PhD
Program in Translational
and Molecular Medicine
DIMET

University of Milano-Bicocca
School of Medicine and school of Science

In vitro generation and *in vivo* characterization of IL-10 engineered T cells suitable for adoptive immunotherapy

Grazia Locafaro **Matr. 716071**

PhD Coordinator: Prof. Andrea Biondi

Tutor: Prof.ssa Giuliana Ferrari

Co-Tutors: Prof.ssa Maria Grazia Roncarolo

Dott.ssa Silvia Gregori

XXVII cycle,
Academic year 2013-2014

The research presented in this thesis was performed at the San Raffaele Telethon Institute for Gene therapy (HSR-TIGET), Department of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, in the laboratory headed by Prof. Maria Grazia Roncarolo under the supervision of Dr. Silvia Gregori.

Table of Contents

Chapter 1

General Introduction

1. Immunological tolerance

Immune system has the unique feature to identify and mount a specific adaptive immune response to eliminate harmful pathogens, while simultaneously minimizing reactions against self and non-harmful antigens (Ag). This process is coordinated by B-cell receptors (BCR) and T-cell receptors (TCR), which are expressed on the surface of B and T cells that recognize with high affinity a specific Ag and their immunologic specificity is the result of random shuffling of the many genes that form the DNA code for the Ag-binding site of these receptors [1,2]. Theoretically, this process could generate up to 10^9 different receptors, included some that could recognize self-Ags. It has been estimated that between 20 and 50% of TCR and BCR derived from V(D)J recombination are potentially self-reactive [3–5] but, since autoimmune diseases affect just 3-8% of the population [6], it stands to reason that there must be a mechanisms that teaches the immune system to guide its strong effectors against foreign pathogens, while ignoring the body's own constituents. Although the regulation of B cells is clearly important for preventing autoimmunity, most of the genetic evidence in humans implicates a role for T cells in maintaining tolerance by the adaptive immune system. It is likely that there are regulatory mechanisms specific to B cells that are also involved in human autoimmune disease but to date the human genetics is still in the early stages of investigation [7]. The regulation of self- and innocuous Ag-reactive T-cell response is achieved by a variety of well studied

mechanisms that occur both during the generation of T lymphocytes from lymphoid progenitors in the thymus and during the differentiation of mature T cells in the periphery (Figure 1). The body of the processes that regulates the establishment and maintainance of immunological tolerance can be classified in two main mechanisms: central and peripheral tolerance. These take place at different time during the maturation of T cells and in different body districts. The first one occurs in the thymus where any immature lymphocyte is subjected to follow a process to become a mature T cell. Although the thymic maturation and selection is a finely regulated process, some self-specific T cell clones can elude this checkpoint and reach the periphery. This is where peripheral tolerance, as a second checkpoint, controls auto-reactive T cell clones (Figure 1).

