantation between identical twins in 1954 (Merrill JP, et al. JAMA. 1956), the practice of solid organ transplantation has spread evolving into the best therapeutic option for patients suffering from end stage renal disease (ESRD) (see table 1), and a life-saving treatment for patients suffering from cardiac, pulmonary and hepatic failure (Sayegh MH, and Carpenter CB. N Engl J Med. 2004).

Table 1. Outcomes comparison among different renal replacement therapies: haemodialysis (HD), peritoneal dialysis (PD) and renal transplantation (RT).

	HD	**PD**	**RT**
1-year mortality[1]	22.5%	12.5%	5.9%
5-year mortality[1]	23.6%	25.4%	6.0%
Quality of life[2,3]	ê	ê	é
Medicare expenditures[1,4]	$ 87˙561	$ 66˙751	$ 32˙914

Notes to Table 1.

1 U.S. Renal Data System, USRDS 2011 Annual Data Report: Atlas of End-Stage Renal Disease in the United States, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, 2011.

2 Cameron JI, et al. Am J Kidney Dis. 2000.

3 Quality of life, measured both as degree of emotional distress and physiological well-being, was significantly superior in renal transplant recipients.

4 Medicare expenditure per person per year in the USA, 2010.

The main reason for the success of transplantation medicine is the dramatic improvement in short-term outcomes: one-year renal graft survival has improved from circa 45% to 95% over the last 50

years, as a result of combined progress in the areas of tissue matching, organ preservation, surgical techniques and more importantly immunosuppression therapy (Lechler RI, et al. Nat Med. 2005).

The introduction of more powerful immunosuppressants has brought about a substantial victory over early acute rejection, which is theoretically the major determinant of graft survival (Hariharan S, et al. N Engl J Med. 2000), and has downsized the impact of the other factors.

The relationship between organ transplantation and immunosuppression is so intimately bound that the evolution of the former is recapitulated by the developments of the latter, which can be traced as follows: from the first studies on prolongation of graft survival by total body irradiation, steroids or chemotherapy drugs in the 1950s, to combination therapy with prednisone and azathioprine in the 1960s (Starlz TE. JAMA. 1984), to the introduction of cyclosporine in the 80s (Calne RY, et al. Lancet. 1979), depleting and non-depleting anti-lymphocyte sera, tacrolimus, mycophenolate mofetil and sirolimus in the 1990s (Halloran PF. N Engl J Med. 2004), and lastly belatacept (Vincenti F, et al. Am J Transplant. 2012).

2. Immunobiology of transplantation.

2.1. Transplantation antigens.

While transplantation between genetically identical individuals (homograft) naturally results in the acceptance of the transplant, transplantation between genetically disparate individuals (allograft) inevitably leads to the rejection of the graft in the absence of effective immunosuppression.

The recipient's immune system is in fact able to recognize and mount the immune response against the antigens (Ag) of the allograft, allo-antigens, which are variants of proteins encoded by highly polymorphic genes.

Among these allo-antigens, there are the proteins of AB0 blood group: these antigens are clinically relevant because they are expressed not only on the red blood cells, which are not transplanted with the graft, but also on the endothelial cells of the graft, and because they are the target of naturally occurring agglutinating antibodies (Abs). Transplantation between AB0 incompatible individuals is not routinely performed as, in the absence of specific removal of these anti-AB0 Abs, it results in a hyperacute rejection within minutes from the revascularization, characterized by local activation of the coagulation and complement cascades and thrombotic infarction of the graft. AB0 matching between donor and recipient is a routine practice and, given the modest Ag variability, it does not represent an obstacle to transplantation.

Another barrier to the acceptance of graft is represented by the proteins of the major histocompatibility complex (MHC), or human leukocytes antigens (HLA) for humans: not only are they the molecules through which Ags are displayed to T cells, but also the most important allo-antigens due to the high proportion of precursor reactive T cells (1-10%), and to the extremely high polymorphism of the genes. Given the minimal probability of HLA matching, transplantation is virtually always performed between HLA not identical individuals, and immunosuppression is required to prevent T cell-mediated rejection. Donor and recipient HLA haplotype and the presence of anti-donor HLA Abs are therefore studied before transplantation, to best allocate the graft. The presence of donor specific anti-HLA Abs is a contraindication to the transplantation because of the

risk of acute antibody-mediated rejection, unless a procedure of desensitization of the recipient is carried out to remove such Abs.

Finally there are a large number of minor histocompatibility Ags (miH), encoded by polymorphic genes outside the MHC regions and partly not identified yet: these antigens together are able to elicit a powerful immune response against the graft and are responsible for the need for immunosuppression even in the presence of a complete HLA matching (Danovitch GM. Handbook of Kidney Transplantation. 2009).

2.2. Allograft rejection.

In the absence of preformed anti-donor Abs, T cells play a central role in the rejection of the graft which occurs in a multi-step process, and which, upon T cell activation, involves B cells and components of the innate immune system (Nankivell BJ and Alexander SI. N Engl J Med. 2010).

2.2.1. T cell recognition of alloantigens.

The presentation of allo-antigens to T cells is carried out by the antigen presenting cells (APCs) through the MHC molecules: class I MHC molecules display peptides to CD8+ T cells; class II MHC molecules, which are only expressed by professional APCs, display peptides to CD4+ T cells. Both donor and recipient APCs are capable of presenting allo-antigens to T cells: in the first case, the donor's dendritic cells (DC) leave the graft and migrate into the recipient's draining lymph nodes and spleen, where they directly present intact MCH-peptide complexes to the recipient's T cells (direct presentation); in the second, it is the recipient's APCs that present processed peptides derived from donor MHC or miH antigens on self MHC molecules (indirect presentation) or present intact donor MHC-peptide complexes acquired on their surface (semi-direct) to the T cells in lymphoid organs (Afzali B, et al. Curr Opinion Organ Transplant. 2008).

2.2.2. T cell activation.

The activation of T cells occurs according to a three-signal model (Halloran PF. N Engl J Med. 2004).

“Signal 1” is provided by the interaction between the peptide-MHC complex displayed by the APC and the cognate receptor of a T cell (TCR), and it is stabilized by the binding of the CD4 or CD8 coreceptors to the MHC molecules.

“Signal 2”, costimulation, is required for the complete activation of T cells and is provided by the interaction between molecules expressed on the surface of the APCs, like CD80 and CD86 or CD40, which respectively bind to CD28 or CD154 on T cells.

Costimulation allows the transduction of signal 1 by the TCR-CD3 complex, which, through the complex activation of different intracellular pathways, including the calcineurin pathway, induces the expression of many molecules, in particular up-regulating the expression of interleukin-2 (IL-2) and its receptor (IL-2R or CD25), whose autocrine interaction, “signal 3”, activates the mechanistic target of Rapamycin (mTOR), induces cell proliferation and clonal expansion, and cell differentiation.

2.2.3. Migration into the graft.

Activated T cells leave the lymphoid organs in response to the chemokines produced by the injured allograft, express adhesion molecules, like LFA-1, adhere to the endothelium, cross the capillary barrier and enter the graft.

2.2.4 Graft rejection.

CD8+ cytotoxic T cells, activated by APCs licenced by CD4+ helper T cells, once migrated into the graft, are able to kill target cells by secreting perforin which perforates cell membranes, and by inducing caspase-mediated apoptosis trough the release of granzymes A and B, and the interaction between Fas ligand and Fas.

Different subsets of CD4+ T cells, which acquire helper functions in response to the presentation of alloantigens, are able to mediate the rejection of the graft (Table 2) (Zelenika D et al. J Immunol. 1998) (Wood KJ and Goto R. Transplant. 2012); T helper 1 cells (Th1) play a major role. They induce cell apoptosis by secreting tumour necrosis factor α (TNF-α), and they release inflammatory cytokines, like interleukin-1 (IL-1), interferon-γ (INF-γ) and TNF-α to attract monocytes, macrophages, natural killer (NK) cells, neutrophils and eosinophiles, which then participate in the destruction of the graft by secreting unspecific mediators such as reactive oxygen species and inflammatory arachidonic acid products, and by amplifying T cell activation. Moreover Th1 activate epithelial cells to acquire a fibroblast phenotype and they promote fibrosis.

B cells, activated by Th cells, participate in the rejection process by producing specific allo-antibodies that recognize donor cells and induce graft damage through the activation of the complement cascade or through the Ab-dependent cell-mediated cytotoxicity carried out by macrophages and NK cells.

The invasion of the graft by T cells and inflammatory cells, the loss of differentiated tissue-specific cells, and the development of fibrosis all cooperate to cause alterations in the architecture of the tissue, and the loss of function of the graft.

Table 2. Summary of the different subsets of CD4+ T helper cells[1].

Subsets	Differentiation factors	Transcription factors	Secreted cytokines	Mechanisms of action
Th1	IL-12	Tbet	IL-1, INF-γ, TNF-α	See the text
Th2	IL-4	GATA-3	IL-4, IL-5, IL-13	Eosinophil attraction
Th9	IL-4, TGF-ß	-	IL-9	Mast cell attraction
Th17	IL-1, IL-6, IL-21, IL-23, TGF-ß[2]	RORγT	IL-17, IL-21, IL-22 (INF-γ)	Neutrophil attraction
Tfh[3]	IL-21	bcl-6	IL-21	B cell maturation

Notes to Table 2.

1 The role of regulatory T cells will be discussed in the dedicated chapter.

2 Transforming growth factor-ß.

3 Follicular helper CD4+ T cells.

3. Currently available immunosuppressive drugs.

The arsenal of immunosuppressive drugs has become wider and more powerful over the years, thus allowing a considerable reduction in the incidence of the early acute rejection, and also some personalization of the immunosuppressive regimen.

The drugs currently available for the clinic and their main characteristics are summarized in Table 3 (Halloran PF. N Engl J Med. 2004) (Taylor AL, et al. Crit Rev Onc. 2005).

Notes to table 3.

1 Immunosuppression; 2 acute rejection; 3 calcineurin; 4 FK Binding Protein 12; 5 thrombotic microangiopathy; 6 inosine monophospathe dehydrogenase; 7 post-transplant lymphoproliferative disease.

Table 3. Drugs currently utilized for transplantation immunosuppression.

Class	Drugs	Indications	Approval	Mechanism of action	Side Effects
Corticosteroids	Methylprednisolone, Prednisolone, Prednisone	IS[1] induction IS maintenance AR[2] treatment	-	decrease pro-inflammatory cytokines synthesis, impair macrophage function; reduce circulating CD4+ T cells	Hypertension, diabetes, dislipidemia, bone avascular necrosis; osteoporosis, cataract, glaucoma
Antimetabolite	Azathioprine	IS maintenance	1968	inhibits cell proliferation	Myelosuppression
CN[3] inhibitors	Cyclosporine,	IS maintenance	1983	inhibits cell activation and IL-2 production; binds to cyclophilin	Hypertension, diabetes; hyperkalemia; neuro-hepato- nephro-toxicity; gingival hypertrophia; hypertricosis; TMA[5]
	Tacrolimus		1994	as above; binds to FKBP12[4]	
mTOR inhibitors	Sirolimus (Rapamycin) (Rapa)	IS maintenance	1999	inhibits cell proliferation; binds to FKBP12	Diabetes, dislipidemia; oedema; delayed wound healing; interstitial lung disease; myelotoxicity; arthralgia; stomatitis; lymphocele; proteinuria
	Everolimus				

Table 3 continued.

Purine synthesis inhibitors	Mycophenolate Mofetil	IS maintenance	2000	inhibits IMD[6] and blocks T and B cell proliferation	Gastro-intestinal symptoms; myelosuppression
	Mycophenoic Sodium		2004	as above; improved bioavailability	
Polyclonal Depleting Ab	Anti-thymocyte globulin	IS induction AR treatment	1998	depletes T cells; polyclonal Ab from horses or rabbit immunized with human thymocytes	Cytokine release syndrome; leukopenia, myelosuppression; PTLD[7]
Monoclonal Depleting Ab	Muromonab	IS induction AR treatment	1992	binds to CD3; depletes T cells; murin monoclonal Ab	
	Campath-1H	IS induction AR treatment	-	binds to CD52, depletes T and B cells; humanized monoclonal Ab	
Not depleting Ab	Daclizumab	IS induction	1997	binds to CD25, blocks IL-2 pathway; humanized monoclonal Ab	Safe (no events added to background)
	Basiliximab		1998	as above, chimeric monoclonal Ab	
	Belatacept	IS maintenance	2012	binds to CD80 and CD86 on APCs and blocks co-stimulation; CTLA-4 fusion protein with human IgG Fc	PTLD[7]

3.1. Phases of clinical immunosuppression for transplantation.

As acute rejection was initially deemed the major detrimental factor for graft survival (Hariharan S, et al. N Engl J Med. 2000), its prevention has been the first concern for transplantation physicians and the newer immunosuppressive strategies have been developed over the last years based on this concept (Sayegh MH, and Remuzzi G. Lancet 2007).

As a consequence of this approach, immunosuppression might be regarded as a "two plus one phases" therapy, composed of the phases of induction, maintenance and, possibly, treatment of acute rejection.

3.1.1. Induction phase.

The induction of IS is meant as the powerful, but short, immunosuppressive treatment, administered in the peri-transplantation phase (from a few hours before to some days after the transplantation), in order to deeply suppress the alloimmune response, prevent AR while graft is healing, and enable the reduction of maintenance IS thereafter.

Two different classes of drugs are utilized as inductive agents: lymphocyte depleting Abs and blocking anti-CD25 Abs, both in combination with corticosteroids.

Thymoglobulins and Campath-1H belong to the first class; although they have different characteristics, summarized in Table 3 (and examined in depth in chapter 5.4.3. for Campath-1H), they share similar effectiveness in preventing AR and prolonging graft survival (Hanaway MJ, et al. N Engl J Med 2011). They induce profound and sustained depletion of lymphocytes, and dramatically reduce the donor specific anti-HLA circulating precursors responsible for the early alloimmune response. Due to the deep IS which they induce and the linked side-effects, their employment was limited to high immunological risk transplantations, such as in complete absence of any HLA-compatibility, presence of anti-HLA Abs, successive-to-the-first transplantation, prolonged ischemia times, not-heart-beating donor, Afro-American or Hispanic recipient. Depleting

Abs have become more common over the last few years since new strategies aimed at the minimization of maintenance IS have appeared.

Daclizumab and Basiliximab are both monoclonal Abs (moAbs) directed against the α chain of the IL-2R, CD25; they halt "signal 3" of the process of T cell activation, by blocking IL-2R and related signal transduction, and prevent the allo-immune response from mounting against the graft. They are safe drugs, but less powerful than the depleting agents (Hanaway MJ, et al. N Engl J Med 2011), and their employment is now usually restricted to recipients at low immunological risk and to older patients.

3.1.2. Maintenance phase.

Following the induction phase, maintenance IS is started and continued in order to prevent graft rejection for the entire survival of the graft, or until malignancies or severe infections incompatible with IS occur in the recipient.

For at least two decades after the introduction of cyclosporine in the 1980s the classic maintenance therapy was a three-drug regimen based on corticosteroids, cyclosporine to inhibit T cell activation through the inhibition of CN, and azathioprine to stop cell proliferation.

As one of the most important side effects of this regimen was the myelotoxicity of azathioprine, when two new classes of immunosuppressants, the mTOR inhibitors and IMD inhibitors, became available in the 1990s, they were initially intended as azathioprine substitutes.

Indeed the IMD inhibitors, which block T cell proliferation by inhibiting purine synthesis, succeeded and have now almost completely replaced azathioprine, due to a similar effectiveness but a safer profile (Sollinger HW. Transpl. 1999).

The utilization of mTOR inhibitors was instead completely rethought as it became apparent that they worsened cyclosporine-induced renal toxicity. Clinical trials with regimens based on mTOR inhibitors and avoidance or suspension of cyclosporine were designed; but after initial enthusiasm, they substantially failed to maintain their promise: not only were the mTOR inhibitors found to be

weaker than cyclosporine at preventing graft rejection, but they also proved to be ill-tolerated drugs, being withdrawn in more than 30% of the patients, and nephrotoxic themselves (Cravedi P, et al. Kidney Int. 2010). Their utilization is currently limited to selected patients, including those who have experienced a malignancy that might benefit from the anti-proliferative effects of the mTOR inhibition.

Tacrolimus is another immunosuppressant introduced in the 1990s: it is a member of the CN inhibitor class, like cyclosporine, with which it shares a similar profile of side effects. Since it proved to be a superior drug at preventing AR than both cyclosporine and Rapa (Ekberg H, et al. N Engl J Med 2007), it has become more popular and its utilization in combination with steroids and IMD inhibitors has by far overtaken that of cyclosporine (U.S. Renal Data System, USRDS 2011 Annual Data Report).

The last of the immunosuppressants approved for the prevention of AR is Belatacept: it is a CTLA-4 IgG Fc fusion protein, which blocks "signal 2", by binding to the costimulatory molecules CD80 and CD86 expressed on the surface of the APCs. Little data is available so far, but in clinical trials, in which the drug was tested as a substitute for cyclosporine in combination with steroids and IMD inhibitors, it showed a similar effect to cyclosporine at preventing AR over a 3-year follow-up (Vincenti F. Am J Transplant. 2012).

3.1.3. Treatment of rejection.

Following the onset of a progressive loss of graft function, in the absence of other obvious causes for it, a clinical diagnosis of graft rejection is made, and a histological confirmation is often not required prior to start an anti-rejection treatment with boluses of methylprednisolone at high dosage. The restoration of the graft function *ex-post* confirms the clinical diagnosis of rejection.

In the absence of a significant improvement of graft function with corticosteroid boluses the histological analysis of the graft is mandatory. If the diagnosis of rejection is pathologically confirmed, the lack of response to corticosteroids supports the possibility of a more severe grade

rejection (Solez K, et al. Am J Transplant. 2008), which is usually characterized by T cell invasion of the small artery walls of the graft (arteritis), and for which a treatment with T cell depleting antibodies is required. T cell ablation is usually effective and, following the reversion of the rejection process, maintenance therapy is then restarted with the adoption of more powerful immunosuppression.

No standardized therapy has been established to treat rejections resistant to T cell ablation: a reasonable approach is to suspect a humoral rejection, which can be treated with therapy targeting anti-donor Abs with plasmapheresis, B cells with rituximab, an anti-CD20 depleting moAb, or the complement cascade with eculizumab, an anti-C5 component moAb (Clatworthy MR. Am J of Transpl. 2011).

4. Transplantation outcomes.

The introduction in clinical practice of new immunosuppressants in the decade 1990-2000, together with the improvement of tissue matching, organ collection and preservation, and surgical technique, has brought about a considerable reduction in the incidence of AR and an increase in the short-term survival rates: for kidney transplantation the incidence of AR within the first year post-transplant has steadily declined from more than 40% in the early 1990s to circa 10% in 2008; and the 1-year graft survival has improved to circa 90% (U S Renal Data System, USRDS 2011 Annual Data Report).

Contrary to predictions, and quite surprisingly, the long-term outcomes have not benefited significantly from such short-term improvements (Meier-Kriesche HU, et al. Am J Transplant. 2004), and are still unsatisfactory (Table 4).

Table 4. Recipient and graft survivals[1].

Organ transplantation	Kidney	Pancreas	Liver	Heart	Lung
1-year graft survival[2] (cohort 2007)	91.7%	74.8%	85.2%	88.3%	81.5%
10-year graft survival[2] (cohort 1998)	44.9%	35.1%	54.8%	54.7%	26.1%
10-year recipient survival (cohort 1998)	62.0%	77.6%	60.4%	56.4%	28.8%

Notes to Table 4.

1 2010 Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data 2000-2009. Department of Health and Human Services, Health Resources and Services Administration, Healthcare Systems Bureau, Division of Transplantation, Rockville, MD; United Network for Organ Sharing, Richmond, VA; University Renal Research and Education Association, Ann Arbor, MI., consulted at the web page http://www.srtr.org/annual_reports/2010/113_surv-new_dh.htm; the data are only relative to deceased donor transplantation performed in the USA.

2 All-cause graft survival, including recipient death with a functioning graft.

4.1. Causes of graft loss and the implication of maintenance immunosuppression.

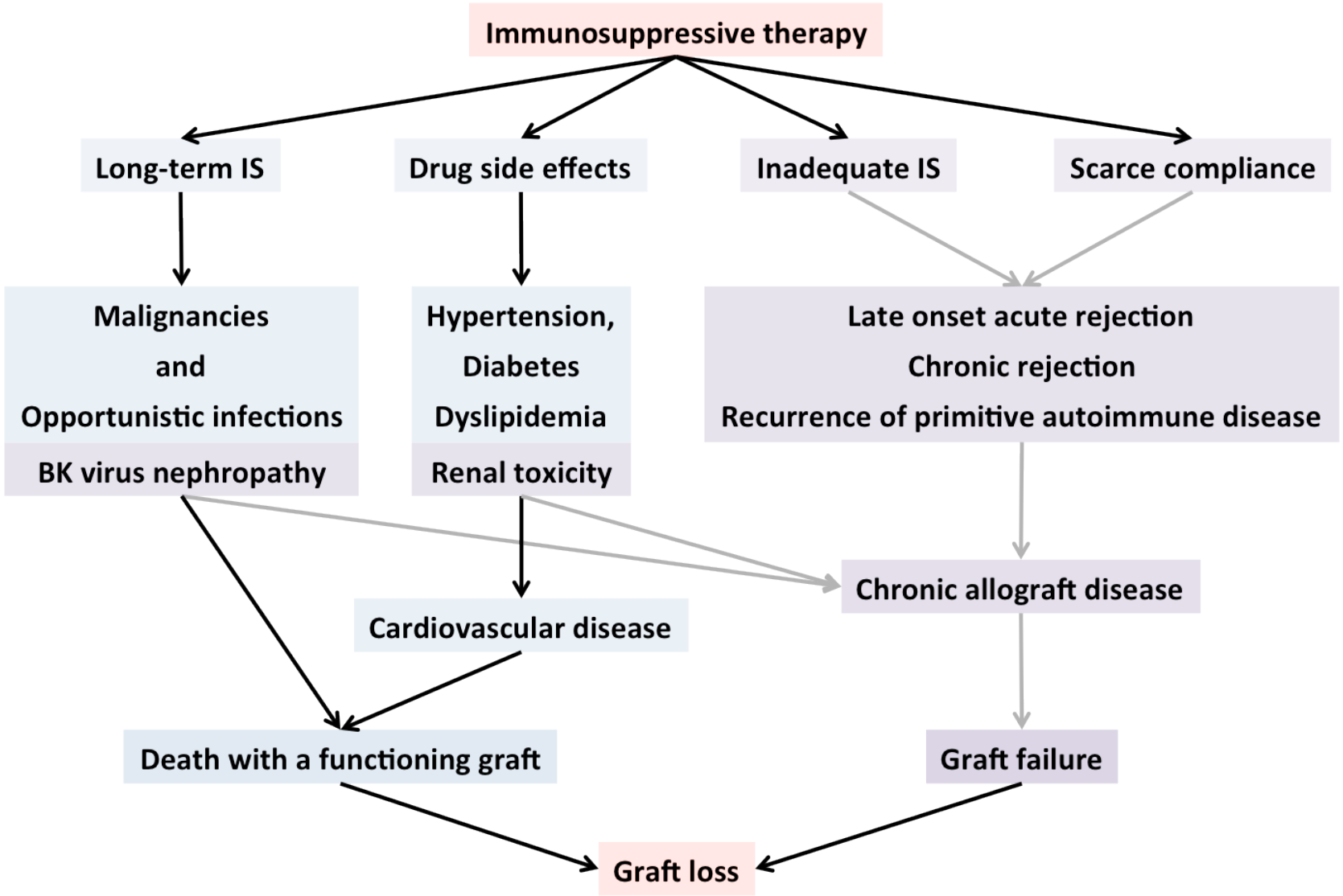
There are several causes of graft loss, which can be grouped into two broad categories: recipient death with a functioning graft and graft failure, the former being mainly determined by cardiovascular diseases (CVD), infections and malignancies, the latter by chronic allograft disease (CAD). Both the two categories of causes contribute equally to the poor long-term graft survival rates, and are associated with the prolonged administration of the immunosuppressive drugs.

Maintenance immunosuppression considerably increases the susceptibility of transplanted patients to opportunistic infections (Fishman JA. Am J Transpl. 2009) and raises 2-4 times the risk of viral-related and unrelated cancers compared to the general population (Engels EA, et al. JAMA. 2011).

Moreover, irrespective of their immunosuppressive properties, corticosteroids and CN inhibitors have severe systemic side effects: they induce hypertension, diabetes and dislipidemia, the main risk factors for CVD, which alone is responsible for circa 30% of the deaths with a functioning graft (U S Renal Data System, USRDS 2011 Annual Data Report: Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, 2011). Some drugs then have organ-specific side effects: the CN inhibitors induce renal chronic toxicity, which accelerates the development of the CAD; mTOR inhibitors may induce proteinuria and have been associated with life-threatening interstitial lung disease (Halloran PF. N Engl J Med. 2004).

Conversely, not only the excess but also the inadequacy of immunosuppression affects transplantation outcomes: late onset of acute rejection, development of chronic rejection and recurrence of the primary autoimmune disease that caused native organ failure, all depend on the inability of current IS strategies to take full control of the immune response. These "immunological" factors, together with hypertension, diabetes and dislipidemia, contribute to the development of chronic allograft disease, by directly causing tissue injuries, such as cellular atrophy, interstitial fibrosis, and arteriosclerosis, which then lead to the failure of the graft (Nankivell BJ, et al. N Engl J Med. 2003).

Figure 1. Implication of maintenance immunosuppression for the loss of the graft.



5. Transplantation tolerance.

5.1. Clinical demand for transplantation tolerance.

The substantial victory over early acute rejection, which was theoretically considered the major determinant of graft survival, has caused a significant improvement in short-term transplantation outcomes, but has unexpectedly failed to positively influence long-term results (Hariharan S, et al. N Engl J Med. 2000).

It is now well recognized that prolonged intense immunosuppression, necessary to prevent the rejection of the graft, has a detrimental impact on long-term survival rates, both for patients and transplants (Meier-Kriesche HU, et al. Am J Transplant. 2004).

Therefore, new immunosuppressive strategies aimed at avoiding the most toxic drugs and at minimizing overall exposure to immunosuppression are currently under investigation.

A short treatment that enables transplantation tolerance, i.e. the specific acceptance of the graft by a fully competent immune system, would represent a solution to the problem:

tolerized recipients would indefinitely maintain their graft, despite being weaned off immunosuppression, thus avoiding exposure to the side effects of immunosuppressive drugs (Kendal AR and Waldmann H. Curr Opinion Immunol. 2010).

5.2. Routes to transplantation tolerance.

Since the discovery in the 1950s that immunological tolerance is an “actively acquired” phenomenon (Billingham RE, et al. Nature. 1953), immunologists have sought to understand and translate this concept into the clinic, being aware that not only transplanted recipients, but also patients suffering from autoimmune diseases, or infections and malignancies, could benefit from therapeutic strategies capable of either harnessing or overriding the mechanisms of tolerance (Waldmann H. Nat Rev. 2010).

The induction of mixed haematopoietic chimerism, following host lymphocyte depletion, has been known for a long time as a reliable strategy to bring about donor-specific transplantation tolerance both in experimental and clinical settings (Wekerle T and Sykes M. Annu Rev Med. 2001).

Central deletion of donor-reactive lymphocyte clones was initially the only mechanism accepted to explain graft tolerance through chimerism (Khan A, et al. Transpl. 1996). The paradigm was the following: first, eliminate pre-existing donor-reactive clones and create "space" for donor bone marrow by ablating host T cells with body irradiation, cytotoxic drugs and depleting antibodies. Second, allow the engraftment of donor haematopoietic stem cells into the host bone marrow compartment, with the subsequent development of mixed haematopoietic chimerism. Third, wait for donor cells to colonize the host's thymus, mature into dendritic cells and mediate negative selection of donor-reactive clones. Tolerance was then deemed to be a merely passive central phenomenon, sustained by the presence of donor dendritic cells in the thymus and by donor haematopoietic cells in the bone marrow compartment; and mixed chimerism, defined as the coexistence of host and donor haematopoietic cells, would be the indicator of such an effective presence.

This knowledge of tolerance, exclusively based on the concepts of central deletion and mixed chimerism, has been revolutionized by the identification of a peripheral active form of tolerance in stringent murine models of transplantation, starting in the 1980s (Hall BM, et al. Transpl. 1984) (Hall BM, et al. J Exp Med. 1985).

The studies carried out by the host laboratory (Therapeutic Immunology Group, headed by Prof. Herman Waldmann) first in Cambridge and then in Oxford have brought to the following discoveries: that host T cell depletion is not an absolute requirement for minor mismatched marrow engraftment, which can instead be obtained, also in thymectomized mice, with the antibody blockade of T-cell co-receptors; that tolerance, so induced, is resistant to the infusion of naïve splenocytes: and, finally, that donor-reactive clones are still present, but anergic, in tolerized recipients (Qin S, et al. J Exp. Med. 1989) (Qin S, et al. Eur J Immunol. 1990). All these findings

were the first indications that allowed the involvement of peripheral mechanisms of active regulation to be uncovered (Bemelman F, et al. J Immunol. 1998).

Moreover, it also became clear that the infusion of bone marrow and the subsequent development of mixed chimerism are not critical for tolerance induced with antibody blockade of T cell co-receptors (Qin S, et al. Eur J Immunol. 1990); but rather, this treatment is capable of "reprogramming" host T cells that become crucial themselves for the maintenance of tolerance, when challenged by the infusion of naïve splenocytes (dominant tolerance) (Cobbold S, et al. Immunol Rev. 1992), and that host T cells are able to actively transfer their suppressive ability to new cohorts of lymphocytes (infectious tolerance) (Qin S, et al. Science 1993).

It is noteworthy that tolerance can be extended towards fully mismatched skin grafts with the addition of the blockade of the co-stimulatory molecule, CD40L, to the co-receptor blockade. Moreover the combined blockade of co-receptors and co-stimulatory molecules was found to enable the development of mixed chimerism upon infusion of standard dose of fully mismatched bone marrow, without any need for the creation of "space" with myelosuppressive conditioning (Graca L, et al. BMC Immunol. 2006).

This form of active peripheral tolerance requires continuous exposure of host T cells to the graft antigens and takes up to five weeks to establish, a period during which the graft is safeguarded by the antibody treatment; but once initiated, tolerance is then self-perpetuating and long-lasting (Scully R, et al. Eur J Immunol. 1994).

Finally, not only is this form of peripheral tolerance dominant and infectious, but it is also defined by the phenomenon of "linked-suppression": an A-strain recipient mouse, tolerized to a B-strain graft, invariably rejects a second graft from a C-strain mouse, but it is instead capable of accepting a graft from a filial 1 (F1) donor cross of the B- and C-strains; after which it accepts a third graft from the C-strain mouse, in the absence of B antigens (Davies JD et al. J Immunol. 1996). This implies that tolerance towards some antigens of the graft can be extended to other antigens when they are presented in closed proximity, if not by the same APC; and that the microenvironment of a tolerated

graft is a site of immune privilege where regulatory T cells operate (Graca L, et al. J Exp Med. 2002).

5.3. Foxp3+ regulatory T cells, crucial mediators of infectious tolerance.

Experiments of selective depletions and adoptive transfers of tolerized T cell subpopulations revealed that, unlike CD8+, CD4+ T cells are crucial for maintenance and contagion of tolerance (Marshall SE et al. Transpl. 1996).

In the wake of the finding that CD4+ T cells expressing the IL-2 receptor α-chain (CD25) maintain self-tolerance (Sakaguchi S, et al. J Immunol. 1995), new studies were conceived to investigate if these CD4+ CD25+ T cells were also responsible for transplantation tolerance. It turned out that naturally occurring CD4+ CD25+ T cells indeed exert a potent immunosuppressive effect, which seems to be independent from antigen specificity or previous exposure to tolerizing treatments (Graca L, et al. PNAS. 2004). However the finding that, following tolerance induction with co-receptor blockade, even CD4+ CD25- T cells are capable of mediating immune suppression (Graca L, et al. J Immunol. 2002), argued against CD25 as a specific marker of such regulatory T cells.

In 2001 it was discovered that loss-of-function mutations of the gene encoding for the transcription factor forkhead/winged-helix protein 3 (Foxp3) are responsible for fatal systemic autoimmune diseases, both in humans suffering from the immune dysregulation, polyendocrinopathy, enteropathy, X-lynked syndrome and in autoimmune-prone scurfy mice (Wildin RS, et al. Nat Gen. 2001) (Bennet CL, et al. Nat Gen. 2001) (Brunkow ME, et al. Nat Gen. 2001). Soon it turned out that Foxp3 is specifically expressed on regulatory CD4+ T cells (Foxp3+ Treg cells), which are predominantly, but not exclusively, CD25+, that Foxp3 exerts a master regulatory function on these cells (Hori S, et al. Science. 2003); and finally that ablation of Foxp3+ Treg cells by specifically targeting Foxp3 causes fatal autoimmunity throughout the lifespan of mice (Kim JM, et al. Nat Immunol. 2007). Two distinct populations of Foxp3+ Treg cells have been identified: one, known as natural Treg cells (nTreg cells), originates in the thymus, has a TCR specificity for self antigens,

and expresses higher levels of the transcription factor Helios, and of the surface protein Neuropilin-1. The other, known as induced Treg cells (iTreg cells), originates in the periphery from conventional CD4+ T cells under different stimuli (see later) and has TCR specificity for both self and not-self antigens. The two populations are thought to have complementary functions, and both are required for preventing the development of autoimmune diseases (Bilate AM and Lafaille JJ. Annu Rev Immunol. 2012).

Given the crucial role of Foxp3+ Treg cells in self-tolerance, their requirement also for transplantation tolerance was soon hypothesized: the discovery of peripheral *de novo* induction of Treg cells with tolerizing protocols corroborated this hypothesis and unveiled a determinant role *in vivo* for TGF-ß (Cobbold S, et al. J Immunol 2004), which had already proven to be able to induce Treg cells *in vitro* (Chen W, et al. J Exp Med. 2003). Only a few years later though, with the generation of Foxp3 reporter transgenic mice, which allows Foxp3+ Treg cells to be selectively depleted or viably sorted, the requirement for these cells as crucial mediators of infectious tolerance could be definitely established (Kendal AR, et al. J Exp. Med. 2011).

Moreover the same study confirms that poised rejecting T cells persist in the tolerated graft, and that the graft itself is a privileged site of active regulation, given that, upon transfer of the tolerated graft into a lymphopenic recipient, the selective ablation of Foxp3+ Treg cells significantly reduces graft survival (Kendal AR, et al. J Exp. Med. 2011).

5.3.1. Not-Foxp3+ subsets of regulatory cells.

Although Foxp3+ Treg cells exert a not redundant suppressive activity, other subsets of regulatory cells have been identified. While the involvement of regulatory B cells, CD8+ T cells and macrophages in infectious tolerance has not been proved yet, a role for some Foxp3- CD4+ T cell subpopulations appears more certain (Gravano DM and Vignali DAA. Cell Mol Life Sci. 2012).

The exposure of CD4+ T cells to IL-10 during TCR stimulation induces a state of anergy in these cells and the emergence of CD4+ CD25- clones, known as type 1 T regulatory cells (Tr1), able to

produce IL-10 and TGF-ß and mediate suppression of both Th1 and Th2 cells (Roncarolo MG, et al. Immunol Rev. 2001).

The exposure of CD4+ cells to IL-4 and TGF-ß in the course of activation expands Th3 regulatory cells (Th3), particularly in the presence of IL-10 and anti-IL-12; Th3 cells secrete a large amount of TGF-ß and suppress both the Th1 and Th2 driven immune response (Weiner HL. Immunol Rev. 2001).

Finally IL-35 converts T cells into induced T regulatory cells (iT(R)35), which in turn through the production of IL-35 exert their suppression activities (Chaturvedi V, et al. J Immunol. 2011).

5.3.2 Mechanisms of action of regulatory T cells.

How these Treg cells operate is still unclear. Experiments *in vitro* have shown that they can secrete different cytokines, like TGF-ß, IL-10 and IL-35, all capable of inducing T cell anergy and of mediating the conversion of naïve T cells into regulatory cells. On the other hand, Treg cells could indirectly mediate suppression by altering the capability of DCs of activating T cells, thus inducing anergy and conversion to a regulatory phenotype. Treg cells physically compete with effector T cells for the antigens displayed by DCs; moreover they down-modulate the expression of co-stimulatory molecules and up-regulate that of inhibitory molecules, like Programmed Death 1 Ligand 1 (PDL1), and of essential amino acid (EAA)-depleting enzymes, like indoleamine 2,3-dioxygenase (IDO). T cells in the graft sense the depletion of EAA caused by such enzymes, in particular tryptophan, and, due to the subsequent inhibition of mTOR signalling, they acquire a regulatory phenotype, rather than becoming activated proliferating effector cells (Gravano DM and Vignali DAA. Cell Mol Life Sci. 2012) (Cobbold SP, Aet al. Proc Natl Acad Sci U S A.).

5.4. Transplantation tolerance in the clinic.

5.4.1. Epithelial graft tolerance through mixed haematopoietic chimerism.

The main hurdle to the widespread clinical application of bone marrow transplantation for the induction of solid organ transplantation tolerance is the requirement, for donor's marrow engraftment, of myelo-suppressive regimens that expose patients to inadmissible risks of severe infections and malignancies.

Indeed the first report of the successful combination of bone marrow and epithelial graft (kidney) transplantation in humans involved patients suffering from multiple myeloma, which had not only caused renal failure, but also represented the rational justification for the risks connected to the conditioning regimen (Bühler LH, et al. Transplant. 2002).

Since this first experience, many efforts have been made to develop less intense, but still permissive, therapeutic protocols, defined as non-myeloablative regimens (Li WH and Sykes M. Nat Rev Immunol. 2012). As summarized in Table 5, the results of these studies are encouraging: stable renal function and transient chimerism were obtained in the majority of patients, with a low incidence of graft versus host disease (GVHD) and a high rate of immunosuppression withdrawal without graft rejection.

Apart from the very small number of patients enrolled, a main concern remains the lack of a definitive proof for the effective requirement for tolerance of haematopoietic chimerism, since no control group without administration of haematopoietic cells was included.

Finally noteworthy is the accompanying infusion of different cell preparations, with the aim of facilitating marrow engraftment by further depleting host lymphoematopoietic cells and preventing GVHD by limiting the expansion of donor host-reactive T cells. In the protocol of the Stanford University, donor CD3+ T cells were administered 11 days after marrow and kidney transplantation; in the protocol of the Chicago Memorial Hospital, a subpopulation of plasmacytoid precursor dendritic facilitating cells (p-preDC FC), which are CD8+ but TCR-, and are prepared together with donor haematopoietic cells (HSC) according to a proprietary undisclosed method,

were administered on the day of the transplantation. Again the effective requirement of such facilitating cells is unclear.

Notes to Table 5.

1 ATG: anti-thymocyte globulins; 2 MMF: Mycophenolate Mofetil; 3 CNI: Calcineurin inhibitors; 4 BM: bone marrow.

Table 5. Clinical trials for graft tolerance through mixed haematopoietic chimerism.

Reference	Scandling JD et al. Am J Transpl. 2012	Leventhal J et al. Sc Transl Med. 2012	Kawai T et al. N Engl J Med. 2008	Fudaba Y et al. Am J Transpl. 2006	Millan MT et al. Transpl. 2002
Previous Reports	Scandling JD et al. N Engl J Med. 2008	-	-	Buhler LH et al. Transpl. 2002	-
Number of Patients	16	8	5	6	4
Multiple Myeloma	No	No	No	Yes	No
Protocol	Stanford University	Chicago Memorial Hospital	Massachussets General Hospital		Stanford University
Irradiation	Total Lymphoid	Total Lymphoid	Thymic	Thymic	Total Lymphoid
Cytotoxic drugs	-	Fludarabine, cyclophosphamde	Cyclophosphamde	Cyclophosphamde	-
Depleting Ab	ATG[1]	-	aCD2, aCD20	ATG[1]	ATG[1]
Initial IS regimen	MMF[2], CNI[3]	MMF[2], CNI[3]	MMF[2], CNI[3]	Steroids, CNI[3]	Steroids, CNI[3]
Organ transplantation	Living donor kidney transplantation				
Bone Marrow tx	CD34+ HSC	HSC	BM[4]	BM[4]	CD34+ HSC
Day of marrow infusion	+11	+1	0	0	+11

Table 5 continued.

Reference	Scandling JD et al. Am J Transpl. 2012	Leventhal J et al. Sc Transl Med. 2012	Kawai T et al. N Engl J Med. 2008	Fudaba Y et al. Am J Transpl. 2006	Millan MT et al Transpl. 2002
HLA-matching	Yes	No	No	Yes	No
Facilitating cells	CD3+ T cells	CD8+ TCR- p-preDCs FC	-	CD3+ T cells	CD3+ T cells
Follow-up, months	12-36	6-20	24-64	24-88	4-13
Functioning organ, n° (%)	16 (100%)	7 (87.5%)	4 (80%)	6 (100%)	4 (100%)
Any chimerism, n° (%)	15 (93%)	8 (100%)	5 (100%)	6 (100%)	3 (75%)
GVHD, n° (%)	0 (0%)	0 (0%)	5 (100%)	3 (50%)	0 (0%)
IS Withdrawal, n° (%)	11 (68%)	5 (62.5%)	4 (80%)	4 (66%)	1 (25%)

5.4.2. Epithelial graft tolerance without chimerism.

Although antibody blockade of T cell co-receptors and co-stimulatory molecules has proven permissive for transplantation tolerance up to the experimental stage of primates, it has never been tested in humans due to legislation limitations that prohibit trialling multiple new agents simultaneously.

Nevertheless, there are different pieces of evidence that transplantation tolerance can also be obtained in humans, without recurring to bone marrow transplantation.

First, given the less immunogenic nature of liver compared to other solid organs and previous anecdotic reports of tolerance in liver recipients, a number of clinical trials have been designed to investigate the feasibility of intentional withdrawal of immunosuppression: the results indicate that tolerance can be achieved in 25% of the recipients, but also that the patients that become tolerant cannot be pre-identified with currently available tests (Orlando G, et al. J Hepatol. 2009).

Second, transplantation tolerance has been described in some solid organ recipients who are found to be not compliant to the immunosuppressive therapy or are forcedly weaned off immunosuppression due to the development of life-threatening infections or malignancies.

Third, long-lasting transplantation tolerance was described in two out of three renal recipients treated with total lymphoid irradiation before the transplantation: although immunosuppression was suspended on the basis of specific lack of response in serial mixed lymphocyte reactions, one patient experienced rejection; moreover severe zoster infections developed in the other two as a consequence of irradiation (Strober S, et al. N Engl J Med. 1989).

These three pieces of evidence not only indicate that transplantation tolerance is achievable, but also that the risks of some strategies are not justified by the potential benefits and that clinical markers to identify tolerized patients are missing.

5.4.3. “Prope tolerance”: the role of Campath-1.

To the best of my knowledge, the only clinical trial conceived to induce and assess tolerance in *humans*, without infusion of bone marrow and body irradiation, is that of Kirk AD and colleagues who treated seven renal graft recipients with Campath-1H and steroids in the peri-transplantation phase, and then completely suspended the IS (Kirk AD, et al. Transpl. 2003).

Campath-1 is a lytic monoclonal antibody directed against the human surface protein CD52 expressed on the surface of B and T cells, which was discovered by Herman Waldmann in the early 1980s in Cambridge in the course of studies aimed at developing lytic anti-human lymphocyte antibodies in the rat, driven by the need of finding a treatment for GVHD. This series of antibodies was first selected above others for their ability to deplete lymphocytes while sparing stem cells, and were named as Cambridge Pathology 1 or Campath-1 antibodies. Campath-1 antibodies were first trialled in patients suffering from refractory haematological malignancies: initial disappointing results in terms of malignant cell clearance, due to the inadequate complement fixation of the IgM (Campath-1M) and IgG2a isotypes, were overcome with the identification of the IgG2b (Campath-1G) isotype that has proven a monoclonal antibody with powerful complement-mediated lytic activity, capable of significantly reducing the bulk of malignant cells. Moreover in a number of different clinical protocols Campath-1 antibodies were also found effective at reducing the incidence of bone marrow rejection and GVHD. At that point the commercial development of the drug, which was also trialled in autoimmune conditions and transplantation, could start; in the meanwhile a humanized version was engineered with the Fc of human IgG1 (Campath-1H) in order to reduce the immunogenicity of the rat antibody.

Campath-1H (alemtuzumab) was approved for the treatment of chronic B cell leukaemia by the Food and Drug Administration in 2001; moreover it is currently under investigation for the treatment of multiple sclerosis, condition for which in a phase III trial it was found more effective than the standard treatment with interferon-ß-1a at preventing relapses and disability over a follow-up of 24 months (Cohen JA, et al. Lancet. 2012) (Coles AJ, et al. Lancet. 2012); and it has

increasingly been used for the induction of immunosuppression in transplantation (Waldmann H and Hale G. Phil Trans R Soc B. 2005).

None of the renal graft recipients recruited in the clinical trial conducted by Kirk AD developed tolerance despite a profound and sustained Campath-1H-induced depletion of peripheral lymphocytes: reversible acute rejection occurred in all the patients, who, after anti-rejection treatment, could be maintained on monotherapy with Rapamycin (Kirk AD, et al. Transpl. 2003).

The choice to trial Campath-1H for the induction of tolerance depended on the findings published a few years before by Calne R et al., who showed that minimization of the IS to monotherapy with cyclosporine was feasible in thirteen renal recipients after T cell depletion with Campath: the authors hypothesized that the initial lymphocyte ablation would avoid organ aggression during the healing phase, at the end of which the graft could have lost immunogenicity and acquired tolerogenic property; the expression “prope tolerance” was coined to define such minimal requirement for IS (Calne R, et al. Lancet. 1998).

Since then, the induction of IS with T cell depleting agents, and Campath-1H in particular, has grown and several clinical trials, including twenty-three randomized trials, have been conducted on the utilization of Campath-1H in transplantation: Campath-1H was found more effective than Basiliximab and as effective as Thymoglobulin in preventing acute rejection (Hanaway MJ, et al. N Engl J Med. 2011); and the induction with Campath-1H has proved permissive for a remarkable minimization of maintenance IS to monotherapy with Cyclosporin (Calne R, et al. Transplant. 1999), Tacrolimus (Tan HP, et al. Am J Transplant. 2009), or Rapamycin (Barth RN, et al. Transpl Int. 2006) (Knechtle SJ, et al. Am J Transplant. 2009).

6. T cell ablation and homeostatic proliferation.

The study of Kirk AD and colleagues has definitely established in humans that T cell ablation, albeit profound and sustained, is alone insufficient for the induction of transplantation tolerance (Kirk AD, et al. Transpl. 2003).

Recent experimental works in mice have identified the emergence of tolerant-resistant T cells through lymphocyte rebound reconstitution as the main reason for failure of T cell ablative therapy to induce tolerance (Wu Z, et al. Nat Med. 2004), which is instead tractable with T cell co-receptors and co-stimulatory molecules blockade.

As for any other system or organ of the body, one of the biological principles that regulate the immune system is that of homeostasis, i.e. the tendency to respond to any perturbation by restoring the original equilibrium. Therefore it is not surprising that, following the development of lymphopenia and irrespective of the cause, whether it be a viral infection, a cytoreductive treatment or body irradiation, a natural process aimed at re-constituting the lymphocyte pool occurs. Both thymic output of new naïve T cells (Berzins SP, et al. J Exp Med. 1998) and peripheral proliferation of surviving T cells in secondary lymphoid organs, known as homeostatic proliferation (HP) (Rocha B, et al. Eur J Immunol. 1989), participate in the process to a different extent (Williams KM, et al. Semin Immunol. 2007).

What is clinically relevant is that reconstitution through HP, whose contribution is largely predominant in adult organisms with a senescent thymus, such as transplant recipients, entails the acquisition of increased immune reactive potential due to the skewing of the lymphocyte repertoire towards a functional memory phenotype (Pearl JP, et al. Am J Transpl. 2005).

The teleologic reason for such conversion would probably be to enhance immune alert and reactivity towards environmental threats in the lymphopenic host; the mechanisms and regulation of it are not fully understood.

If the molecular trigger for HP is still unknown, it is well established instead that peripheral T cells are able to sense the “empty space” and subsequently to start proliferating and carry on until such

space is filled: it has therefore been hypothesized that lymphocyte homeostasis would be regulated by competition, or lack of it as in a lymphopenic environment, for limiting survival and proliferation resources. Competition would also explain why the reconstitution process does not bring about an excessive repopulation, which is prevented by termination of lymphocyte proliferation and extensive cell death once homeostasis has been re-established (Stockinger B, et al. Immunol. 2004).

Repopulation kinetics has been studied *in vivo* by analysing either the dilution of intracellular fluorescent dye 5,6-carboxyfluorescin diacetate succinimyl esterdilution (CFSE) in labelled T cells adoptively transferred into lymphopenic hosts, or by detecting the uptake of the thymidin analogues bromodeoxyuridin (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU) by dividing cells. With the utilization of such methods different patterns of proliferation were found for different T cell subsets with different signalling requirements: recovery of CD8+ T cells is generally faster than that of CD4+ T cells; both naïve CD4+ and naïve CD8+ T cells usually undergo slow HP, which means one division per 24-36 hours; whereas memory T cells undergo faster HP, one division per 6-8 hours; and regulatory T cells present an intermediate pattern (Tchao NK and Turka LA. Am J Transplant. 2012).

Under lymphopenic conditions naïve T cells require two crucial signals to initiate HP: a low-affinity TCR stimulation with self peptide-MHC complexes displayed on APCs, signal that is otherwise unable to promote cell division in a replete host (Ernst B, et al. Immunity. 1999) (Goldrath AW and Bevan MJ. Immunity. 1999), and the availability of the key cytokine IL-7 (Schluns KS, et al. Nat Immunol. 2000). TCR stimulation is instead unnecessary for memory T cells (Murali-Krishna K, et al. Science. 1999), which only need IL-7, and IL-15 at least for CD8+ T cells (Ku CC, et al. Science. 2000). Once initiated, HP then proceeds independently from co-stimulatory signals (Prlic M, et al. J Immunol. 2001) and from the presence of allo-antigens which, albeit not required, can further accelerate the reconstitution by inducing the expansion of the few

antigen-specific clones, which in the end would significantly contribute to the composition of post-lymphopenia T cell repertoire (Maury S, et al. Blood. 2001) (Gorski J, et al. Blood. 2007).

Studies on transgenic mice knocked-out for the genes encoding either IL-7 (IL-$7^{-/-}$) or its receptor (IL-$7R^{-/-}$) have clearly shown that IL-7 signalling exerts a crucial and not redundant role for homeostasis of conventional T cells (Tan JT, et al. PNAS. 2001): through the binding to its receptor, a heterodimer composed of a specific α chain (CD127) and the common γ chain (CD132) shared by the receptors for other cytokines such as IL-2, IL-4, IL-9, IL-15, IL-21 (Ozaki K and Leonard WJ. J Biol Chem. 2002), IL-7 induces T cell proliferation and, by up-regulating the expression of the anti-apoptotic genes such as bcl-2, promotes T cell survival (Schluns KS and Lefrançois L. Nat Rev. 2003).

IL-7 is produced at a constant rate by stromal cells and dendritic cells in the bone marrow, thymus and secondary lymphoid organs where HP occurs (Guimond M, et al. Nat Immunol. 2009); in a lymphopenic environment the reduced consumption of IL-7 by the fewer number of surviving T cells determines a transient increase in the availability of IL-7 per single lymphocyte, which not only induces T cell proliferation but also causes down-modulation and redistribution of the expression of IL-7R. The suppression of IL-7R on T cell surface concurs to the termination of HP by limiting the responsiveness to IL-7, and to the maintenance of a diverse TCR repertoire by allowing the greatest possible number of different T cell clones to experience IL-7 (Park JH, et al. Immunity. 2004). Moreover the redistribution of IL-7R selectively on a small population of effector T cells preserves these cells from apoptosis, allowing them to differentiate into long-lasting memory T cells (Kaech SM, et al. Nat Immunol. 2003).

Unlike for conventional T cells, IL-7 pathway does not affect the homeostasis of regulatory T cells which instead rely on TCR interaction with self peptide-MHC complexes and IL-2 for both survival and proliferation, but not on IL-7 (Le Campion A, et al. J Immunol. 2012) for which they do not even express the receptor (Seddiki N, et al. J Exp Med. 2006), insomuch as the absence of CD127 on the cell membrane has been utilized as cell sorting criterion to improve the purity of human

CD4+ CD25+ Treg cells (Liu W, et al. J Exp Med. 2006). Moreover being unaffected by the IL-7 pathway, Treg cells are in the optimal condition to contribute to the control of HP of conventional T cells: indeed, beside the mechanism of competition for limiting resources, HP is influenced by Treg cells which reduce cell division rate, enhance cell apoptosis and limit the expansion of effector/memory T cells capable of producing pro-inflammatory cytokines (Shen S, et al. J Clin Inv. 2005).

The expansion of memory T cells in the course of HP is determined by several factors: memory T cells proliferate preferentially in a lymphopenic environment, are more resistant to ablative treatments than naïve T cells (Pearl JP, et al. Am J Transpl. 2005), and finally they can result from the conversion of proliferating cells with a naïve phenotype. Interestingly this conversion is determined by the act of proliferating, and not by the condition of lymphopenia *per se*, given that T cells incapable of proliferate maintain their phenotype even in a lymphopenic environment, and the naïve phenotype is restored as soon as the reconstitution process halts (Goldrath AW, et al. J Exp Med. 2000). These emergent memory T cells are characterized by reduced expression of CD62L, or L-selectin, which is required for homing into lymphoid tissues, and increased expression of CD44, required instead for entry into peripheral tissues, site of the immune response; in addition they produce greater amounts of immune mediators, such as INF-γ, and react prompter to allo-antigens presentation compared to conventional T cells. It is worth highlighting that while T cell conversion to a memory phenotype is only transient in irradiated or T cell ablated hosts, it is indefinitely maintained in genetically lymphopenic hosts, such as recombination activation gene knock-out (RAG.1$^{-/-}$) mice (Mombaerts P, et al. Cell. 1992) and severe combined immunodeficiency (SCID) mice (Bosma MJ, Carroll AM. Annu Rev Immunol. 1991) (Cho BK, et al. J Exp Med. 2000), in which naïve T cells are capable not only of classic HP, but also of a faster IL-7-independent "endogenous proliferation" (EP).

However the implications for the clinic of this phenomenon, which apart from genetically lymphopenic mice has been identified only in wild-type neonate mice (Min B, and Paul WE. Sem Immunol. 2005) are still unknown.

Conversely clinical implications of HP have been extensively studied over the last years since it was found an increased incidence of several autoimmune diseases in the course of HP in humans and in experimental animals (Coles A, et al. Clin Neurol Neurosurg. 2004) (King C, et al. Cell. 2004). This increased susceptibility to autoimmune reactions has partially been ascribed to the accidental peripheral expansion of self-reactive clones with high TCR affinity to self-peptide.

In addition clinical interest has been elicited by the idea that HP could be therapeutically exploited to induce a powerful immune response towards either virus-infected or malignant target cells. For patients suffering from malignancies, the development of lymphopenia caused by the administration of chemotherapeutic drugs could be regarded as an opportunity to vaccinate against the tumour: in this condition the exposure of surviving proliferating T cells to tumour antigens would expand reactive clones capable of breaking tumour tolerance and clearing malignant cells. For patients suffering from acquired immunodeficiency syndrome (AIDS) the administration of IL-7 could antagonize lymphopenia caused by HIV infection, by stimulating CD4+ cells survival and proliferation. Clinical trials currently in progress have been reviewed by Mackall CL and colleagues (Mackall CL, et al. Nat Rev Immunol. 2011).

6.1 Homeostatic proliferation and implications for transplantation.

Following the publication of the paper of Kirk AD and colleagues (Kirk AD, et al. Transpl. 2003) different laboratories have experimentally addressed the issue of the failure of T cell ablative therapy to enable transplantation tolerance, and have identified HP, with the accompanying emergence of memory T cells, as a the main barrier to overcome.

Moxham VF and colleagues discovered that HP elicits the rejection of grafts that would otherwise be accepted by replete hosts: in a murine model of kidney transplantation, transferred T cells, which are allowed to expand through HP in RAG.1$^{-/-}$ mice, become capable of rejecting even low immunogenic grafts, and they express CD44 and produce INF-γ (Moxham VF, et al. J Immunol. 2008).

Wu Z and colleagues found that not only HP can accelerate graft rejection, but it also hinders therapeutic induction of transplantation tolerance: in a murine model of fully mismatched cardiac transplantation co-stimulatory molecule blockade fails to induce tolerance both in T cells undergoing homeostatic proliferation and in resting T cells previously expanded through HP. Most of the experiments were carried out in SCID mice that received syngenic T cells, but similar results in terms of graft rejection were also obtained in conditions closer to the clinic with the use of wild-type mice treated with depleting anti-CD4 and CD8 antibodies (Wu Z, et al. Nat Med. 2004).

Neujahr DC and colleagues discovered that the barrier of HP to the induction of transplantation tolerance might be overcome by the infusion of CD4+ CD25+ regulatory T cells in addition to the blockade of co-stimulatory molecules. The authors, who adopted a murine model of fully mismatched cardiac transplantation and induced lymphocyte ablation with depleting antibodies, also showed that endogenous Treg cells hardly expand in the course of HP, as they proliferate slower than conventional T cells (Neujahr DC, et al. J Immunol. 2006).

The finding that Treg cells are capable of taking control of HP and enable transplantation tolerance raises the possibility that therapeutic expansion of Treg cells from within might be as effective as

their infusion from without, but with the remarkable advantage of avoiding the safety and logistic issues of translating a cell therapy into the clinic.

A first, maybe not completely aware, effort in this direction was the combination of T cell depletion with Rapa, which, despite exerting its immunosuppressive action primarily by preventing the proliferation of activated T-cells, can selectively induce Treg cells by inhibiting mTOR (Battaglia M, et al. Blood. 2005) (Zeiser R, et al. Blood. 2008). Indeed in a recent clinical trial on renal graft recipients, Rapa was found capable of enriching for circulating Treg cells compared to cyclosporin, but this was insufficient to protect the graft from chronic injury (Ruggenenti P, et al. Transplant. 2007).

7. Aim of the thesis.

I investigated whether immunosuppressive protocols built on T cell ablation could enable transplantation tolerance, with the aim to take long-lasting advantage of currently available induction treatments with anti-lymphocyte depleting sera.

I followed two different approaches.

First, I sought to overcome the emergence of tolerant-resistant T cells through homeostatic proliferation by therapeutically guiding the reconstitution process, in order to limit the expansion of effector T cells and favour the enrichment of regulatory T cells.

To this purpose I identified two crucial pathways for homeostatic proliferation and T cell activation: IL-7 signalling and mTOR activity; and I evaluated whether their blockade, respectively with a blocking antibody directed against IL-7Rα (Sudo T, et al. Proc Natl Acad Sci U S A. 1993) and with Rapa, might induce tolerance upon T cell depletion in a stringent murine model of skin transplantation.

Second, I sought to obtain transplantation tolerance for epithelial (skin) graft through the administration of donor bone marrow and the establishment of mixed haematopoietic chimerism.

In order to make such approach clinically practicable and patient friendly, I studied protocols to induce donor marrow engraftment built on T cell depletion, without recurring to any conditioning regimens.

MATERIALS AND METHODS.

1. Reagents, cell media and solutions.

- **Distilled water** (dH_2O) (Gibco, 10977);
- **milliQ water** (mQH_2O);
- **10X Dulbecco's Phosphate Buffered Saline** (10X PBS) without Ca^{2+} and Mg^{2+} (Lonza, BE17-515F);
- **1X PBS** (PBS) either prepared by sterile dilution 10X DPBS with mQH_2O or purchased from PAA Laboratories (H15-002);
- **RPMI 1640+2 mM L-Glutamine** (RPMI) (PAA Laboratories, R15-802);
- **Ultra Low IgG Fetal Bovine Serum** (FBS) (Gibco, 16250);
- **100X Penicillin/Streptomycin** (PAA Laboratories, P11-010);
- **100 mM Sodium Pyruvate Solution** (PAA Laboratories, S11-003);
- **1 M HEPES Buffer Solution** (PAA Laboratories, S11-001);
- **2-mercaptoethanol**;
- **Bovine Serum Albumin** (BSA) (Sigma-Aldrich, A9647);
- **Ethylenediaminetetraacetic disodium salt** (EDTA) (AnalR, 100938B);
- **R10-medium:** RPMI, 10% v/v FBS, 1X Penicillin/Streptomycin, 1 mM Sodium Pyruvate Solution, 10 mM HEPES Buffer Solution, 50 μM 2-mercaptoethanol;
- **Tris-Buffered Ammonium Chloride** (TBAC): 1 part of 0.17 M TRIS, 9 parts of 0.15 M Ammonium Chloride (NH_4Cl), adjusted to pH 7.2 with 4 M HCl and made sterile by filtering through a 0.2 μm filter.

2. Monoclonal antibodies used in this thesis for *in vivo* administration.

The following monoclonal antibodies were conveniently diluted in sterile PBS, filtered through a 0.2 μm filter and stored at 4°C. They were administered *in vivo* at the dosages indicated in the text:

- **Campath-1G**: rat anti-human CD52 IgG2b, depleting in vivo (Hale G, et al. J. Immunol. Methods. 1987);
- **A7R34**: rat anti-IL-7 Receptor α IgG1, blocking *in vivo* (Sudo T, et al. Proc Natl Acad Sci U S A. 1993);
- **YTS177.9.6.1**: rat anti-mouse CD4 epitope A IgG2a, blocking *in vivo* (Qin SX, et al. Eur J Immunol. 1990);
- **YTS105.18.10**: rat anti-mouse CD8 Lyt2 IgG2a, blocking *in vivo* (Qin SX, et al. Eur J Immunol. 1990);
- **MR1**: hamster anti-mouse CD154, blocking *in vivo* (Noelle RJ, et al. Proc Natl Acad Sci USA. 1992);
- **5D2**: mouse anti-mouse CD20 IgG2a, depleting *in vivo* (Genetech);
- **FD441**: rat anti-mouse LFA-1 (CD11a/CD18) IgG2b, blocking *in vivo* (Benjamin RJ, et al. Eur J Immunol. 1988).
- **YTS191.1.2**: rat anti-mouse CD4 epitope A IgG2b, depleting *in vivo* (Cobbold SP, et al. Nature. 1984);
- **YTA3.1.2**: rat anti-mouse CD4 epitope B IgG2b, depleting *in vivo* (Qin S, et al.. Eur J Immunol. 1987);
- **YTS169.4.2.1**: rat anti-mouse CD8 Lyt2 IgG2b, depleting *in vivo* (Cobbold SP, et al. Nature. 1984);
- **YTS156.7.7**: rat anti-mouse CD8 Lyt3 IgG2b, depleting *in vivo* (Qin S, et al. Eur J Immunol. 1987);
- **1D11**: mouse anti-human TGF-ß IgG1, blocking *in vivo* (Dasch JR, et al. J Immunol. 1989);
- **13C4**: mouse IgG1 isotype control.

3. Rapamycin.

Rapamycin (Rapa) (Calbiochem, 553210) was dissolved in 100% ethanol to a concentration of 1 mg/ml; aliquots of 30 µl and 150 µl were made and stored at -20°C. Carboxy-methyl-cellulose sodium salt (Sigma-Aldrich, C5013) was diluted to a concentration of 0.2 g/dl in dH_2O, and stored at 4°C. Stock aliquots of Rapa were freshly diluted with carboxy-methyl-cellulose sodium salt solution to final concentration of either 30 µg/ml or 150 µg/ml on the day of the injections.

Before the first administration, mice were weighed in order to establish the amount of drug to be administered thereafter (Zeiser R, et al. Blood 2008).

The following table (Table 6) was utilized to calculate the amount of Rapa to inject into each mouse

Table 6. Rapamycin dosage.

Rapa Dilution	30 µg/ml	150 µg/ml		
Dosages	0.1 mg/kg	0.5 mg/kg	1.0 mg/kg	1.5 mg/kg
20 g	65	65	135	200
25 g	85	85	170	250
30 g	100	100	200	300
40 g	135	135	270	400
Body Weight	**Injection Volume [µl]**			

4. Experimental animals.

The following mice strains were used in this thesis:

- **CBA/ca** (CBA): H-2^k haplotype with CBA minors;
- **Campath mice** (CP1H): H-2^k haplotype with CBA minors, transgenic (tg) for the expression of human CD52, the target of Campath, on the surface of T cells (Gilliland LK, et al. J Immunol. 1999);
- **B10.BR**: H-2^k haplotype with B10 minors;
- **C57BL/6** (B6): H-2^b haplotype with B6 minors;
- **B6.Foxp3hCD2** (B6.hCD2.KI): H-2^b haplotype with B6 minors; (Komatsu N, et al. Proc Natl Acad Sci U S A. 2009);
- **BALB/k**: H-2^k haplotype with BALB minors;
- **(B6 x BALB/k) F1** H-$2^{b,k}$ haplotype with B6 and BALB minors;
- **Thy1.1**: H-2^k haplotype with CBA minors; allele variants expressing the CD90.1 antigen on the surface of lymphocytes;
- **CBA/Rag**.$1^{-/-}$ H-2^k haplotype with CBA minors; genetically T cell deficient mice due to the knock-out of rag.1 gene (Mombaerts P, et al. Cell. 1992)

All mice were bred and maintained under specific pathogen-free conditions in the animal facility at the Sir William Dunn School of Pathology (Oxford, UK).

5. *Ex vivo* collection, preparation, characterization and separation of immune cells.

5.1. Animal killing.

Animal killing was humanely performed by exposure of the experimental mice to rising concentration of carbon dioxide until respiratory arrest; death was then confirmed by dislocation of the neck, in accordance with the Schedule 1 of the Animal (Scientific Procedures) Act 1986.

5.2. Tissues collection.

5.2.1. Blood collection.

Peripheral blood samples were taken by severing, with a sterile size 24 scalpel blade (Swann-Morton, 0311), a lateral tail vein of mice, which had been pre-heated with an infra-red lamp to cause vasodilatation. Typically four drops of blood per mouse (50-100 µl) were collected into an Eppendorf tube conditioned with 30 µl of Heparin Sodium Salt 100 U/mg (heparin) (MP Biomedicals, 101931).

In addition, blood was taken by means of trans-thoracic puncture of the heart of killed mice with insulin syringes (Terumo) conditioned with heparin.

5.2.2. Thymus, lymph nodes and spleen collection.

Mice were killed in accordance with Schedule 1. After a neck to pubis incision of the skin, the thymus was exposed by cutting the ribs and opening the chest cage. Axillary and subiliac nodes (Van den Broeck W, Derore A, Simoens P. J Immunol Methods. 2006) were then exposed by dissecting the subcutaneous tissues. Finally the spleen was exposed by incising the left flank along the edge of the bottom rib. The exposed organs were isolated, excised and collected into cold R10 medium.

5.2.3. Bone marrow collection.

Mice were killed in accordance with Schedule 1. Hind limb bones were exposed, isolated from surrounding tissues, dissected and collected into cold R10 medium. The bones were washed with 70% v/v ethanol for 1 minute and rinsed with R10 medium. The ends of the bones were cut and the BM was sterilely flushed with R10 medium from the shafts of the bones into a sterile Petri dish, using a 25-gauge needle (Terumo) attached to a 20 ml syringe (Terumo).

5.3. Preparation of cell suspensions.

Single cell suspensions were sterilely prepared. The collected spleen and lymph nodes and the flushed bone marrow were passed trough a sterile 70 μm nylon cell strainer (Cell Strainer, BD Falcon) by applying gentle pressure with the plunger of a 2 ml syringe (Terumo). The strainer was washed with 5 ml of R10 medium and the cell suspension was collected into a Falcon tube and pelleted by centrifugation at 1200 rpm for 5-7 minutes.

5.4. Lysis of red blood cells.

For blood samples, red blood cells were removed by means of osmotic lysis: blood was mixed with 9 parts of dH2O and incubated for 30 seconds; 1 part of 10X PBS was then added to re-equilibrate osmolarity to physiological levels and to stop the lysis; finally white blood cells were pelleted by centrifugation at 1200 rpm for 5 min. In instances of incomplete lysis, the protocol was repeated once or twice.

For single-cell suspensions obtained from spleen, lymph nodes and bone marrow, red blood cells were lysed with TBAC: cells were resuspended in 2 to 3 ml of TBAC and incubated for 1 to 2 minutes at room temperature (RT); 7 ml of R10 medium were then added to stop the lysis; cells were then pelleted by centrifugation at 1200 rpm for 7 minutes, and washed with R10 medium.

5.5. Cell counting.

10 µl of the single-cell suspension obtained as described above were diluted 1 in 10 or 1 in 100 with 0.4% Trypan Blue solution (Sigma-Aldrich, T8154) and live cells were counted using a haemocytometer. The following formula was applied to calculate the total number of cells (Cell n°) in suspension:

Cell n° = counted cells/μm^2 x 10 x 1000 x Dilution Factor x Volume of cell suspension in ml

5.6. Fluorescence-activated cell sorting (FACS) analysis.

The following buffers were prepared and stored at 4°C.

Blocking buffer, utilized to block Fc and non-specific immunoglobulin binding sites, was prepared according the following recipe: BSA 1%, Heat Inactivated [45 min, 56°C] Normal Rabbit Serum (HINRS) 5%, sodium azide (NaN3) 0.1% in PBS 1X.

Washing buffer, utilized to remove the excess of antibodies, was prepared according the following recipe: BSA 0.1%, NaN3 0.1% in PBS 1X.

Fixing buffer was prepared fresh by mixing Vol./Vol. the following stock solutions:

- solution A: BSA 1%, NaN3 0.1% in PBS 1X;
- solution B: formalin 4% in PBS 1X.

Single cell suspensions were obtained and red blood cells were lysed as previously described. Staining was performed in FACS tubes or round-bottomed 96-well plates (Falcon, 353910) using 0.2-0.5x10^6 cells/well.

In order to prevent unspecific binding, the cells were resuspended and incubated for 10 min at 4°C with 50 µl of blocking buffer containing 10 µg/ml of an anti-mouse Fc receptor gamma antibody (2.4G2.D5) (Unkeless JC. J Exp Med. 1979) grown and purified in the Therapeutic Immunology Group laboratory (Sir William Dunn School of Pathology, Oxford, UK).

For cell surface labelling, the cells were centrifuged at 1200 rpm for 5-7 min, resuspended and incubated for 30-60 min. at 4°C in the dark, with 50 µl of blocking buffer containing the primary antibodies appropriately diluted. If required, a further labelling step was similarly performed either using secondary antibody (see table below) or streptavidin conjugates (BD Pharmingen, 554061 and 554067). Between each step, cells were washed with 100-500 µl of washing buffer. Finally cells were fixed with 200 µl of fixing buffer and stored at 4°C in the dark until acquisition of the data.

For intracellular labelling of FoxP3, FoxP3 Staining Buffer Set (eBioscience) was utilized. After the labelling of cell surface markers, permeabilization was performed incubating the cells for 30-60 min with 50 µl of the following freshly prepared solution: 1 part of Fixation/permeabilization Concentrate (eBioscience, 00-5123-43) and 3 parts of Fixation/permeabilization Diluent (eBioscience, 00-5223-56). Cells were then washed with 1X Permeabilization Buffer (eBioscience, 00-8333-56) and resuspended and incubated for 30-60 min at 4°C in the dark with 50 µl of the same buffer containing the anti-FoxP3 antibody. Cells were finally washed with 1X Permeabilization Buffer, resuspended with fixing buffer and stored at 4°C.

Samples were acquired with a 4-colour FACSCalibur flow cytometer (Becton Dickinson) with dual laser excitation (488nm and 633nm). CellQuest software (Becton Dickinson) was used to set parameters of acquisition. Acquired data were analyzed using FlowJo software, version 9.5 (Tree Star, Ashland, OR, USA).

Forward and side scatter gating was used to exclude dead cells. Relevant isotype controls (either eBioscience or BDPharmingen) or unstained samples were used to discriminate labelled and unlabelled cells.

In the following table (**Table 7**) the antibodies utilised for FACS experiments are enlisted:

Antigen	Isotype	Clone	Manufacturer	Catalogue N°
CD3e	Armenian Hamster IgG	145-2C11	BD Pharmingen	553066/7
CD19	Rat IgG2a,k	1 D3	BD Pharmingen	550992
CD45R/B220	Rat IgG2a,k	RA3-6B2	BD Pharmingen	553090
CD11b	Rat IgG2b,k	m1/70	BD Pharmingen	557686
F4/80	Rat IgG2a,k	BM8	BD Pharmingen	11-4801
CD49b	Rat IgGM	DX5	LifeTechnologies	A14741
Ly-6C (GR1)	Rat IgG2b,k	RB6-8C5	BD Pharmingen	553128
CD4	Rat IgG2a,k	H129.19	BD Pharmingen	553652
CD4	Rat IgG2a,k	RM4-5	BD Pharmingen	553051/2
CD8	Rat IgG2a,k	53-6.7	BD Pharmingen	553033/7
CD45.2	Mouse IgG2a,k	104	BD Pharmingen	560695
H-2-K^{b}D^b	Mouse IgG2a,k	28-8-6	BD Pharmingen	553576
CD44	Rat IgG2b,k	IM7	BD Pharmingen	553132
CD62L	Rat IgG2a,k	MEL-14	BD Pharmingen	553152
CD25	Rat IgG1λ	PC61.5	eBioscience	13-0251
CD122	Rat IgG2b,k	TM-ß 1	BD Pharmingen	559884
CD127	Rat IgG2b,k	SB/199	BD Pharmingen	561533
CD90.1	Mouse IgG1k	OX-7	eBioscience	12-0900
CD117	Rat IgG2b,k	ACK2	BD Pharmingen	17-1172
FoxP3	Rat IgG2a,k	FJK-16s	eBioscience	17/53-5773
Rat IgG2a Fc	Mouse IgG2b,k	RG7/1.30	BD Pharmingen	558067
Rat IgG2b Fc	Mouse IgG2b,k	RG7/11.1	BD Pharmingen	553900

Unless differently stated, all the primary antibodies are directed against mouse antigens.

5.7. Cell separation.

5.7.1. T-cell separation.

T cells were sterilely separated as already reported (Graca L, et al. Proc Natl Acad Sci USA. 2004). Buffer 1 was prepared according to the following recipe: 1X PBS**,** 0.1% BSA**,** 2mM EDTA**;** pH was adjusted to 7.4, and the buffer was filtered and stored at 4°C.

Spleens and nodes were collected and pooled together. A single-cell suspension was obtained, and erythrocytes were lysed with TBAC. The retrieved cells were counted and incubated for 45 minutes at 4°C with the following rat monoclonal antibodies: M5/114 and 187.1, respectively directed against mouse H-2-I-A/I-E and kappa chain. Cells were then washed twice with R10 medium and resuspended with Buffer 1 at 10^7 cells per 1 ml of buffer. Sheep anti-rat IgG magnetic beads (Dynabeads, Invitrogen, 110.35) were added to the cell suspension at 50 µl per 10^7 cells, and incubated for 45 minutes on a rotator at 4°C. Cell suspension was then placed on a magnet for 5 minutes, and unbound cells were collected and transferred to a new sterile tube: this step was repeated to remove all the beads and the cells bound to them. Cell purity was checked by FACS analysis.

5.7.2. Hematopoietic stem cell separation.

Hematopoietic stem cells (HSC) were separated with the Lineage Cell Depletion Kit (Miltenyi Biotec, 130-090-858) and the autoMACS Separator: isolation was performed by negative selection, depleting the cells expressing the following lineage antigens: CD5, CD45R, CD11b, GR-1, 7-4 and Ter-119 antigens (Lagasse E, et al. Nat Med. 2000). The following buffers were sterilely prepared and stored at 4°C until the use.

Running buffer: BSA 0.5%, EDTA 2mM in PBS; **rinsing buffer**: EDTA 2mM in PBS.

Single cell suspensions were obtained from donor's bone marrow. No red blood cell lysis was performed. The cells were counted and resuspended with 40 µl of running buffer and 10 µl of biotin antibody cocktail per 10^7 cells and incubated for 15 min at 4°C. 30 µl of running buffer and 20 µl of

anti-biotin microbeads per 10^7 cells were then added for a further 15 min. The cells were then washed and resuspended in 500 µl of running buffer. HSC were isolated with autoMACS Separator, using the Deplete programme, washed in sterile PBS and counted. The purity of the separated population was checked by FACS.

5.8. Cell labelling with carboxyfluorescein diacetate succinimidyl ester for *in vivo* study of cell proliferation.

Cell labelling was performed sterilely. Cells were washed twice with PBS to remove all the proteins in the supernatant, counted and resuspended at 10^7 cells per ml in PBS. A 10 mM aliquot of carboxyfluorescein diacetate succinimidyl ester (CFSE) (CellTrace CFSE Cell Proliferation Kit, Molecular Probes, C34554), previously prepared and stored at -80°C, was thawed and diluted 1 to 30000 with pre-warmed PBS. Cell suspension and diluted CFSE were mixed Vol./Vol. and incubated for 8 minutes at RT. Cold FBS was added to a final concentration of 10% and cell suspension was incubated for 10 minutes on ice to quench CFSE. CFSE labelled cells were washed twice with R10 medium and twice with PBS, resuspended to $50x10^6$ cells/ml in PBS, and finally adoptively transferred into recipient mice at a dose of $10x10^6$ cells/mouse. The mice were killed seven days later, and the spleens were collected for FACS analysis (Quah BJC, Warren HS and Parish CR. Nature Protocols. 2007) and proliferation study (FlowJo. Data Analysis Software for Flow Citometry. Proliferation Tutorial).

5.9. *In vivo* labelling of proliferating cells with 5-ethynyl-2'-deoxyuridine and FACS analysis.

5-ethynyl-2'-deoxyuridine (EdU) (Invitrogen, E10187), a nucleoside analog to thymidine, was utilized for the *in vivo* measure of DNA synthesis, and hence cell's ability to proliferate. Similarly to bromodeoxyuridine (BrdU), EdU is incorporated into cell's DNA during DNA synthesis (S-phase); but unlike BrdU, the detection system is based on a "click reaction", a copper catalyzed covalent reaction between an alkyne (EdU) and an azide (dye azide conjugated to a fluorochrome).

The main advantage of this system is that DNA denaturation is not required to expose and detect EdU, and the risk of signal alteration and antigen destruction is avoided.

Upon arrival, EdU was dissolved with dH_2O to a concentration of 2.4 mg/ml; aliquots were made and stored at -20°C. On the day of the administration to the experimental mice, EdU solution was further diluted with water to a final concentration of 0.8 mg/ml and it was made available *ad libitum* to the mice as drinking water (5 ml of solution/mouse/day). The bottles were covered with aluminium foil to protect EdU from light; the solution was considered stable for 48 hours and changed every other day for six days. At the end of this period, mice were killed, the spleens were collected and a single cell suspension freed from red blood cells was obtained. EdU labelled T cells were detected with Click-iT EdU AF488 Flow Citometry Assay Kit (Invitrogen, C35002), adopting a modified version of the manufacturer's protocol, optimized for FoxP3 staining. Labelling for cell surface antigens and then for FoxP3 were performed according to regular protocols, avoiding PE conjugates as recommended by the manufacturer. The cells were washed and resuspended with Click-iT fixative and incubated for 15 min. at RT in the dark; they were then washed again, resuspended with 1x Click-iT saponin-based permeabilization and wash buffer and incubated for a further 15 min. In the meanwhile, Click-iT reaction cocktail was prepared according to the proportions provided in the handbook, but considering a volume of 200 µl per sample. The cells were washed, mixed with Click-iT reaction cocktail and incubated for 30 min. at RT in the dark. After a final wash step, FACS analysis was performed.

6. Animal experimental procedures.

All procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986. Skin grafting, bone marrow transplantation and T-cell adoptive transfer were all performed between sex-matched mice.

6.1 General anaesthesia and analgesia.

For the induction of general anaesthesia, Xylazine (Rompun, Bayer) and Ketamine (Ketaset, Fort Dodge) were utilized. The two anaesthetics were diluted together with sterile PBS at a final concentration of 1 mg/ml and 10 mg/ml, respectively; the mixture of the two drugs was stored at 4°C, and upon filtration was administered intraperitoneally (i.p.), at a dosage of 70 µl/10 g of body weight, using a BD Micro-Fine insulin syringe (BD Medical).

For the induction of analgesia, Buprenorphine (Vetergesic, Champion Alstoe) was utilized. The analgesic was diluted in sterile PBS to a final concentration of 0.03 mg/ml, filtered and administered subcutaneously at the dosage of 100 µl.

Before proceeding to skin grafting, the depth of general anaesthesia was evaluated by assessing the tail and pedal withdrawal reflexes. If required, supplemental doses of anaesthetic solution were administered.

6.2. Skin grafting.

Skin grafting was performed using a modification of a previously described technique (Billingham RE and Medawar PB. J Exp Biol. 1951). The lateral thoracic wall of an anaesthetised recipient mouse was shaven and the graft bed was prepared. Full thickness tail-skin of 0.5 to 1 cm^2 was placed in the bed and fastened using the following dressings: a paraffin gauze (Smith & Nephew, UK) directly applied to the graft, then a cotton gauze, and finally a plaster bandage (Gypsona, Smith & Nephew). An autoclip was used to attach the top edge of the cast to the skin at the back of the neck. Seven days after the grafting, the bandage was removed and grafts were observed for

signs of rejection three times a week for the first two months, and weekly thereafter. Graft rejection was defined as absence of viable donor skin; graft acceptance as presence of viable donor skin one hundred days after transplantation. When performing re-grafts, these were transplanted contra-laterally.

6.3. Cell adoptive transfer.

The following populations of cells were adoptively transferred into recipient mice:

- CD3 enriched, CFSE labelled Thy1.1 lymphocytes (10^7 cells per mouse);
- CD117 enriched, lineage antigens negative (Lin^-) HSC ($0.5\text{-}1x10^6$ cells per mouse).

The cells were sterilely washed, resuspended in PBS to a convenient concentration and kept on ice until injection. Recipient mice were pre-heated with an infra-red lamp (IMS) to cause vasodilatation and the cells were injected into the lateral tail vein.

6.4. Bone marrow transplantation.

Bone marrow (BM) donors were depleted of T cells, two to five days before BM collection, with an i.p. injection of 1 mg of each of the following monoclonal antibodies: YTS191.1 and YTA3.1 to deplete CD4+ cells, and YTS169.4 and YTS156.7 to deplete CD8+ cells (Cobbold S, Martin G. and Waldmann H. Eur. J. Immunol. 1990).

On the day of the transplantation, donors were killed in accordance with Schedule 1; femurs and tibias were collected and treated as previously described to obtain a single cell suspension. Red blood cells lysis was not performed. Cells were counted, washed twice in R10 medium and twice in PBS, resuspended in PBS at a final concentration of $125x10^6$ cells/ml, and kept on ice until injection. Vasodilatation was obtained as described and $25x10^6$ BM cells were injected into the lateral tail vein of each recipient.

7. Statistical analysis.

Statistical analysis was performed using Graphpad Prism software, version 5.0d. Log-rank Mantel-Cox test was used to compare graft survival curves; and, unless otherwise specified, 1way-ANOVA followed by Bonferroni's post-tests for comparison among the groups. P values less than 0.05 were considered statistically significant: $p>0.05$ not significant (NS); $p<0.05$ *; $p<0.01$ **; $p<0.001$ ***.

RESULTS 1/2:

“Therapeutic guidance of homeostatic proliferation towards transplantation tolerance”.

1. Introduction.

A major challenge in organ transplantation is improvement of long-term outcome without incurring the numerous side-effects inherent in current immunosuppressive regimens (2010 Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data 2000-2009) (Meier-Kriesche HU, et al. Am J Transplant. 2004).

An increasingly adopted strategy has been to utilize anti-lymphocyte depleting antibodies in the peri-transplant phase as induction therapy to safeguard the graft during the healing process, after which the requirements for immunosuppression are less intense (Calne R, et al. Transplant. 1999). Particularly valuable as an inductive agent is Campath-1H (Hanaway MJ, et al. N Engl J Med. 2011), a humanized monoclonal antibody against human-CD52 (hCD52) (Waldmann H and Hale G. Phil Trans R Soc B. 2005), which effectively depletes lymphocytes and enables a considerable reduction of maintenance immunosuppression (Tan HP, et al. Am J Transplant. 2009) (Barth RN, et al. Transpl Int. 2006) (Knechtle SJ, et al. Am J Transplant. 2009). While the long-term benefits of Campath-1H induction have yet to be established, it is already clear that lymphocyte depletion alone, albeit profound and sustained, does not induce graft acceptance (Kirk AD, et al. Transpl. 2003).

Harnessing mechanisms of tolerance after lympholytic induction could, in principle, allow us to build on its benefits. It would appear however, that the lymphocyte reconstitution, which follows depletion, is detrimental to numerous tolerising strategies due to an unfavourable rebound proliferation of residual lymphocytes. In part, this rebound is mediated through feedback control by interleukin-7 (IL-7) (Schluns KS, et al. Nat Immunol. 2000) (Tan JT, et al. PNAS. 2001) in a process often referred to as homeostatic proliferation (Bourgeois C, et al. J Immunol. 2005). This results in effector/memory T cells emerging preferentially (Rocha B, et al. Eur J Immunol. 1989) (Cho BK, et al. J Exp Med. 2000) (Goldrath AW, et al. J Exp Med. 2000) (Pearl JP, et al. Am J Transpl. 2005), with the lymphocyte population as a whole acquiring resistance to induction of tolerance (Moxham VF, et al. J Immunol. 2008) (Wu Z, et al. Nat Med. 2004). As an infusion of

regulatory T cells could overcome this tolerance-refractory state (Neujahr DC, et al. J Immunol. 2006), I investigated whether one could therapeutically guide reconstitution to favour the emergence of endogenous regulatory T cells at the expense of conventional T cells (Tconv). To this end I considered targeting two signalling pathways, namely IL-7/IL-7-Receptor (IL-7R) and mTOR signalling. Not only are both involved in homeostasis and activation of Tconv, but mTOR inhibition also favours the induction of Foxp3+ regulatory T-cells (Treg) (Battaglia M, et al. Blood. 2005) (Zeiser R, et al. Blood 2008) (Ruggenenti P, et al. Transplant. 2007). Moreover as IL-7R is poorly expressed in Treg (Seddiki N, et al. J Exp Med. 2006), I expected operational selectivity in the quality of reconstitution following IL-7R blockade.

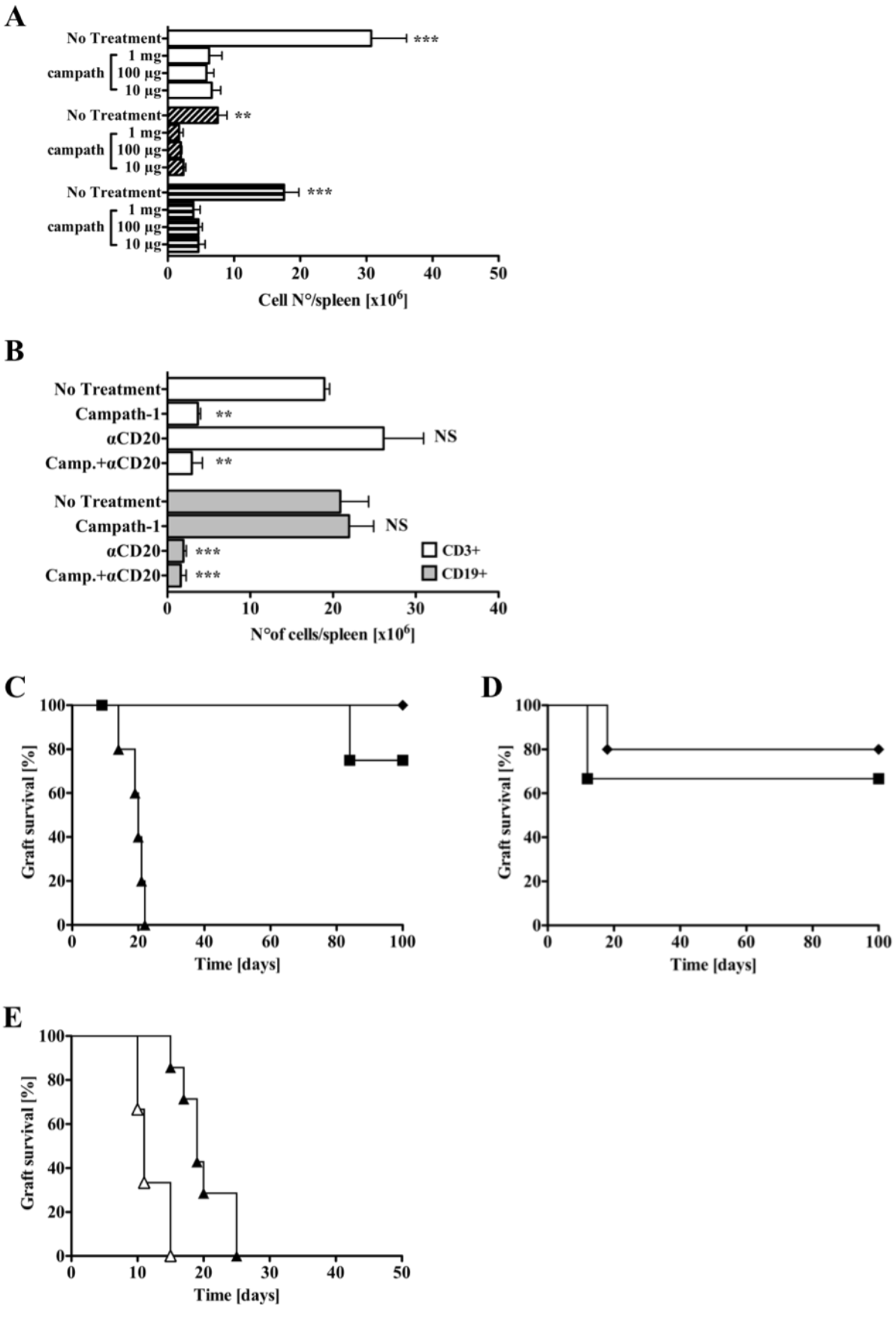
2. Specific methods: dosage of monoclonal antibodies and Rapamycin.

For T cell depletion mice were treated with Campath-1, a rat IgG2b anti-hCD52 (Waldmann H and Hale G. Phil Trans R Soc B. 2005), 1 mg i.p. on days -3 and -1. For B cell depletion mice received a mouse IgG2a anti-murine CD20 (αCD20), 250 μg i.p. on days -3 and -1; this antibody was obtained from Genentech. For co-receptor blockade, mice were given YTS177.9, a rat IgG2a anti-mouse CD4 (αCD4) (Cobbold S, et al. Eur. J. Immunol. 1990), and YTS105.18, a rat IgG2a anti-mouse CD8 (αCD8) (Cobbold S, et al. Eur. J. Immunol. 1990), 1 mg of each i.p. on days 0, 2, 5. For IL-7R blockade, mice received A7R34, a rat IgG1 αIL-7R (Sudo T, et al. Proc Natl Acad Sci U S A. 1993), 7 i.p. injections of 0.4 mg from day -3 to 14. Campath-1, αCD4, αCD8 and rat αIL-7R were produced in host laboratory as previously described. For TGF-ß neutralization, mice were given 1D11, a mouse IgG1 αTGF-ß (Dasch JR, et al. J Immunol. 1989), (or the relevant isotype 13C4) 7 i.p. injections of 2 mg from day -3 to 21; these antibodies were kindly provided by Genzyme. Rapa (Calbiochem) was dissolved in 100% ethanol and stored at -20°C; on the day of the injection a stock aliquot was diluted with 0.2% carboxymethycellulose sodium salt (Sigma-Aldrich) and administered i.p.; unless otherwise specified, mice received 7 injections of 1.5 mg/kg from day -3 to 14 (Zeiser R, et al. Blood 2008).

3. Lymphocyte depletion is insufficient for transplantation tolerance.

Lymphocyte depletion with Campath-1 has increasingly been adopted as induction therapy in organ transplantation (2010 Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data 2000-2009). However, following lymphocyte depletion maintenance immunosuppression, albeit with some reduction, is still required (Kirk AD, et al. Transpl. 2003) as reconstitution through homeostatic expansion continues to replenish cells capable of rejection (Wu Z, et al. Nat Med. 2004). In order to study the effect of lymphocyte reconstitution on graft survival, we utilized, as recipients of MHC mismatched skin grafts, hCD52-Tg mice (Gilliland LK, et al. J Immunol. 1999), in which treatment with Campath-1 was particularly effective at depleting T-cells (Figure 2A and 2B). As also seen in the clinic, T-cell depletion in mice delayed, but never prevented, graft rejection (Figure 2C); moreover additional B-cell depletion could not provide any further benefit (Figure 2E). However, as anticipated (Neujahr DC, et al. J Immunol. 2006), tolerance induction through co-receptor antibody blockade was not hindered by T-cell depletion (Figure 2C and 2D).

Figure 2.


A
No Treatment
campath
1 mg
100 µg
10 µg
***
**
***
0
10
20
30
40
50
Cell N°/spleen [x10^6]
B
No Treatment
Campath-1
αCD20
Camp.+αCD20
**
NS
**
NS
***
***
CD3+
CD19+
0
10
20
30
40
N°of cells/spleen [x10^6]
C
Graft survival [%]
Time [days]
D
Graft survival [%]
Time [days]
E
Graft survival [%]
Time [days]

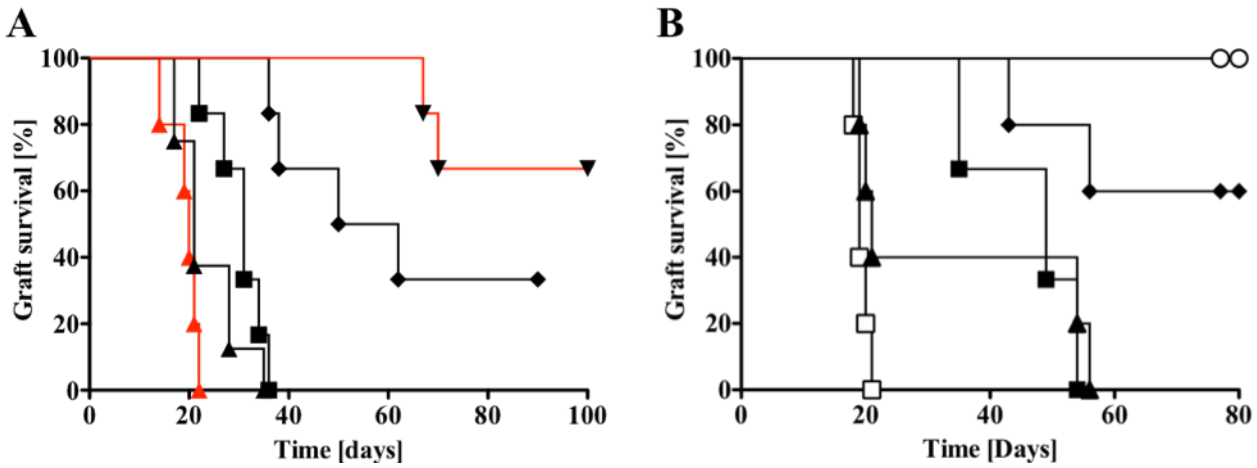
Legend to Figure 2. T-cell depletion is insufficient to promote long-term graft survival, but does not hinder the induction of tolerance with co-receptor blockade. (A) hCD52-Tg mice untreated or treated with Campath-1, two i.p. injections on days -3 and -1 at the dosage indicated in the figure, were euthanized on day 7 (3-5 mice per group); spleens were collected, vital cells counted and characterized by FACS. Absolute numbers were calculated as product of numbers of spleen vital cells and percentage of CD3+ (white bars), CD8+ (diagonally striped bars) and CD4+ T-cells (longitudinally striped bars). Shown are means ±SD of absolute numbers of cells per spleen. **(B)** Same experiments as in A with spleen cells collected from hCD52-Tg mice not treated (n°3), treated with Campath-1 (n°3), with αCD20 (n°3), or with both drugs (n°4) on day 7. Shown are means ±SD of absolute numbers per spleen of CD3+ cells (white bars) and CD19+ cells (grey bars). **(C)** Fully mismatched B6 skin grafts were transplanted to (hCD52-Tg×CBA/Ca)F1 heterozygous mice under the cover of different treatments. Co-receptor blockade with αCD4 and αCD8 (◆, n°5) induced graft acceptance in all the treated mice (MST >100 days). Depletion of T-cells with Campath-1 (☐, n°5) was insufficient to prevent graft rejection (MST 20 days). Treatment with αCD4 and αCD8 following depletion with Campath-1 (■, n°5) induced long-term graft survival (MST >100 days, p=0.0049 compared to Campath-1, p=0.26 vs αCD4 and αCD8). 1 mouse treated with Campath-1, αCD4 and αCD8 was censored in the survival analysis on day 9 due to surgical failure. **(D)** The mice that had accepted the first skin graft under the cover of Campath-1, αCD4 and αCD8 (■, n°3), or αCD4 and αCD8 (◆, n°5) received a second B6 skin graft 100 days after the first graft. The majority of the mice in both the groups accepted also the second grafts, and there was no difference in graft survival between the two groups (MST >100 days for both the groups, p=0.6). **(E)** Fully mismatched B6 skin grafts were transplanted to hCD52-Tg mice under the cover of αCD20 alone (△, n°6) or in combination with Campath-1 (☐; n°5). Lymphocyte antibody depletion could not prevent graft rejection in any mice; however treatment with αCD20 and Campath-1 significantly delayed rejection compared to αCD20 alone (MST 19 and 11 days respectively, p=0.0015)

4. A short-course treatment with αIL-7R blocking antibody and Rapamycin promotes long-term graft survival following T cell depletion.

Having established that tolerance is possible after lymphocyte depletion, and given that αCD4 and αCD8 reduce the proliferation of reconstituting T cells (Neujahr DC, et al. J Immunol. 2006), I explored whether another more clinically feasible route to induce tolerance might be therapeutically skewing the reconstitution process so as to give an advantage to regulation. Two strategies seemed plausible. The first takes account of the established role of IL-7 signalling through IL-7R in promoting homeostatic expansion of immune cells (Schluns KS, et al. Nat Immunol. 2000) (Tan JT, et al. PNAS. 2001). Treg express less IL-7R (Seddiki N, et al. J Exp Med. 2006), and therefore could gain competitive benefit following IL-7R blockade. The second, the inhibition of mTOR signalling, derives from selective effects of the mTOR inhibitor on immune function, as well as the known impact on induction of Treg (Battaglia M, et al. Blood. 2005) (Zeiser R, et al. Blood 2008).

The combination of Campath-1 and short-course IL-7R blocking antibody (αIL-7R) and Rapa proved very effective with extended graft survival in virtually all the recipients (MST 56 days, $p=0.001$ vs Campath-1+Rapa), and, in a separate experiment with a prolonged course of Rapa, indefinite graft survival in two thirds of the mice (MST>100 days) (Figure 3A). The synergy between αIL-7R and Rapa was also observed when small numbers of T-cells were adoptively transferred into genetically lympho-deficient mice, where Campath-1 had not been used to purge T-cells (Figure 3B).

Figure 3.



Legend to Figure 3. The combination of αIL-7R and Rapa promotes long-term graft survival.

(A) (hCD52-Tg×CBA/Ca)F1 heterozygous mice were transplanted with a fully mismatched B6 skin graft under the cover of different treatments. All the mice treated with Campath-1 and αIL-7R (▲, n°7) rejected their graft (MST 21 days), as did mice treated with Campath-1 and Rapa (■, n°6) (MST 31 days, p=0.11 vs Campath-1 and αIL-7R). A short-course treatment with αIL-7R and Rapa following T cell depletion with Campath-1 (◆, n°6) significantly extended graft survival (MST 56 days, p=0.001 vs Campath-1 and αIL-7R or Rapa). In a separate experiment a prolonged administration of Rapa (10 injections over the first 35 days) (▼, n°6) promoted long-term graft survival with 4 mice accepting the graft for more than 100 days (MST>100 days). **(B)** Fully mismatched B6 skin grafts were transplanted to CBA.Rag1$^{-/-}$ mice 3 days after the adoptive transfer of syngeneic (CBA/ca) CD3-enriched cells. A group of mice did not receive T-cells (○, n°4) and accepted the grafts indefinitely (MST>75 days); whereas mice that received T-cells without any treatment (□, n°5) promptly rejected the grafts (MST 19 days). Treatment with αIL-7R (▲, n°5) did not delay graft rejection (MST 21 days, p=0.09 vs No Treatment). Treatment with Rapa (■, n°3) significantly extended graft survival (MST 49 days, p=0.01 vs No Treatment), but could not prevent the rejection of the graft. The combination of αIL-7R and Rapa (◆, n°5) promoted long-

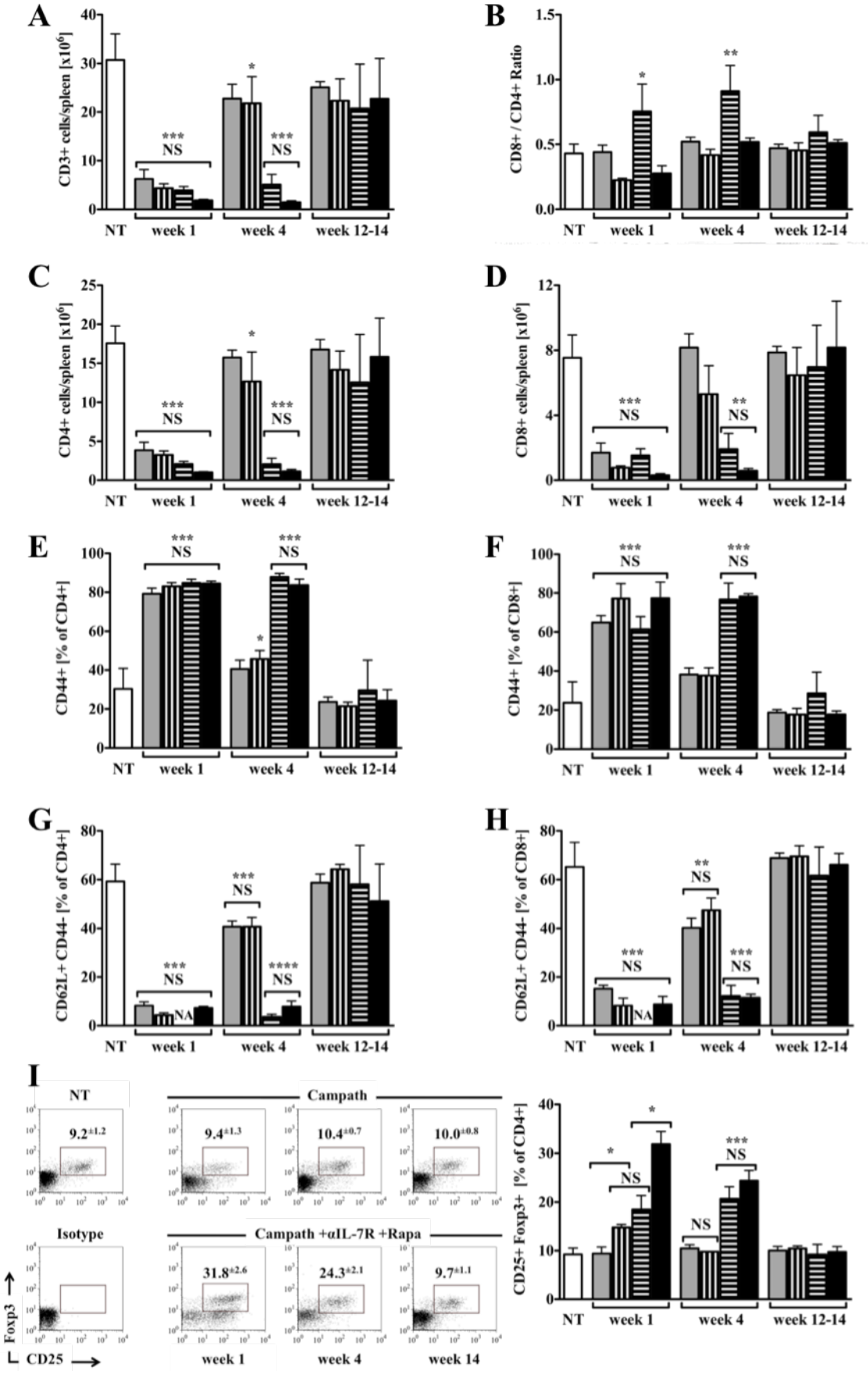
term graft survival, and induced graft acceptance in 3 mice (MST>75 days, p=0.02 and 0.04 vs αIL-7R alone and Rapa alone, respectively). Data are aggregated results of 3 different experiments; within each experiment all the mice of different groups received the same number of cells: 6.5, or 7.5 or $9.5x10^6$.

5. The combination of αIL-7R antibody blockade and Rapamycin favourably shapes T cell reconstitution.

5.1. Treatment with αIL-7R and Rapa delays T cell reconstitution.

In order to establish the extent to which αIL-7R and Rapa could influence T cell reconstitution, I treated hCD52-Tg mice with Campath-1 alone and in combination with either αIL-7R or Rapa, or both the drugs. Splenic T cell counts reached the nadir 1 week following treatment with Campath-1, irrespective of any additional drug ($p<0.001$ vs untreated group). Thereafter T cell reconstitution started and was complete by week 4 in mice treated with Campath-1 alone, and proceeded similarly in mice that received αIL-7R. In contrast reconstitution was delayed at week 4 in mice that received Rapa, alone or in combination with αIL-7R ($p<0.01$ vs untreated group); nevertheless even in these mice T cell counts returned to basal levels by week 12-14, i.e. 10-12 weeks after Rapa was stopped (Figure 4A, 4C, 4D). The addition of αIL-7R to Rapa prevented the imbalance between reconstituting CD8+ and CD4+ cells at weeks 1 and 4 (Figure 4B). Following depletion, and irrespective of any additional drug, the emerging pattern of T cell phenotypes was significantly skewed towards an effector/memory composition characterized by more CD44+ cells and fewer CD62L+. This skewing was transient and disappeared once T cell reconstitution was complete (by week 4 for mice treated with Campath-1±αIL-7R, and by week 12-14 for those treated with Campath-1+Rapa±αIL-7R) (Figure 4E-H). Finally the addition of αIL-7R to Campath-1 induced a significant increase in the proportion of CD25+ Foxp3+ Treg at week 1; Rapa had a similar effect, which extended through to week 4; the combination of αIL-7R and Rapa further increased the proportion of reconstituting Treg, which however returned to basal levels by week 12-14. These data suggest that the treatment with αIL-7R and Rapa following depletion with Campath-1 might promote graft acceptance by delaying T cell reconstitution, maintaining a normal CD8/CD4 ratio and enriching for Treg in the early reconstitution period.

Figure 4.


A
CD3+ cells/spleen [x10⁶]
B
CD8+ / CD4+ Ratio
C
CD4+ cells/spleen [x10⁶]
D
CD8+ cells/spleen [x10⁶]
E
CD44+ [% of CD4+]
F
CD44+ [% of CD8+]
G
CD62L+ CD44- [% of CD4+]
H
CD62L+ CD44- [% of CD8+]
I
NT
Campath
Isotype
Campath +αIL-7R +Rapa
9.2±1.2
9.4±1.3
10.4±0.7
10.0±0.8
31.8±2.6
24.3±2.1
9.7±1.1
Foxp3
CD25
week 1
week 4
week 14
CD25+ Foxp3+ [% of CD4+]
NT
week 1
week 4
week 12-14
NS
NA

Legend to Figure 4. A short-course αIL-7R and Rapa delays T cell reconstitution after depletion with Campath-1 and enriches for reconstituting regulatory T cells. T cell reconstitution was studied in hCD52-Tg mice treated with Campath-1 alone (grey bars) or in combination with either αIL-7R (vertically striped bars) or Rapa (horizontally striped bars) or both drugs (black bars) (3-6 mice per group per time point). Spleens were collected 1, 4 and 12-14 weeks after depletion; vital cells were counted and characterized by FACS. Statistical analysis was performed with 1-way ANOVA followed by Bonferroni's test for comparison of the different groups of mice at a given time point and not treated mice (NT, white bars). Results are represented as mean and standard deviation. **(A, C, D)** CD3+, CD4+ and CD8+ splenic T cells. Absolute numbers were obtained as product of vital cell counts per spleen and relative FACS percentage. **(B)** Ratio of CD8+ vs CD4+ cells. **(E, F)** Percentage of CD44+ effector/memory T cells among CD4 and CD8 subpopulations. **(G, H)** Percentage of CD62L+ CD44- naïve T cells among CD4 and CD8 subpopulations. NA: data not available. **(I)** Percentage of CD25+ Foxp3+ cells among CD4 subpopulation.

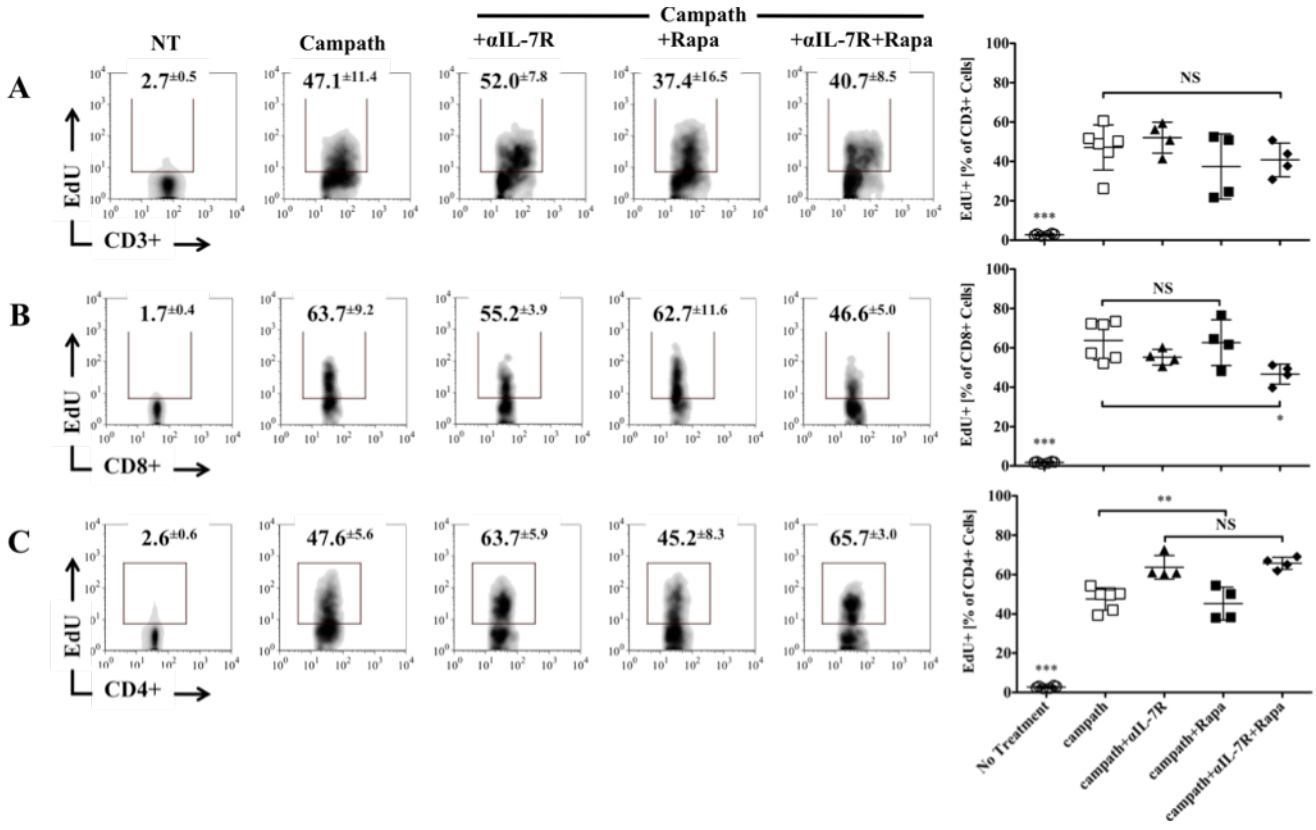
5.2 Treatment with αIL-7R and Rapa reduces proliferation of conventional T cells and enriches for reconstituting regulatory T cells.

I next investigated whether the changes in T cell reconstitution induced by αIL-7R and Rapa were dependent on modulation of T cell proliferation. First I studied proliferation according to EdU incorporation in T cells. The proportion of EdU-labelled cells among splenic T cells was significantly higher in all the groups of mice treated with Campath-1 compared to untreated mice (Figure 5A, 5B, 5C). While EdU incorporation into CD3+ cells seemed unaffected by αIL-7R and/or Rapa (Fig.5A), the data demonstrated a significant reduction in the proportion of CD8+ EdU+ cells (46.6%±5.0 vs 63.7%±9.2; $p<0.05$) (Figure 5B) and an increase in CD4+ EdU+ cells (65.7%±3.0 vs 47.6%±5.6; $p<0.01$) (Fig.5C) with the combination of αIL-7R and Rapa compared to Campath-1 alone. These findings suggest that treatment with αIL-7R and Rapa preferentially blocks CD8+ cell proliferation. The response to treatments of different CD4+ subsets was not studied in this experiment.

The study above was conducted in circumstances where the transition from lymphocyte depletion to reconstitution was not obvious, as all T cells expressed hCD52 and were targets for depletion as long as Campath-1 was circulating. In order to get a clear window on the reconstitution phase, I studied proliferation by transferring CFSE-labelled T cells from Thy1.1+ donors, which did not carry hCD52, into syngenic hCD52-Tg recipients. In accordance with data on reconstitution (Fig.4), Thy1.1+ T-cells transferred into hosts treated with Campath-1 acquired an effector/memory phenotype characterized by higher CD44 expression and lower CD62L, irrespective of any additional drug (Figure 6A and 6B). A significantly higher proportion of Thy1.1+ CD4+ Foxp3+ cells was found in mice treated with Campath-1 compared to controls (23.4%±5.3 vs 6.7%±1.7, $p<0.001$); the addition of αIL-7R or Rapa further enriched for Treg (35.6%±4.0 and 39.0%±4.5, $p<0.01$ vs Campath-1); and the combination of the three drugs was particularly effective at favouring Treg emergence (52.4%±3.7, $p<0.01$ vs Campath-1+αIL-7R or Campath-1+Rapa) (Fig.6C).

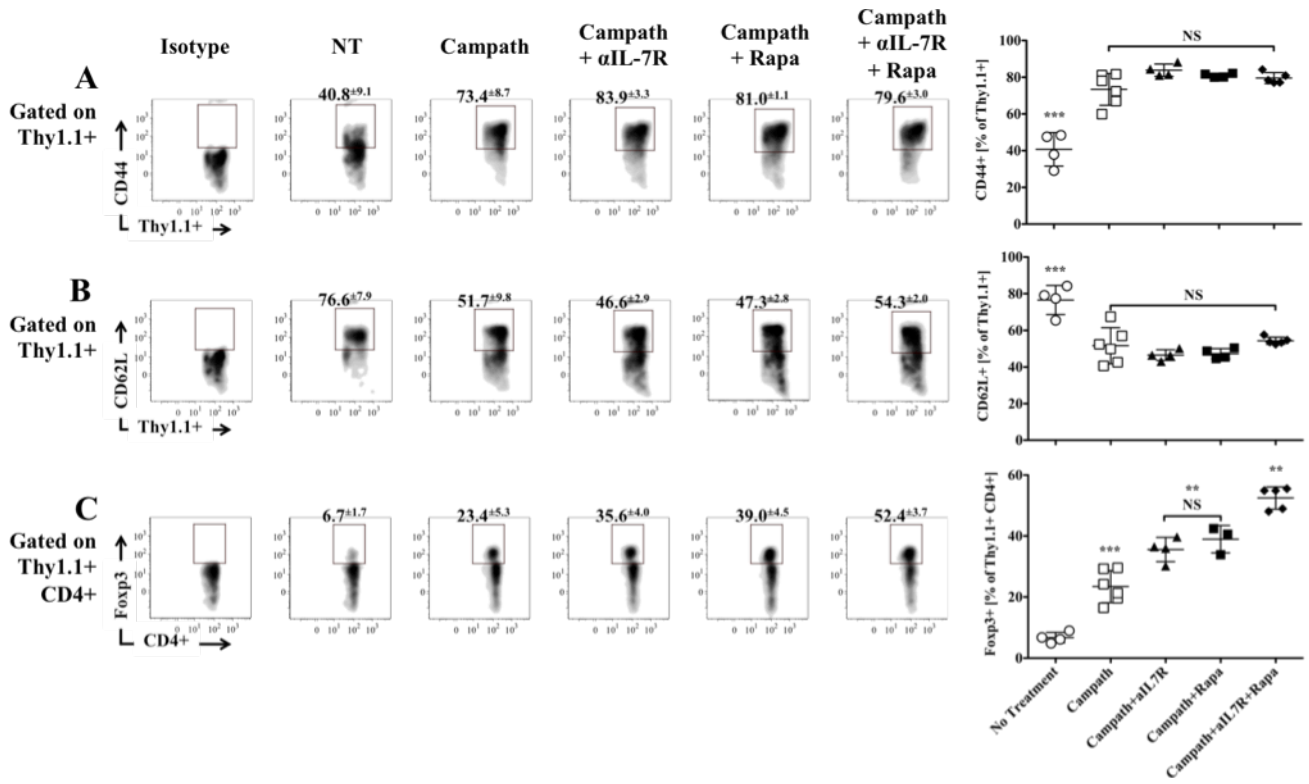
I studied the extent of proliferation of T-cell subsets by analysing CFSE dilution with the FlowJo proliferation platform. Two parameters were considered to better characterize T-cell proliferation: the division index, the mean number of divisions of the whole cell population; and the proliferation index, the mean number of divisions of the cells that have divided at least once (FlowJo, Tree Star, Inc., Ashland Oregon U.S.A. Proliferation Tutorial. Last accessed on 13 February 2013, at http://www.flowjo.com/home/tutorials/proliferation.html). The division index of Thy1.1+ CD8+ and CD4+ cells was increased when the cells had been transferred into mice depleted with Campath-1 compared to that into replete controls (1.5±0.3 vs 0.1±0.1, $p<0.001$ for CD8+; 1.2±0.1 vs 0.1±0.09, $p<0.001$ for CD4+). The addition of αIL-7R or Rapa significantly decreased this index, and the combination of the two drugs further reduced it to levels similar to those found in controls (0.3±0.1, $p>0.5$ for CD8+; 0.2±0.05, $p>0.5$ for CD4+ vs controls) (Figure 7A and 7B, left diagrams). The proliferation index of Thy1.1+ CD8+ cells was not affected by any treatment. Whereas the proliferation index of Thy1.1+ CD4+ cells was increased in depleted mice treated with αIL-7R and Rapa (3.4±0.5 vs 1.8±0.2 in mice treated with Campath-1; $p<0.001$) (Figure 7A and 7B, right diagram). The division index of Thy1.1+ CD4+ Foxp3+ and Foxp3- cells was similar after treatment with Campath-1 (1.0±0.2 vs 1.2±0.2, $p>0.05$); whereas the addition of αIL-7R and Rapa selectively reduced the division index of Foxp3- cells (0.8±0.1 vs 0.1±0.01, $p<0.001$) (Figure 7C). Taken together these results indicate that the combination of αIL-7R and Rapa reduces the homeostatic expansion of CD8+ and CD4+ Tconv, but does not hinder that of Treg, which emerge preferentially under this treatment.

Figure 5.



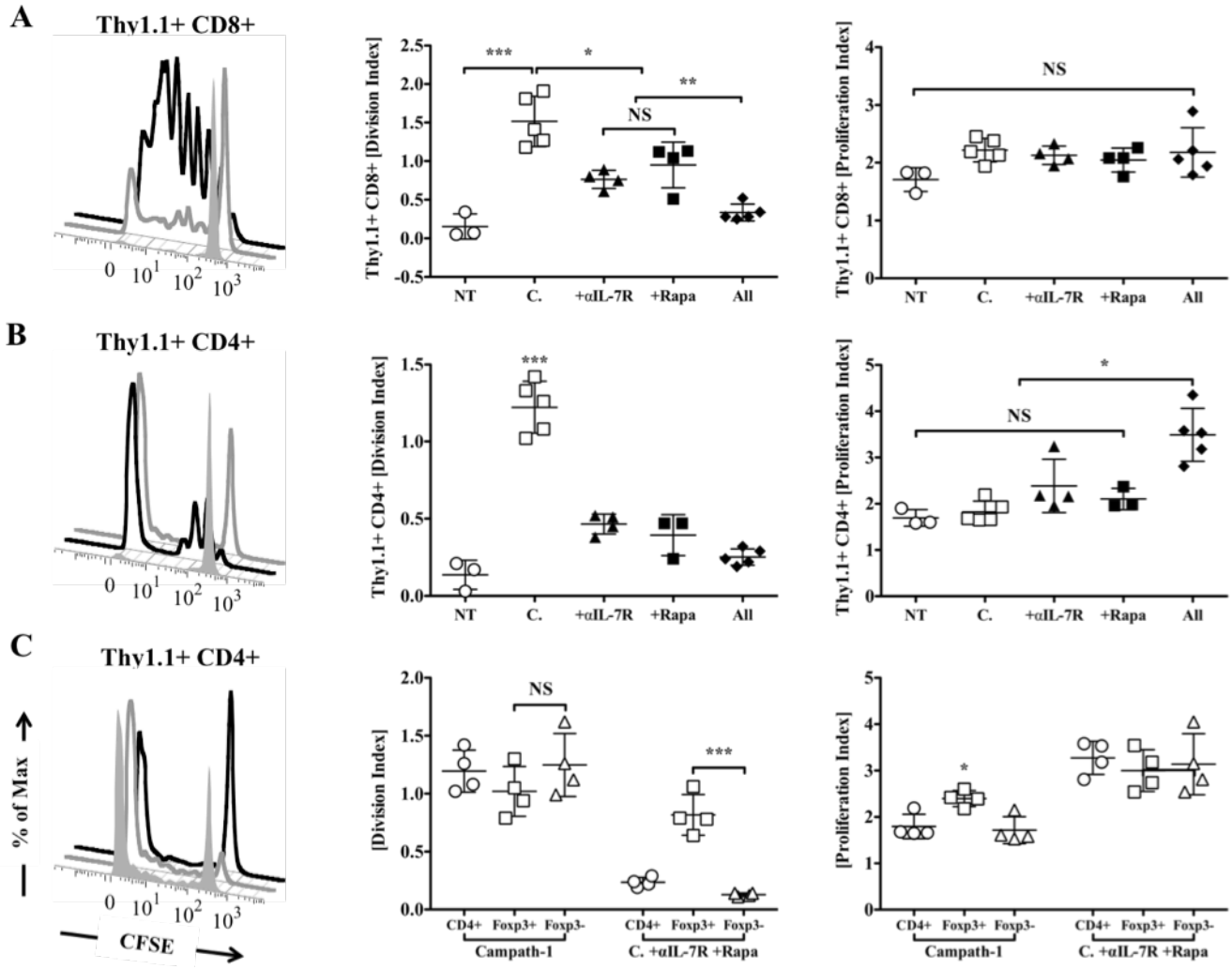
Legend to Figure 5. Treatment with αIL-7R and Rapa reduces the proportion of EdU-labelled CD8+ cells, while increases that of CD4+ cells. EdU dissolved in drinking water at 0.8 mg/ml was administered to hCD52-Tg mice for 6 days from day 1 after T-cell depletion with Campath-1. The following groups were included: no treatment (○, n°6); Campath-1 (□, n°6); Campath-1+αIL-7R (▲, n°4); Campath-1+Rapa (■, n°4); Campath-1+αIL-7R+Rapa (◆, n°6); on day 7 spleens were collected and the proportion of EdU+ T-cells was determined by FACS. **(A, B and C)** Percentage of EdU-labelled cells among CD3+, CD8+, and CD4+ populations.

Figure 6.


Isotype
NT
Campath
Campath + αIL-7R
Campath + Rapa
Campath + αIL-7R + Rapa
A
Gated on Thy1.1+
CD44
Thy1.1+
40.8±9.1
73.4±8.7
83.9±3.3
81.0±1.1
79.6±3.0
B
Gated on Thy1.1+
CD62L
Thy1.1+
76.6±7.9
51.7±9.8
46.6±2.9
47.3±2.8
54.3±2.0
C
Gated on Thy1.1+ CD4+
Foxp3
CD4+
6.7±1.7
23.4±5.3
35.6±4.0
39.0±4.5
52.4±3.7
CD44+ [% of Thy1.1+]
CD62L+ [% of Thy1.1+]
Foxp3+ [% of Thy1.1+ CD4+]
NS
***
**
No Treatment
Campath
Campath+αIL7R
Campath+Rapa
Campath+αIL7R+Rapa

Legend to Figure 6. Treatment with αIL-7R and Rapa enriches for reconstituting regulatory T-cells. 10^7 CFSE labelled CD3 enriched Thy1.1+ cells were adoptively transferred into syngenic hCD52-Tg (Thy1.2+) mice. The following groups were included: no treatment (○); Campath-1 (□); Campath-1+αIL-7R (▲); Campath-1+Rapa (■); Campath-1+αIL-7R+Rapa (◆) (3-6 mice per group). 7 days later splenic cells were collected and analyzed by FACS. **(A)** Expression of the memory marker CD44 on Thy1.1+ cells. **(B)** Expression of the naivety marker CD62L on Thy1.1+ cells. **(C)** Percentage of Foxp3+ cells in the Thy1.1+ CD4+ subset.

Figure 7.

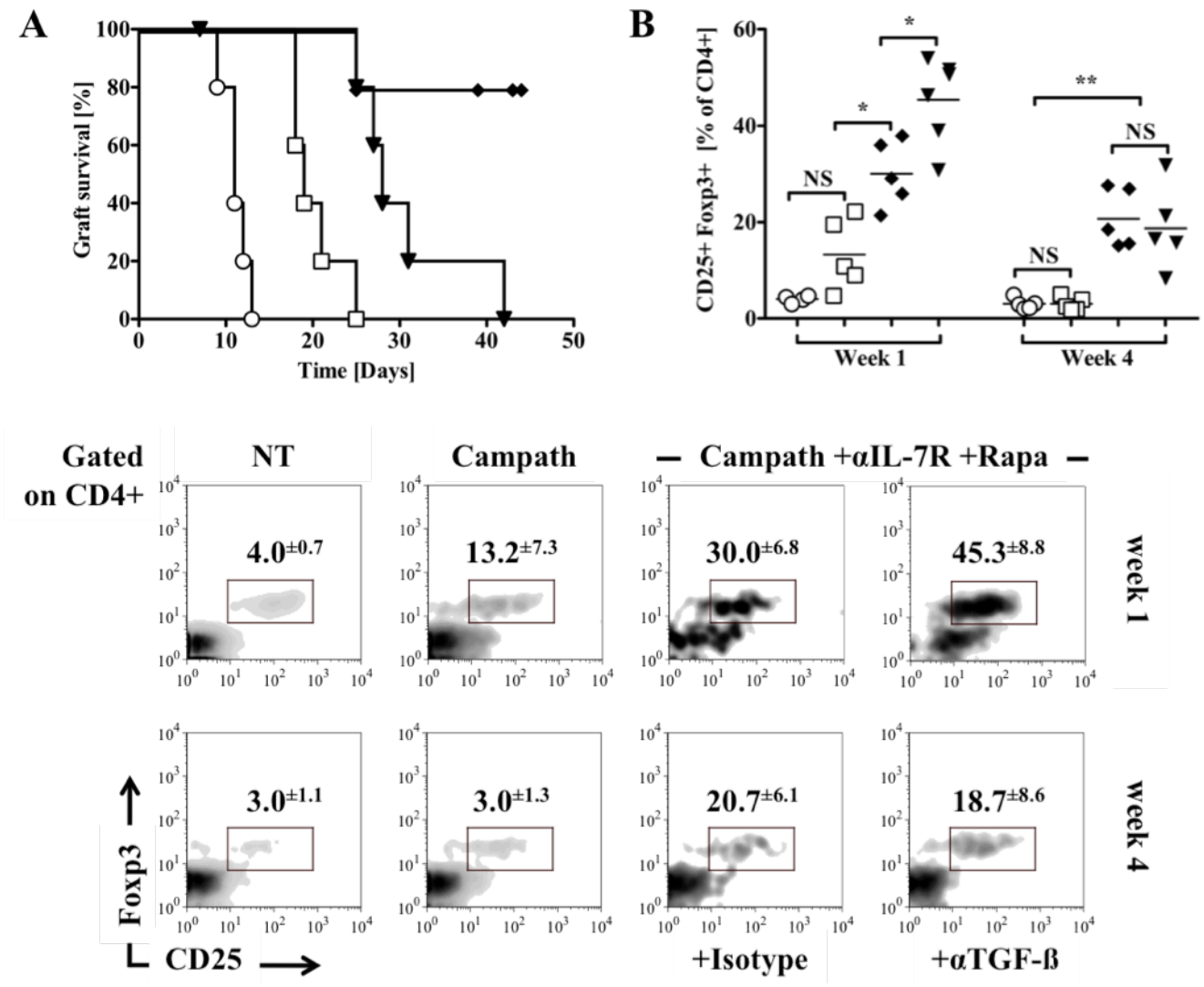

A
Thy1.1+ CD8+
Thy1.1+ CD8+ [Division Index]
Thy1.1+ CD8+ [Proliferation Index]
NT
C.
+αIL-7R
+Rapa
All
NS
B
Thy1.1+ CD4+
Thy1.1+ CD4+ [Division Index]
Thy1.1+ CD4+ [Proliferation Index]
C
Thy1.1+ CD4+
% of Max
CFSE
[Division Index]
[Proliferation Index]
CD4+
Foxp3+
Foxp3-
Campath-1
C. +αIL-7R +Rapa

Legend to Figure 7. Treatment with αIL-7R and Rapa selectively reduces the expansion of CD8+ and conventional CD4+ T-cells, but not that of regulatory T-cells. Data are obtained from the same experimental mice described in Figure 6: no treatment (○); Campath-1 (□); Campath-1+αIL-7R (▲); Campath-1+Rapa (■); Campath-1+αIL-7R+Rapa (◆) (3-6 mice per group). Analysis of cell division and proliferation was performed according to CFSE dilution analysed with FlowJo proliferation platform. Division index is the mean number of divisions of the whole population of cells; proliferation index is the mean number of divisions of the cells that divided at least once. **(A and B, histograms)** Representative histograms of CFSE dilution of Thy1.1+ CD8+ or CD4+ cells in mice not treated (shaded), treated with Campath-1 (black line), treated with Campath-1+αIL-7R+Rapa (grey line). **(A and B, left diagrams)** Division index of Thy1.1+ CD8+ and CD4+ cells. **(A and B, right diagrams)** Proliferation index of Thy1.1+ CD8+ and CD4+ cells. Abbreviations: NT: no treatment; C.: Campath-1; +αIL-7R: Campath-1+αIL-7R ; +Rapa: Campath-1+Rapa; All: Campath-1+αIL-7R+Rapa. **(C, histogram)** Representative histogram of CFSE dilution of Thy1.1+ CD4+ (shaded), CD4+ Foxp3- (black line) and CD4+ Foxp3+ cells (grey line) in mice treated with Campath-1+αIL-7R+Rapa. **(C, diagrams)** Division index (left) and proliferation index (right) of Thy1.1+ CD4+ (○) Foxp3- (△) and Foxp3+ (□) cells in mice treated with Campath-1 alone or Campath-1 in combination with αIL-7R and Rapa. Abbreviations: +αIL-7R+Rapa: Campath-1+αIL-7R+Rapa.

6. TGF-ß neutralization limits graft survival after short-course IL-7R blocking antibody and Rapamycin.

Finally, since treatment with αIL-7R and Rapa favoured the emergence of Treg and promoted long-term graft survival following T cell depletion, I asked whether TGF-ß, a cytokine crucial for both the induction of peripheral Treg (Chen W, et al. J Exp Med. 2003) and transplantation tolerance (Daley SR, et al. J Immunol. 2007), might play a role also in this setting. I found that in mice receiving B6 skin grafts under the cover of Campath-1, αIL-7R and Rapa, TGF-ß neutralization limited the survival-time of the grafts, all being rejected within 6 weeks after transplantation (MST 28 days vs >40 days in mice that received the isotype control; $p=0.03$) (Figure 8A). The proportion of reconstituting Treg favoured by treatment with αIL-7R and Rapa was unaffected by TGF-ß blockade, which surprisingly even appeared to enhance it at week 1 (45.3%±8.8 vs 30.0%±6.8 $p<0.05$ at week 1; 18.7%±8.6 vs 20.7%±6.1 $p>0.05$ at week 4) (Figure 8B). This result does not exclude a need for TGF-ß in driving conversion to induced Treg, as it currently cannot be distinguished between induced and natural Treg in the expanding Foxp3+ subset. Suffice it to say that TGF-ß exerts a non-redundant role in maintaining long-term graft survival following a treatment with Campath-1, αIL-7R and Rapa.

Figure 8.


A
Graft survival [%]
Time [Days]
B
CD25+ Foxp3+ [% of CD4+]
NS
*
*
**
NS
NS
Week 1
Week 4
Gated on CD4+
NT
Campath
Campath +αIL-7R +Rapa
4.0±0.7
13.2±7.3
30.0±6.8
45.3±8.8
week 1
3.0±1.1
3.0±1.3
20.7±6.1
18.7±8.6
week 4
Foxp3
CD25
+Isotype
+αTGF-ß

Legend to Figure 8. TGF-ß neutralization reduces graft survival in T-cell depleted mice treated with αIL-7R and Rapa. Fully mismatched B6 skin grafts were transplanted to hCD52-Tg mice under the cover of different treatments. Recipient mice were monitored 3 times/week for graft rejection, and bled at weeks 1 and 4 after transplantation to measure CD4+ CD25+ Foxp3+ cells by FACS. **(A)** Graft survival. Depletion of T-cells with Campath-1 (□; n°5) delayed graft rejection compared to no treatment (○; n°5) (MST 19 and 11 days respectively; $p<0.0001$). The combination of αIL-7R and Rapa improved survival compared to treatment with Campath-1 alone: MST >40 days in the Isotype control group (◆; n°5) and 28 days in the αTGF-ß group (▼; n°6) ($p<0.0042$ vs Campath-1). However TGF-ß neutralisation significantly limited such improvement compared to the Isotype control ($p=0.032$). **(B)** Proportion of CD4+ CD25+ Foxp3+ Treg cells in the peripheral blood at weeks 1 and 4 after transplantation (same symbols as for panel A). One mouse in the αTGF-ß group was censored in the survival analysis due to surgical failure on day 7; four mice in the Isotype control group were euthanized on days 39-44 while still carrying a viable graft. NT: no treatment.

7. Discussion.

In this study I have been able to demonstrate, for the first time, that indefinite survival of MHC mismatched skin grafts can be achieved following T cell depletion without the need for haematopoietic chimerism, by use of a protocol based on guiding lymphocyte reconstitution. Clinical experience with Alemtuzumab has indicated preferential survival of CD4+CD25+ regulatory T cells in patients (De Serres SA, et al. J Am Soc Nephrol. 2012) (Cox AL, Eur. J. Immunol. 2005), and also in hCD52-transgenic mice (Hu Y, et al. Immunology. 2009). Clearly, that differential impact of lymphocyte ablation was insufficient to prevent graft rejection (Kirk AD, et al. Transpl. 2003). My aim in this study was to build on this differential lymphocyte ablation so as to prevent unfavourable effector-memory T cell reconstitution during homeostatic proliferation (Moxham VF, et al. J Immunol. 2008) (Wu Z, et al. Nat Med. 2004), while favouring endogenous Treg, which can promote tolerance (Kendal AR, et al. J Exp Med. 2011). Previous studies in lymphopenic mice have indicated that sufficient naïve Treg made available from the outset were able to control rejection responses mediated through homeostatic expansion (Neujahr DC, et al. J Immunol. 2006) (Graca L, et al. Proc Natl Acad Sci USA. 2004).

The targeting of IL-7 signalling was based on the known role of IL-7 in promoting survival and proliferation of Tconv but not Treg (Tan JT, et al. Proc Natl Acad Sci USA. 2012) (Seddiki N, et al. J Exp Med. 2006). Antibody blockade of IL-7R has proven effective at preventing and treating certain experimental autoimmune diseases (Hartgring SA, et al. Arthritis Rheum. 2010) (Liu X, et al. Nat Med. 2010) (Penaranda C, et al. Proc Natl Acad Sci USA. 2012) (Lee LF, et al. Proc Natl Acad Sci USA. 2012) (Willis CR, et al. J Inflamm. 2012), and graft-versus-host disease (Chung B, et al. Blood. 2007). I then added a further intervention aimed at favouring Treg, the mTOR inhibitor Rapa, a clinically available immunosuppressant, known to block proliferation of activated T-cells (Halloran PF. N Engl J Med. 2004), and to selectively enhance Treg expansion (Battaglia M, et al. Blood. 2005). A short-course combination of αIL-7R and Rapa promoted long-term graft survival in hCD52-Tg mice treated with Campath-1 more effectively than either drug alone (Figure 3A). The

same combination could also delay graft rejection in lymphocyte-deficient mice injected with syngeneic T-cells (Figure 3B), a system that provides a more stringent test of a tolerising protocol (Wu Z, et al. Nat Med. 2004) as transferred T-cells rapidly repopulate by homeostatic expansion and a substantial proportion acquire a functional memory-like phenotype (Cho BK, et al. J Exp Med. 2000). These data are consistent with the finding that IL-7 antibody neutralization could improve graft survival in replete mice, when combined with co-stimulation blockade (Wang Y, et al. Am J Transplant. 2006).

As IL-7 signalling regulates T-cell metabolism (Jacobs SR, et al. J Immunol. 2010), and influences expression of the PD1 co-inhibitory receptor (Penaranda C, et al. Proc Natl Acad Sci USA. 2012), it cannot be excluded that extended graft survival following IL-7R blockade did not exploit additional immunosuppressive mechanisms. What can be excluded, however, is long-term immunosuppression from persistent depletion, as T cell counts eventually returned to normal levels, even after combined treatment with αIL-7R and Rapa (Figure 4). Moreover neither of the two drugs prevented some T cells from acquiring the typical memory-like phenotype, which, consistent with previous results (Goldrath AW, et al. J Exp Med. 2000), was only transient and dependent on the reconstitution stage. Conversely I found, as initially hypothesized, that αIL-7R and Rapa are particularly effective at enriching for Treg in the early reconstitution period. This enrichment of Treg may have been sufficient to achieve the regulatory dominance of Treg as seen in previous studies (Neujahr DC, et al. J Immunol. 2006) (Graca L, et al. Proc Natl Acad Sci USA. 2004). Moreover the addition of αIL-7R was capable of restraining the expansion of CD8+ T-cells, which are induced by Rapa when given alone. It has recently been shown that under lymphopenic conditions, inhibition of mTOR with Rapa promotes memory CD8+ T cells (Araki K, et al. Nature. 2009) (Pearce EL, et al. Nature. 2009) (Li Q, et al. Immunity. 2011) (Ferrer IR, et al. Am J Transplant. 2011). As Rapa-induced memory CD8+ T-cells highly express IL-7R (Araki K, et al. Nature. 2009), antibody blockade of this receptor might minimize this disadvantageous property of Rapa.

Measures of proliferation *in vivo* with two different approaches (Figure 5, 7) allowed us to confirm that treatment with αIL-7R and Rapa constrained CD8+ T cell proliferation. The effect on CD4+ T cells is more complex: αIL-7R and Rapa reduced the proportion of CD4+ cells entering cell division, but those cells that escaped this block proliferated more than reconstituting CD4+ cells in mice treated with Campath-1 alone. Consistent with the working hypothesis, I found that Treg are included within this set of CD4+ cells whose proliferative capacity is unaffected by αIL-7R and Rapa.

Finally we have identified a role for TGF-ß by showing that its neutralization shortened graft survival using the combination protocol (Figure 8). TGF-ß has already proven to be important for development and maintenance of dominant tolerance induced with co-receptor and co-stimulation blockade (Daley SR, et al. J Immunol. 2007) (Cobbold SP, et al. J Immunol. 2004). As Treg are essential for tolerance (Kendal AR, et al. J Exp Med. 2011) and are peripherally induced by TGF-ß (Chen W, et al. J Exp Med. 2003), graft loss following TGF-ß blockade might be due to decreased conversion to induced Treg.

We cannot easily corroborate this, as induced and natural Treg are not reliably distinguished with current antibody probes. Other explanations are also possible including accelerated homeostatic proliferation and differentiation of rejecting T-cells following TGF-ß blockade (Surh CD, and Sprent J. Nat Immunol. 2012) or abrogation of secondary pro-tolerogenic pathways such as leukocytes production of anti-inflammatory adenosine (Regateiro FS, et al. Eur J Immunol. 2011).

RESULTS 2/2:

“Donor bone marrow transplantation for epithelial graft tolerance without any need for myelo-ablation”.

1. Introduction.

I have already highlighted how a short-course treatment capable of reprogramming the immune system and inducing transplantation tolerance would provide a considerable improvement in long-term transplantation outcomes by both limiting recipient exposure to immunosuppression, and its intrinsic side-effects, and achieving indefinite graft acceptance (Kendal AR and Waldmann H. Curr Opinion Immunol. 2010).

One route to obtain this result relies on combined bone marrow (BM) and epithelial organ transplantation. In particular the development in the recipient of mixed, donor and host, haematopoietic chimerism following BM transplantation has been known for a long time as an essential and reliable step for the induction of donor-specific epithelial graft tolerance, both in experimental and clinical settings (Ildstad ST, and Sachs DH. Nature. 1984) (Wekerle T and Sykes M. Annu Rev Med. 2001).

The main hurdle to the widespread clinical application of this procedure is the requirement for myelo-suppressive regimens that would enable the engraftment and thriving of donor BM in the host, regimens that expose patients to inacceptable risks of severe side effects.

Since the first report of a successful combination of BM and epithelial (kidney) graft transplantation in humans (Bühler LH, et al. Transplantation. 2002), many efforts have been made to develop less intense therapeutic protocols, named as non-myeloablative regimens (Li WH and Sykes M. Nat Rev Immunol. 2012). Nevertheless these “non-myeloablative” regimens, that have already been utilized in humans for the combined transplantation of living donor kidney and BM, are based on total or thymic lymphoid irradiation, lymphocyte antibody depletion and cytotoxic drugs (see Table 5) (Bühler LH, et al. Transplantation. 2002) (Millan MT, et al. Transplantation. 2002) (Scandling JD, et al. N Engl J Med. 2008) (Scandling JD, et al. Am J Transplant. 2012) (Leventhal J, et al. Sci Transl Med. 2012) (Fudaba Y, et al. Am J Transplant. 2006) (Kawai T, et al. N Engl J Med. 2008).

The use of body irradiation and cytotoxic drugs is anchored to the assumption that creation of “space” in the host is required for donor BM engraftment, unless mega-doses of donor BM are

administered to the recipient. This concept, though, has progressively been brought into question by the new recent experimental findings, that have established that allogenic haematopoietic stem cells (HSC) home and survive in host BM niches for up to 30 days after the transfer into untreated mice (Fujisaki J, et al. Nature. 2011); and that engraftment of conventional doses of BM can be achieved with a treatment wholly based on co-receptor and co-stimulation antibody blockade (Graca L, et al. BMC Immunol. 2006).

I therefore asked whether a more patient-friendly and clinical-practicable protocol based on T cell depletion might be permissive for donor BM engraftment and induction of transplantation tolerance without the use of any myelo-suppressve treatments or BM mega-doses.

2. Specific methods.

2.1. Dosage of monoclonal antibodies and Rapa utilized for recipient mice.

For co-receptor and co-stimulation blockade, mice were given YTS177.9, a rat IgG2a anti-mouse CD4 (αCD4) (Cobbold S, et al. Eur. J. Immunol. 1990), and YTS105.18, a rat IgG2a anti-mouse CD8 (αCD8) (Cobbold S, et al. Eur. J. Immunol. 1990), and MR1, a hamster anti-mouse CD154 (αCD40L), 1 mg of each Ab i.p. on days 0, 2, 5 (Noelle RJ, et al. Proc Natl Acad Sci USA. 1992).

For T cell depletion mice were treated with Campath-1, a rat IgG2b anti-hCD52 (Waldmann H and Hale G. Phil Trans R Soc B. 2005), 1 mg i.p. on days -3 and -1.

For IL-7R blockade, mice received A7R34, a rat IgG1 αIL-7R (Sudo T, et al. Proc Natl Acad Sci U S A. 1993), 7 i.p. injections of 0.4 mg from day -3 to 14.

For the blockade of the chemotactic molecule LFA-1, mice received FD441, a rat anti-mouse LFA-1 (CD11a/CD18) blocking (αLFA1), 7 i.p. injections of 1 mg from day -3 to 14 (Benjamin RJ, et al. Eur J Immunol. 1988).

All the antibodies were produced in our laboratory as previously described.

Rapa (Calbiochem) was dissolved in 100% ethanol and stored at -20°C; on the day of the injection a stock aliquot was diluted with 0.2% carboxymethycellulose sodium salt (Sigma-Aldrich) and administered i.p.; unless otherwise specified, mice received 7 injections of 1.5 mg/kg from day -3 to 14 (Zeiser R, et al. Blood 2008).

2.2. Bone marrow and haematopoietic stem cell transplantation.

BM and spleen donors were depleted of T cells two to five days before organs collection with an i.p. injection of 1 mg of each of the following antibodies: YTS191.1 (Cobbold SP, et al. Nature. 1984) and YTA3.1 (Qin S, et al. Eur J Immunol. 1987) to deplete CD4+ cells, and YTS169.4 (Cobbold SP, et al. Nature. 1984) and YTS156.7 (Qin S, et al. Eur J Immunol. 1987) to deplete CD8+ cells (Cobbold S, et al. Eur. J. Immunol. 1990). All the antibodies were produced in our laboratory as previously described.

On the day of the transplantation, femurs and tibias were collected and flushed, and a single cell suspension was obtained. For BM transplantation, vital cells were counted with Trypan blue, washed in PBS and injected e.v. at around $25x10^6$ cells per recipient. For haematopoietic stem cell (HSC) transplantation, HSC were separated from whole bone marrow cell suspension with the Lineage Cell Depletion Kit (Miltenyi Biotec, 130-090-858) and the autoMACS Separator by negative selection for the following lineage antigens: CD5, CD45R, CD11b, GR-1, 7-4 and Ter-119 antigens. HSC-enriched suspension was washed in PBS and injected e.v. at $0.8\text{-}3.0x10^6$ cells per recipient.

BM transplantation was carried out under sterile conditions and mice were kept under specific pathogen-free conditions, in so much as no antibiotic therapy was necessary in the peri-transplant phase.

2.3. Adoptive transfer of spleen cells.

Spleens were collected from the same T-cell depleted donors described in paragrph 2.2.. A single-cell suspension was obtained, and erythrocytes were lysed with Tris-buffered ammonium chloride. Vital cells were counted with Trypan blue, washed in PBS and injected e.v. at around $25x10^6$ cells per recipient.

2.4. Chimerism assessment by means of FACS analysis.

Blood, bone marrow and spleen cells were collected and prepared for FACS analysis as already described in the appropriate paragraphs of Materials and Methods. The following primary anti-mouse antibodies (all from BD Pharmingen) were used:

- anti-H-$2K^bD^b$ (28 8 6) in order to measure the engraftment of cells of B6 origin;
- anti-CD45.2 (104), anti-CD3 (145-2C11), anti-CD19 (1 D3), anti-CD11b (m1/7) to define the lineage of chimeric cells, respectively for whole white blood cells, T cells, B cells, macrophages.

3. Experimental model of BM transplantation in advance of skin grafting.

In order to assess whether the immunosuppressive treatments tested and based on T cell depletion were capable of inducing mixed haematopoietic chimerism upon BM transplantation, and consequently donor epithelial graft tolerance, I developed the experimental model represented in Figure 9.

First, recipient mice were hCD52-tg mice (H-2K^k) (Gilliland LK, et al. J Immunol. 1999) pre-treated with Campath-1 to deplete T cells. Further additional treatments employed to foster BM engraftment were indicated in Table 8.

Second, donor mice were MHC fully mismatched B6 mice, whose haematopoietic cells could be easily identified according to MHC specificity (H-2K^b): donors had been treated 2 to 5 days before BM collection with a mixture of CD4 and CD8 depleting antibodies in order to prevent GVHD (Cobbold S, Martin G. and Waldmann H. Eur. J. Immunol. 1990).

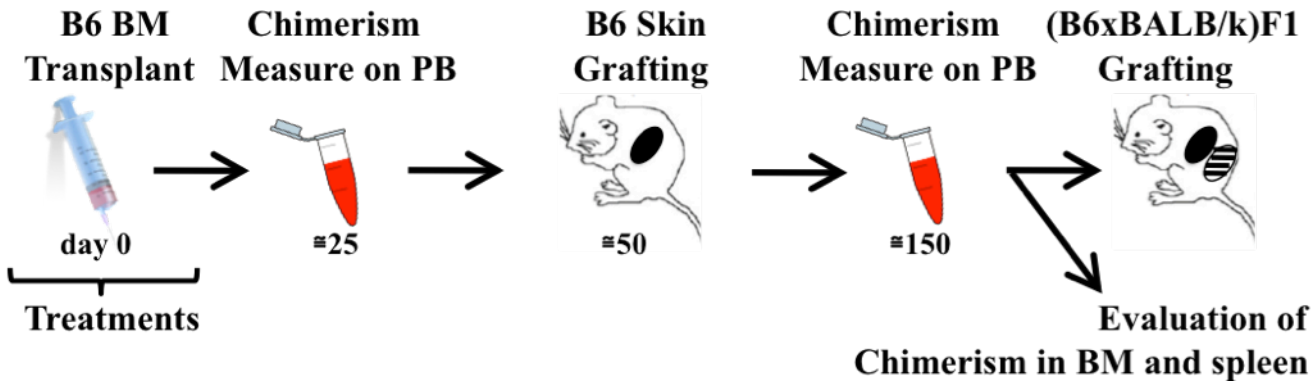
On day 0 BM transplantation was carried out; and in order to confirm that the experimental conditions were functional, a comparison group of mice treated with co-receptor and co-stimulation blocking antibody, a protocol already known to be effective (Graca L, et al. BMC Immunol. 2006), was included.

On day 25 after BM transplantation the development of mixed haematopietic chimerism was assessed on peripheral blood of recipient hCD52-tg mice by means of FACS analysis identification and quantification of H-2K^b+ cells.

On day 50 after BM transplantation donor-specific tolerance for epithelial graft was tested by performing skin grafting from syngeneic B6 donors: the acceptance and survival of the graft would be the first indicator of induction of tolerance with the therapeutic protocol adopted.

On day 150 after BM transplantation the mice belonging to the same therapeutic group that had accepted the B6 skin graft were divided into 2 sub-groups: one euthanized to re-evalutae the presence of chimerism on peripheral blood, and also in the spleen and BM, the other re-grafted with skin graft from (B6xBALB/k)F1 donors to test for linked-suppression.

Figure 9.



Legend to Figure 9. Experimental design for allogenic bone marrow transplantation in advance of epithelial (skin) grafting of donor type. (A) Under the cover of different treatments (see Table 8) hCD52-Tg mice (H-2^k) received fully mismatched BM ($25x10^6$ cells e.v. per recipient) collected from B6 or B6hCD52KI donors (H-2^b) depleted of T cells 2-5 days before. Recipient mice were bled around 25 and 150 days after BM transplantation, and the presence of haematopoietic chimerism was assessed on peripheral blood by FACS. Moreover 50 days after BM transplantation recipient mice received skin grafts of donor (H-2^b) type; grafts were periodically checked and considered accepted if still viable 100 days after the transplantation. The experiments that resulted in graft acceptance were repeated for confirmation. The mice that accepted the graft in the 1st series of experiments were euthanized and the presence of chimerism was studied also in the spleen and bone marrow. The mice that accepted the graft in the 2nd series of experiments were re-grafted with a second transplant from (B6xBALB/k)F1 donors to look for linked-suppression, and were followed up for a further 50 days.

4. Bone marrow engraftment can be induced with a protocol based on T cell depletion without any need for myelo-ablation.

I tried to build on the immunosuppressive potential of T cell depleting treatment in order to establish a protocol that would enable bone marrow engraftment following transplantation without recurring to any myelo-ablative regimen, either based on lymphoid irradiation or cyto-reductive drugs.

As control group I included mice treated with co-receptor and co-stimulation blocking antibodies, αCD4, αCD8 and αCD40L: this strategy developed in the host laboratory some years ago (Graca L, et al. BMC Immunol. 2006) allowed me to verify that the experimental procedures of BM collection and transplantation worked, and to analyse the characteristics of hematopoietic chimerism in experimental mice compared to controls.

As shown in Table 8, co-receptor and co-stimulation antibody blockade was always successful at inducing BM engraftment, and donor skin graft acceptance when performed later in time. On the contrary T cell depletion, albeit profound and sustained, was alone insufficient for BM engraftment as none of the mice treated with Campath-1 developed chimerism following the transplantation of fully mismatched BM.

When it came to decide what drug to combine with Campath-1, I reasoned that Rapa would suit in the protocol not only for its effective immunosuppressive activity (Ramsey H, et al. Transpl Int. 2013), but also for the capability of inducing Tregs (Battaglia M, et al. Blood. 2005) which had proven fundamental for BM engraftment under conditioning regimens (Pilat N, et al. Am J Transplant. 2010). Indeed the addition of Rapa to Campath-1 was effective at least in one of four treated mice: not only this mouse became chimeric but also indefinitely accepted the skin graft provided later in time (Table 8 and Figure 10).

Therefore I hypothesized that an additional treatment to Rapa was necessary and I tested three different monoclonal blocking antibodies for their ability to target different key steps of the allo-immune response. First an αIL-7R blocking antibody was used to guide T cell reconstitution

following lymphocyte depletion (see Chapter 3 of this Thesis) and further favour the emergence of Treg (Pilat N, et al. Am J Transplant. 2010). Second an αLFA1 blocking antibody was used to inhibit the chemotaxis of T cells and the activation of NK cells, cells that, unlike for epithelial graft, exert a relevant role in BM rejection (Benjamin RJ, et al. Eur J Immunol. 1988). Third an αCD40L blocking antibody was used to block co-stimulation and inhibit "signal 2" of the allo-immune response (Noelle RJ, et al. Proc Natl Acad Sci USA. 1992).

While the first two strategies were ineffective, the short-course combination of Rapa and αCD40L following T cell depletion with Campath-1 proved to be successful at inducing BM engraftment and consequently donor-specific transplantation tolerance (Table 8 and Figure 10): these data have demonstrated for the first time that a protocol based on T cell depletion can enable BM engraftment without any need for myelo-ablation.

Many aspects of this experiments deserve to be highlighted.

First, as for the control group, also in mice treated with Campath-1, Rapa and αCD40L hematopoietic chimerism significantly increased over time and became clearly detectable with FACS on peripheral blood by day 150 (Figure 10). It is also noteworthy that the levels of chimerism on day 150 in mice treated with Campath-1, Rapa and αCD40L, were similar, albeit more variable, to the levels in control mice treated with αCD4, αCD8 and αCD40L: in both the groups circa 15% of peripheral blood leukocytes were of donor origin (Figure 10).

Second, chimerism so obtained not only was present in peripheral blood, but also in the bone marrow and spleen of recipient mice (Figure 11A and 11B) and it was of multi-lineage nature (Figure 11): cells of donor origin were found to belong to the T lineage defined by CD3 surface expression, to the B lineage defined by CD19 expression, and to the macrophage lineage defined by CD11b expression (Pilat N, et al. Am J Transplant. 2010).

Finally, transplantation tolerance developed by chimeric mice was based on peripheral active regulation, and not merely on central passive depletion, as proved by the phenomenon of linked-

suppression (Davies JD, et al. J Immunol. 1996) to third-party (multiple minor) antigens incorporated in a second graft, also bearing the tolerated antigens, and transplanted 150 days later.

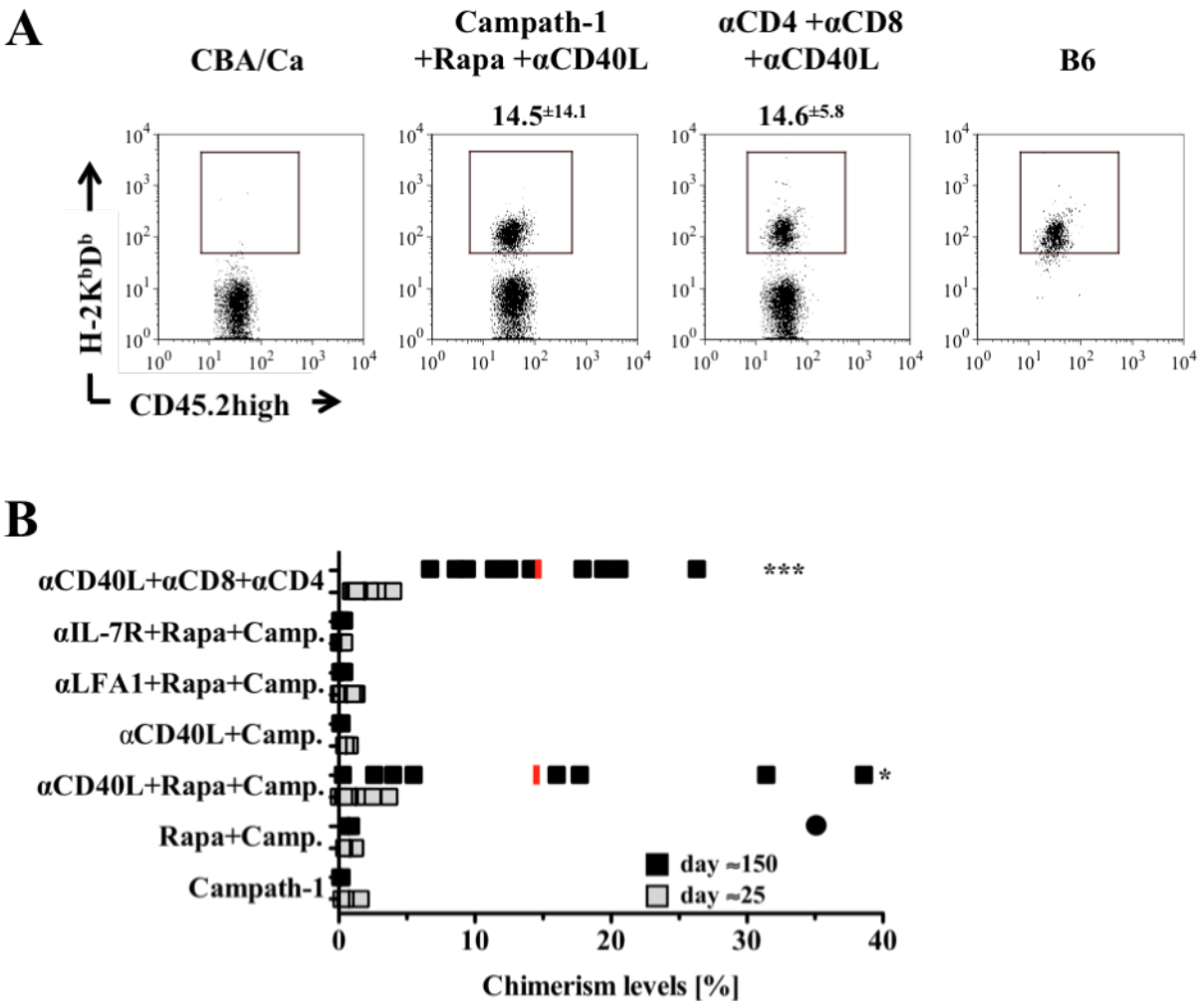
On the other hand, also some questions rose from the analysis of these data, questions that I tried to address in the following series of experiments. First, as chimerism seems to develop and increase over time after skin grafting, is BM transplantation necessary at all for epithelial graft tolerance? Second, would it be possible to get similar result in terms of tolerance, avoiding BM transplantation or substituting it with other sources of donor antigens? Third, would this protocol based on Campath-1, Rapa and αCD40L be permissive also for the simultaneous transplantation of donor BM and epithelial graft graft, as routinely required in the clinic of cadaveric donor transplantation?

Table 8. Therapeutic protocols investigated for BM and skin graft acceptance.

Treatment	hCD52-Tg recipients (n°)	B6 skin graft acceptance for >100 days (MST)	*Chimerism on PB on day 150 after BM Tx	(B6×BALB/k)F1 skin graft acceptance for >50 days
Campath-1	3	0/3 (12 days)	0/3	-
Campath-1 + Rapa	**4**	**1/4 (73.5 days)**	**1/4**	**-**
Campath-1 + Rapa + αLFA1	8 (5+3)°	0/8 (13 days)	0/8	-
Campath-1 + Rapa + αIL-7R	4	0/4 (12 days)	0/4	-
Campath-1 + Rapa + αCD40L	**9 (4+5)°**	**8/9 (>100 days)**	**7/8**	**3/4[#]**
Campath-1 + αCD40L	4	0/4 (15 days)	0/4	-
αCD4 + αCD8 + αCD40L	**11 (5+6)°**	**11/11 (>100 days)**	**11/11**	**6/6**

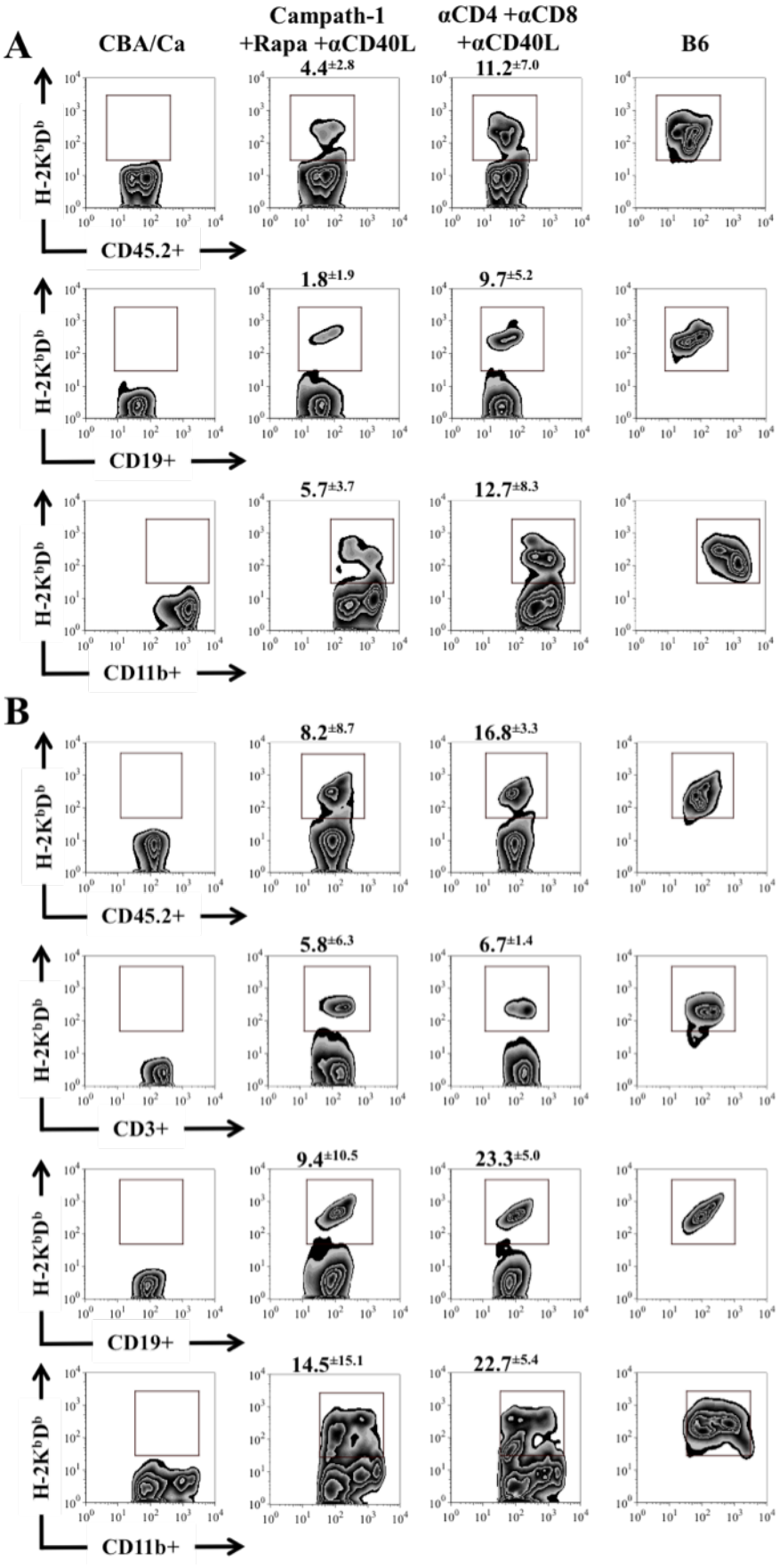
Notes to Table 8. Antibody and immunosuppressant dosage: Campath-1 1 mg i.p. on days -3 and -1 in respect to the day of BM transplantation (day 0); Rapa 1.5 mg/kg i.p., 7 injections from day -3 to day 14; αCD40L 1 mg i.p. on days 0, 2, 4, 6; αLFA1 1 mg i.p., 7 injections from day -3 to day 14; αIL-7R 0.4 mg i.p., 7 injections from day -3 to day 14; αCD4+αCD8+αCD40L 1 mg of each antibody i.p., 7 injections from day -3 to day 14. **Abbreviations:** MST (mean survival time), PB (peripheral blood), BM (bone marrow), Tx (transplantation). **Notes:** ° Experiments repeated twice. * A mouse was considered chimeric if >1% of CD45.2^{high} blood cells were positive for H-2K^{b}D^{b} labelling by FACS analysis. [#] One mouse in this group was found dead after the second skin transplantation and was censored in the survival analysis.

Figure 10.


A
CBA/Ca
Campath-1
+Rapa +αCD40L
αCD4 +αCD8
+αCD40L
B6
14.5±14.1
14.6±5.8
H-2K^bD^b
CD45.2high
B
αCD40L+αCD8+αCD4
αIL-7R+Rapa+Camp.
αLFA1+Rapa+Camp.
αCD40L+Camp.
αCD40L+Rapa+Camp.
Rapa+Camp.
Campath-1
***
*
day ≈150
day ≈25
0
10
20
30
40
Chimerism levels [%]

Legend to Figure 10. Bone marrow engraftment can be achieved with a protocol based on T-cell depletion, Rapa and co-stimulation blockade with αCD40L. (A) Representative FACS plots of chimerism measure circa on day 150 in mice treated with Campath-1, Rapa and αCD40L, and mice treated with αCD4, αCD8 and αCD40L (results are from different experiments, and in each a CBA/Ca and a B6 mouse was included as negative and positive control for H-2K^{b}D^b antigens). Numbers are mean ±SD. **(B)** Levels of chimerism on peripheral blood 25 and 150 days after BM transplantation in mice treated as indicated in the figure. Each box represents a single mouse; red line is mean. Chimerism significantly increased over time in mice treated with Campath-1, Rapa and αCD40L, as did in mice treated with αCD4, αCD8 and αCD40L. The mouse in the Campath-1+Rapa group that indefinitely accepted the skin graft was also the only of the group highly chimeric on day 150 (black circle).

Figure 11.


A
CBA/Ca
Campath-1 +Rapa +αCD40L
αCD4 +αCD8 +αCD40L
B6
4.4±2.8
11.2±7.0
1.8±1.9
9.7±5.2
5.7±3.7
12.7±8.3
H-2KbDb
CD45.2+
CD19+
CD11b+
B
8.2±8.7
16.8±3.3
5.8±6.3
6.7±1.4
9.4±10.5
23.3±5.0
14.5±15.1
22.7±5.4
CD3+

Legend to Figure 11. Chimerism achieved without myelo-suppression is of multi-lineage nature. Representative FACS plots for chimerism measures in bone marrow **(A)** and spleen **(B)** from mice that had accepted B6 skin graft (see Table 8), and from CBA/Ca and B6 controls. Numbers are mean percentage ± SD of H-2K^{b}D^b+ cells. A mouse was considered chimeric if the percentage of H-2K^{b}D^b positive cells was greater than 1%. Multi-lineage chimerism was defined by the presence of chimerism in all the lineages studied both in bone marrow and spleen. Multi-lineage chimerism could be detected in 5 out of 5 mice treated with blocking αCD4, αCD8 and αCD40L; and in 2 out of 3 mice treated with Campath-1, αCD40L and Rapa.

5. Bone marrow transplantation and engraftment is necessary for the development of tolerance.

Having found that a protocol based on T cell depletion, Rapa and co-stimulation blockade is permissive for the engraftment of allogenic bone marrow, and that skin graft of donor type is accepted when performed later in time, I wished to establish whether BM transplantation was really necessary for tolerance, or it can be avoided or substituted with other source of antigens from the same donor origin.

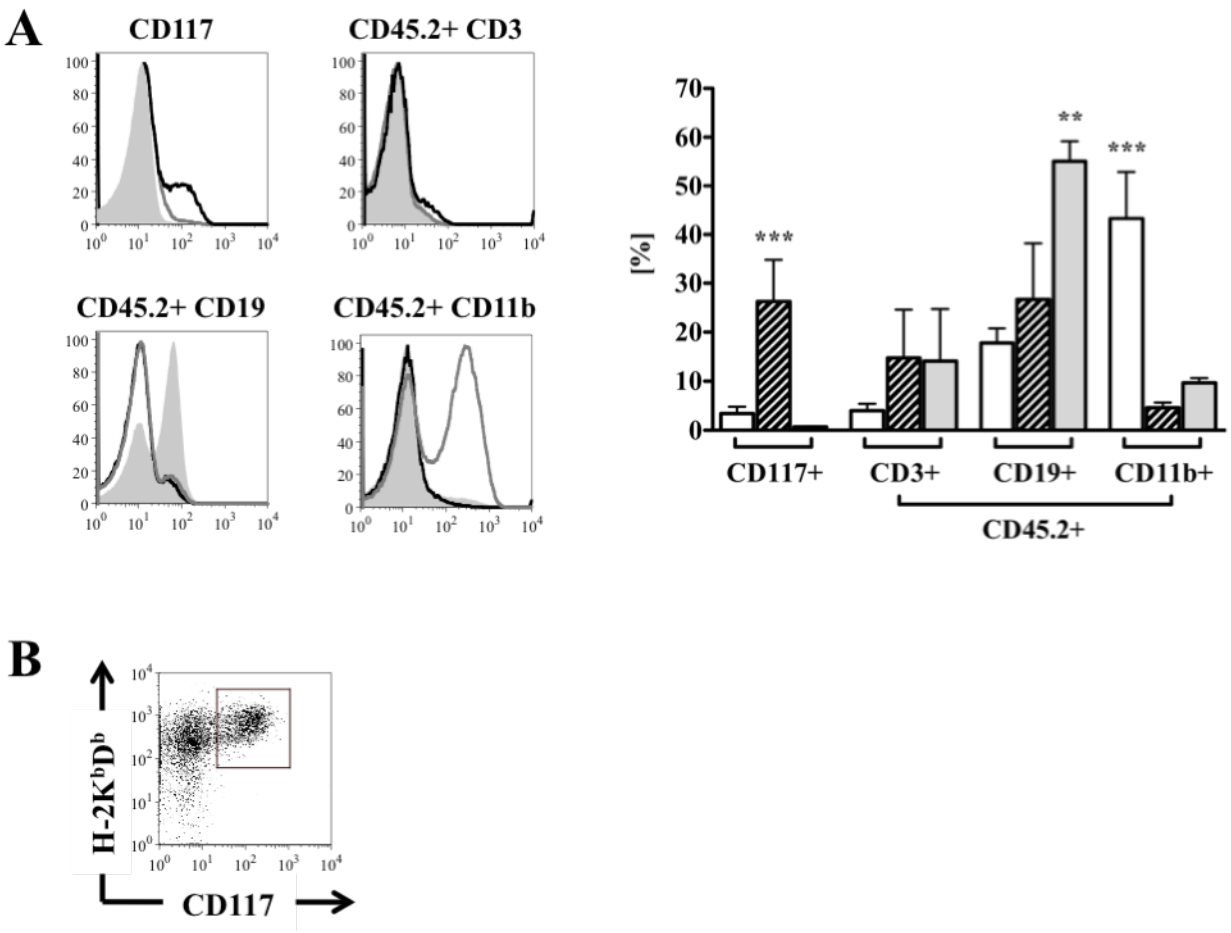
First, three different allogenic cell suspensions were prepared from T cell depleted B6 donor mice and characterized before injection into recipient hCD52-tg mice: whole BM preparation (BM), HSC-enriched preparation (HSC), and splenocytes (Spleen) (Figure 12A). HSC preparation, composed by marrow cells negatively sorted for classic lineage markers, resulted significantly richer in pure CD117+ HSC compared to the other preparations, and significantly poorer in antigen presenting cells (APCs), whether be it CD19+ B cells or CD11b+ dendritic cells, respectively compared to Spleen preparation and BM preparation (Figure 12A). Therefore HSC preparation contained less cells capable of direct antigen presentation, but nevertheless was highly allogenic as all the HSC cells expressed the murine MCH antigens H-2K^bD^b (Figure 12B). Moreover it is noteworthy that very low levels of CD3+ T cells were present in all 3 the cell preparations, as result of the pre-treatment of donor mice with T cell depleting antibodies (Figure 12B).

Second, an experimental model very close to that described in Figure 9 was adopted, but in this case all the recipient mice were treated with Campath-1, αCD40L and Rapa, the protocol that had already proven to be permissive for BM engraftment (Table 8). Under the cover of this treatment mice received either no cells, or cells preparations as described in Figure 12: BM, HSC, or spleen. Chimerism was assessed 25 and 125 days after cell transfer; B6 skin grafts were performed 50 days after cell transfer (Figure 13A). All the mice that had received spleen cells rapidly rejected the donor skin graft and did not become chimeric, thus indicating that the spleen preparation, devoid of HSC and rich in APCs, had primed the recipient immune system (Figure 13B and 13C). Similarly

the majority of mice that had received no cells rejected their graft albeit with a slight delay, compared to mice injected with spleen cells, and did not become chimeric, thus demonstrating that treatment with Campath-1, Rapa and CD40L was alone insufficient for the induction of transplantation tolerance (Figure 13B and 13C). Finally all the mice that had received HSC and BM cells (1st experiment) accepted the skin grafts and developed some level of chimerism detectable with FACS on day 125 (Figure 13B and 13C).

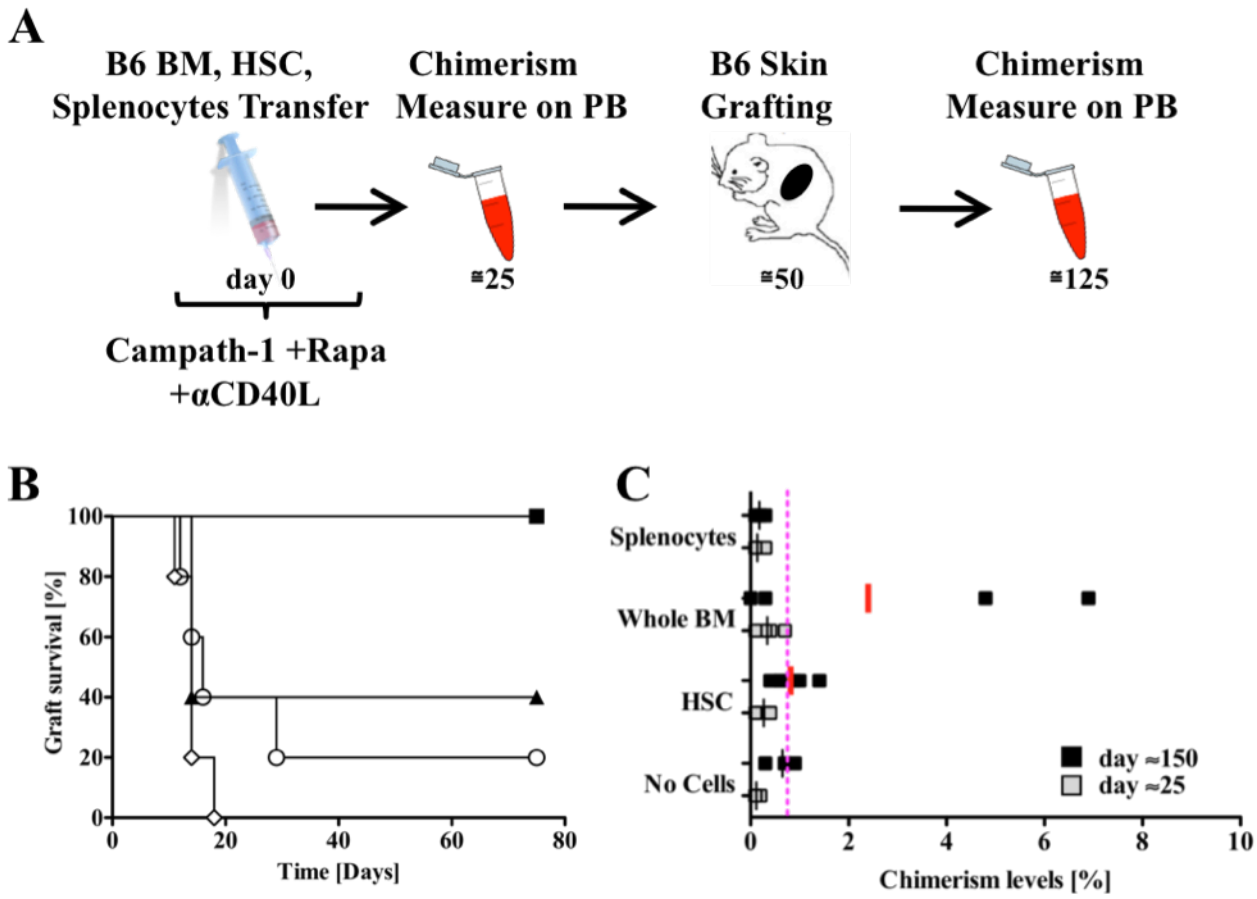
I have to make clear that a 2nd experiment for mice that had received BM cells did not work, conceivably for technical reason, as this result was unique among a total of 4+2 identical experiments (Table 8 and Figure 13).

Figure 12.



Legend to Figure 12. Hematopoietic stem cells sorting and characterization. BM and spleens were collected from B6 donors depleted of T cells 2-5 days before. BM cell suspension was divided into two aliquots. Before adoptive transfer, one BM aliquot was negatively depleted for lineage markers to enrich for haematopoietic stem cells. Then the following three preparations were analyzed before injection into hCD52-Tg recipients: whole bone marrow cells (BM), HSC-enriched bone marrow preparation (HSC), spleen cells (Spleen). **(A, histograms)** Representative histograms for expression of CD117, and CD45.2+ CD3, CD19 and CD11b in HSC (black line), BM (dark grey line) and Spleen (shaded) preparations. **(A, diagram)** Mean percentage ± SD of CD117+, CD45.2+ CD3+, CD19+ and CD11b+ cells in BM (white bars), HSC (striped bars) and Spleen (grey bars). **(B)** H-2K^{b}D^b expression by CD117+ HSC.

Figure 13.



Legend to Figure 13. Bone marrow engraftment under the cover of Campath-1, Rapa and αCD40L is necessary for the induction of transplantation tolerance.

(A) A similar experimental design to that described in Figure 1 was utilized with two differences. First, all the hCD52-Tg recipient mice were treated with the combination Campath-1, Rapa and αCD40L with the same schedule as in Table 1. Second, recipient mice either did not received any cell transfer or different cell preparations from T-cell depleted B6 donors: HSC, BM and Spleen. Data are aggregated results of two experiments. **(B)** All the mice that had received the transfer of spleen cells under the cover of the above mentioned combination treatment (◇, n°5) rapidly rejected B6 skin grafts when performed 50 days after cell injection (MST 14 days), as did the majority of mice that had not received cells (○, n°5) (MST 16 days, p=0.22). Of the mice that had received BM (▲, n°5), 2 out of 2 of the 1st experiment accepted, whilst 3 out of 3 of 2nd experiment

rejected the grafts (cumulative MST 14 days). Finally all the mice that had received HSC (■, n°6) accepted the grafts (MST>75 days, p=0.006 vs No Cells). **(C)** None of the mice that had received either no cells or Spleen developed chimerism on day 25 and 125. On the contrary 2 out of 2 mice that had received BM and had accepted the skin graft proved to be chimeric on day 125, whilst none of the mice that had received BM and had rejected the graft was found chimeric. 3 out of 6 mice that had received HSC had detectable, albeit low, levels of chimerism in peripheral blood on day 125, and in 2 of those chimerism was of multi-lineage nature (data not shown).

6. Tolerance could not be induced when simultaneous transplantation of donor bone marrow and skin graft was performed.

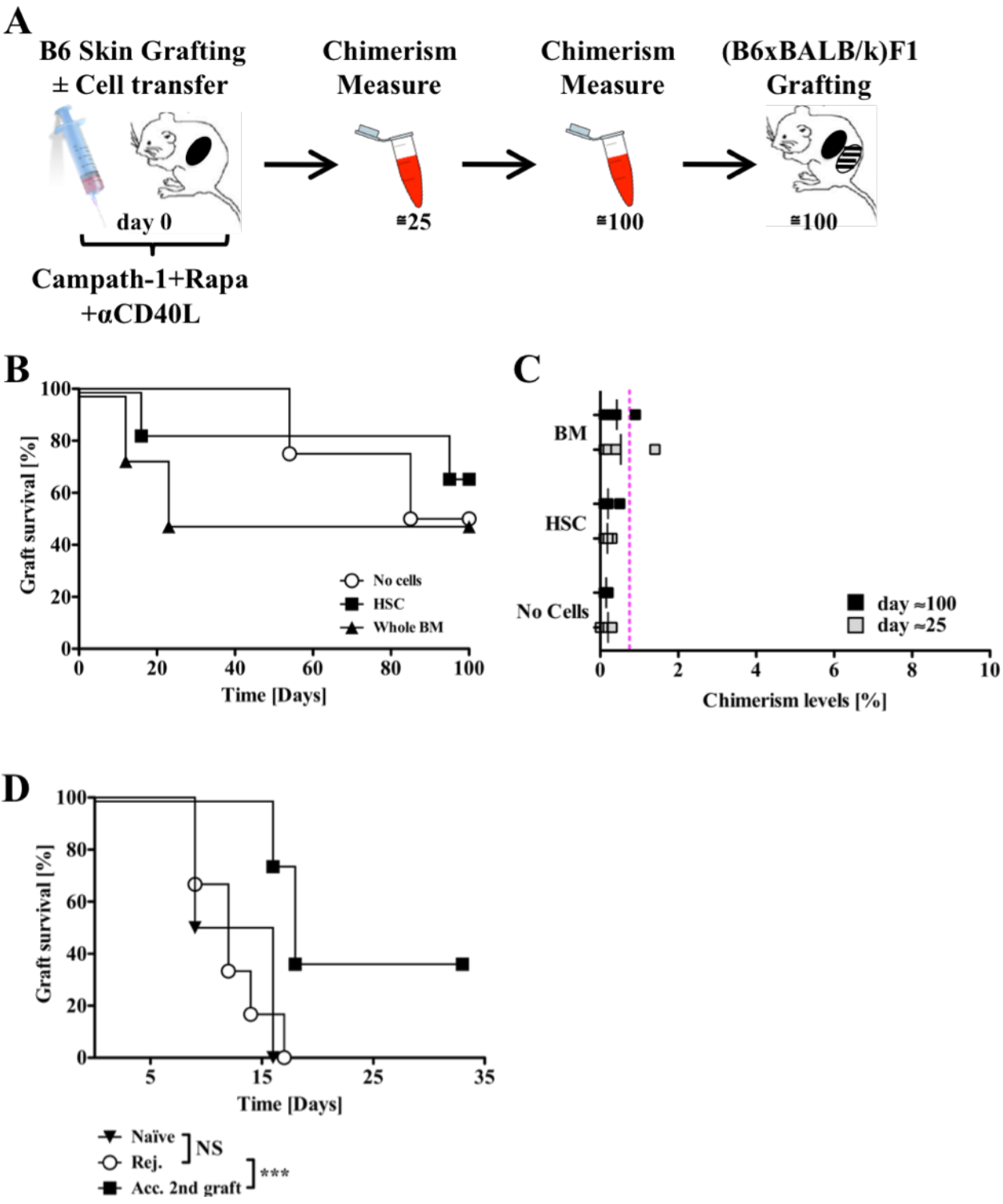
Finally, having identified a therapeutic protocol based on T cell depletion that enables allogenic BM to engraft and mixed haematopoietic chimerism to develop in the course of time, and having demonstrated that BM engraftment in advance of skin grafting is necessary for donor-specific epithelial graft tolerance, I investigated whether the same protocol would be effective also for simultaneous transplantation of bone marrow and skin graft, in view of a potentially wider clinical application. Indeed the strategy based on provision of donor BM and epithelial graft at separate time could only be applied to living donor transplantation as it does not fit the logistic requirements for cadaveric donor transplants, which represent the vast majority.

As indicated in Figure 14A, under the cover of Campath-1, Rapa and CD40L, recipient hCD52-tg mice simultaneously received fully miss-matched B6 skin grafts and no cells, or B6 BM or HSC suspensions, prepared as described in Figure 12; chimerism was checked by FACS on peripheral blood 25 and 100 days after the simultaneous transplantation; finally mice were re-grafted on day 100 with (B6xBALB/K)F1 to check for linked-suppression (Figure 14A).

Irrespective of the administration of no cells, or BM or HSC preparations, treatment with Campath-1, Rapa and CD40L prolonged the survival of the skin graft and circa 50% of the mice in each group accepted the graft for more than 100 days (Figure 14B). Interestingly none of the mice developed a significant level of chimerism either on day 25 or on day 100 following the transplantation, thus indicating the lack of BM engraftment (Figure 14C). Finally no linked-suppression phenomenon could be found in the mice, including those that had accepted the first graft tended to reject a second graft from (B6xBALB/K)F1 (Figure 14D).

I concluded that treatment with Campath-1, Rapa and CD40L could not enable the engraftment of BM when provided simultaneously with the epithelial graft and that prolonged graft survival was not based on peripheral regulation, but rather on the prolonged immunosuppression established with the inductive treatment employed.

Figure 14.


A
B6 Skin Grafting
± Cell transfer
Chimerism
Measure
Chimerism
Measure
(B6xBALB/k)F1
Grafting
day 0
≅25
≅100
≅100
Campath-1+Rapa
+αCD40L
B
Graft survival [%]
Time [Days]
No cells
HSC
Whole BM
C
BM
HSC
No Cells
day ≈100
day ≈25
Chimerism levels [%]
D
Graft survival [%]
Time [Days]
Naïve
Rej.
Acc. 2nd graft
NS
***

Legend to Figure 14. Simultaneous BM transplantation and skin grafting under the cover of Campath-1, Rapa and αCD40L. (A) Under the cover of Campath-1, Rapa and αCD40L, hCD52-Tg mice received B6 skin grafts and either no cell transfer or HSC or BM from T-cell depleted B6 donors. Data are aggregated results of two experiments. **(B)** Irrespective of any cell transfer, the combination treatment promoted long term graft survival: no cells (○, n°4): MST 92.5 days; HSC (■, n°6): MST >100 days BM (▲, n°4): MST 61.5 days (NS for all comparison). **(C)** Mice did not develop any chimerism in peripheral blood detectable with FACS analysis. **(D)** 100 days after BM and skin transplantation from B6 donors, all the mice were re-grafted with (B6×BALB/k)F1 skin grafts. The mice that had rejected the first B6 graft rapidly rejected also the second challenge graft (○, n°6), as did hCD52-Tg mice (▼, n°4) (MST 12 and 12.5 days, p=0.93). Also the mice that had accepted the first graft (■, n°8) tended to reject the second challenge graft, but significantly later in time (MST 18 days, $p<0.001$).

7. Discussion.

In this study I have been able to demonstrate for the first time that the engraftment of allogenic bone marrow can be obtained with a protocol based on T cell depletion without any need for myeloablation (Table 8 and Figure 10); and that tolerance obtained through BM transplantation under this protocol was based on peripheral regulation as proved by the presence of linked-suppression (Table 8).

The use of conditioning regimens to produce myelo-ablation is dictated by the concept that "space" in the recipient BM is needed to allow donor BM to engraft; however this concept has already been brought into questions by two studies: the first have demonstrated that donor HSC survive in host BM niche up to 30 days without any facilitating treatment (Fujisaki J, et al. Nature. 2011); the second that BM engraftment and HSC in mice treated exclusively with co-receptor and co-stimulation blocking antibodies (Graca L, BMC Immunol. 2006).

The protocol I have established have proven equally effective as the one by Graca L et al., but it is based on the cobination of two drugs commercially available, Campath-1 and Rapamycin, and one experimental agent, αCD40L, for which a recently approved counterpart already exists, Belatacept (Vincenti F, et al. Am J Transplant. 2012), a co-stimulation blocking fusion protein of CTLA-4 and IgG Fc. If the use of belatacept proved to be as effective as that of αCD40L, this protocol would be ready to be directly translated into the clinic for trialling.

Another relevant aspect of this study is that a full characterization of the development of mixed haematopoietic chimerism, which is the direct proof of allogenic BM engraftment, is provided. First, BM transplantation and chimerism were necessary for transplantation tolerance (Table 8, Figure 13); second, the level of chimersim increased over time (Figure 11) up to becoming easily detectable with FACS (Figure 10 and Figure 11) not only in the peripheral blood, but also in the bone marrow and the spleen; finally chimerism was of multi-lineage nature. On day 150 7 out of 8 mice treated with Campath-1, Rapa and αCD40L presented macro-chimerism, i.e. a more than 1% of total blood cells were of donor origin: mean percentage was circa 15% and highest percentage

38% (Figure 10B). This level of chimerism (1-40%) that is inadequate for treatment of blood malignancies but is necessary and sufficient for the induction of epithelial graft tolerance, bears at least 3 advantages.

The immune system of the host remains fully competent, and therefore the risk of opportunistic infective diseases or malignancies should be much more limited compared to that in fully chimeric recipients, in whom the whole blood cells are of donor type, and there are concerns regarding the ability of host APCs to harness donor T cells.

On the other side, also the risk of graft versus host disease is prevented, as donor and recipient lymphocytes, both present at similar levels, mutually keep at bay (Starzl TE. New Eng J Med 2008).

Finally, transplantation tolerance so obtained is based on peripheral regulation as demonstrated by the presence of linked suppression (Davies JD. J Immunol. 1996). It has already been shown that tolerance trough haematopoietic chimerism is not an exclusive result of central deletion, but the type of mechanism underlying the acceptance of the graft depends on the level of chimerism: the higher the chimerism is, the more tolerance relies on central mechanisms; the lower the chimerism is, the more tolerance relies on peripheral regulation (Bemelman F, et al. J Immunol. 1998).

The fact that transplantation tolerance induced with this protocol is based on peripheral regulation, represents a major advantage, as by definition it is self-perpetuating as old cohorts of tolerant regulatory T cells keep recruiting newer cohorts of lymphocytes: a short-course treatment is able to re-programme the immune system to accept foreign antigens and its benefits theoretically last life-long (Waldmann H. Nat Rev. 2010).

CONCLUSIONS.

The aim of the thesis was to establish clinically feasible therapeutic protocols that enable the induction of transplantation tolerance.

A short-course treatment capable of inducing tolerance, i.e. the specific acceptance of the graft by a fully competent immune system, would be deeply welcome in transplantation clinic, as it potentially allows recipients to be weaned off current life-long immunosuppression with its intrinsic side effects, which represent the major limiting factor for long-term patient and graft survivals (2010 Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data 2000-2009).

It has already been demonstrated that transplantation tolerance is possible in humans, and that even adult and mature immune systems can be experimentally re-programmed to accept not-self antigens with a short-course treatment based on co-receptor and co-stimulation antibody blockade. Although this treatment has proven successful up to the experimental stage of primates, it has never been tested in humans due to legislation limitations that prohibit trialling multiple new agents simultaneously (Kendal AR and Waldmann H. Curr Opinion Immunol. 2010).

I therefore sought to harness the mechanisms of tolerance after treatment with anti-lymphocyte depleting antibodies, which instead have increasingly been utilized in the clinic, in the peri-transplant phase as induction therapy to safeguard the graft during the healing process (2010 Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data 2000-2009). However not only lymphocyte depletion alone, albeit profound and sustained, does not induce graft acceptance (Kirk AD, et al. Transpl. 2003), but tolerance induction after depletion has proven to be less tractable than in a replete immune system (Wu Z, et al. Nat Med. 2004).

First, I have been able to demonstrate, for the first time, that building on the benefits of T cell depletion indefinite survival of MHC mismatched skin grafts can be achieved by use of a protocol based on guiding lymphocyte reconstitution towards regulation (see chapter Results 1/2).

Second, I have also established a protocol based on lymphocyte depletion that enables allogenic bone marrow engraftment without any need for myelo-ablation; the consequent development of mixed haematopoietic chimerism was necessary for the induction of transplantation tolerance towards donor epithelial grafts when performed later in time (see chapter Results 2/2).

The relevance of these findings consists not only in the first experimental demonstration that transplantation tolerance is possible after lymphocyte depletion, but also in the implications for translation into the clinic. Indeed the two strategies I have developed are based on combination treatments that entail the use of two drugs commonly employed in the clinic, the depleting antibody Campath-1 and the immunosuppressant Rapamycin, and one experimental agent, respectively a αIL-7R blocking antibody and a αCD40L blocking antibody. As αIL-7R has already proven to be effective for the experimental treatment of other immune conditions (see Chapter Results 1/2), and αCD40L has a counterpart already approved by the FDA as trasnplantation immunosuppression, named belatacept (Vincenti F, et al. Am J Transplant. 2012), I optimistically see both the protocols to be trialled in primates and humans soon.

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