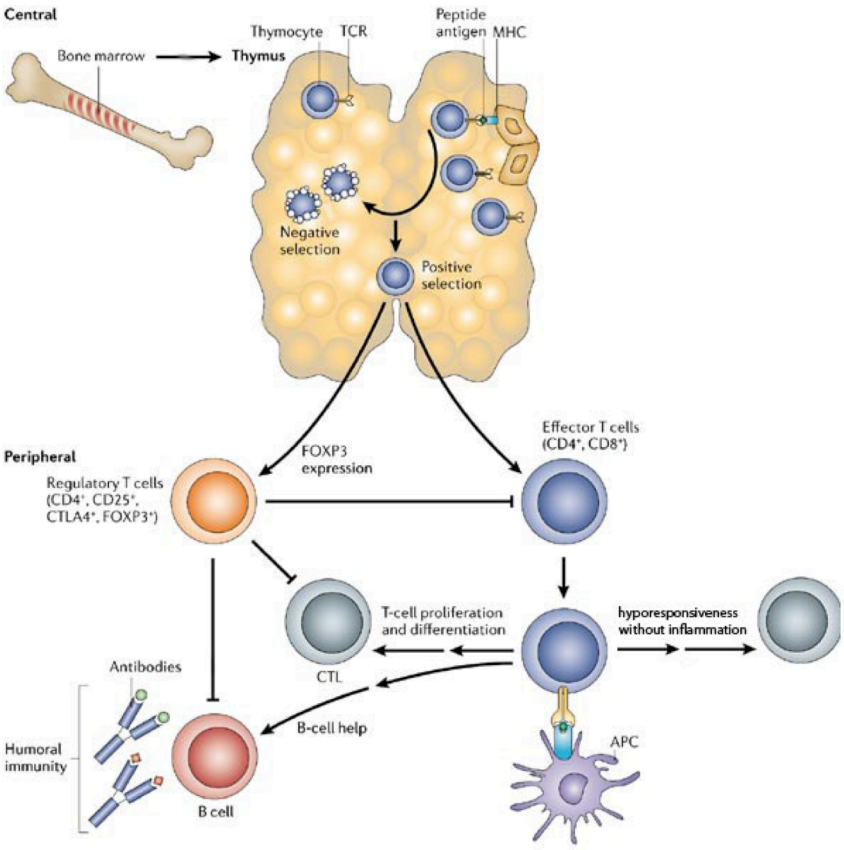


Figure 1: Central and peripheral tolerance mechanisms. Central tolerance takes place in the thymus (top panel), where thymocytes undergo a maturation and selection process that deletes self-reactive thymocytes (negative selection), preserving functional thymocytes (positive selection). In the cortex, thymocytes interact with MHC/peptide complex and receive positive signals whether they are specific and reactive. Conversely, weak reactive thymocytes die by apoptosis. Strongly self-reactive thymocytes are then deleted in the medulla when they recognize tissue-restricted self-peptides in complex with MHC that are transcribed by the gene AIRE expressed by medullary thymic epithelial cells. Mature thymocytes develop into effector $CD4^+$ and $CD8^+$ T cells and migrate in the periphery where they are controlled by several mechanisms of peripheral tolerance. These mechanisms include suppression by T regulatory cells and hyporesponsiveness when lymphocytes recognize specific Ag in the absence of costimulation originated by inflammation *(Modified from Gregersen P.K. & Behrens T.W., Nature Reviews Genetics, 2006).*

1.1 Central tolerance

T lymphocytes derive from common lymphocyte precursors originated from hematopoietic stem cells in the bone marrow that migrate to the thymus where T lymphocytes mature and are selected, generating a pool of effector T (Teff) cells able to successfully protect the body from pathogens without threatening its own components. T cell precursors that enter the thymus at the cortico-medullary junction are double negative cells (they lack the expression of CD4 and CD8 co-receptors) and do not express the TCR [8]. After several differentiation stages, $CD4^-CD8^-$ cells express a final TCR, with a single specificity, and become CD4 and CD8 double positive (DP) cells [9]. DP cells that express a TCR with no affinity for the major hystocompatibility complex (MHC) are deleted in the cortex, a mechanism that lead to the loss of 80-90% of thymocytes. Conversely, DP cells that express TCRs with low-affinity for MHC/self-ligands complexes undergo positive selection and become either $CD4^+$ or $CD8^+$ cells. A further selection, termed negative selection then occurs to eliminate any $CD4^+$ or $CD8^+$ cell with TCR specific for MHC/self-Ags complexes. Negative selection relies on the interaction between $CD4^+$ or $CD8^+$ cells with the medullary thymic epithelial cells (mTEC) and the dendritic cells (DCs) that either are resident or migrate from the periphery. mTEC and DCs express co-stimulatory molecules and, when a thymocyte interacts with high affinity to the MHC/Ag complex is deleted (clonal deletion) [10]. Overall, it has been estimated that about 50-70% of the positive selected T cells are

subjected to negative selection (Figure 1). The importance of this mechanism has been underlined by studies performed in mice lacking co-stimulatory molecules or medullary MHC molecules, in which self-reactive T cells escape the negative selection and reach the periphery, where they give rise to systemic inflammation resembling graft-versus-host-disease (GvHD) [11]. mTEC play a central role in the establishment of dominant tolerance through the induction of thymic-derived T regulatory (tTreg) cells, a subpopulation of $CD4^+$ T cells, characterized by the constitutive expression of CD25 and of the transcription factor forkhead box protein 3 (FOXP3) (Figure 1), tTreg cells are generated from self-reactive $CD4^+$ precursors that receive an intermediate intensity stimulus from the thymic antigen presenting cells (APCs), which does not lead to clonal deletion [12]. T lymphocytes that escape negative selection are released from the thymus and reach the periphery, where they will become Teff cells or will be subjected to peripheral tolerance.

1.2 Peripheral tolerance

Peripheral tolerance is operational to control self-reactive T cell clones that escaped thymic negative selection. The mechanisms underlying peripheral tolerance can be intrinsic (ignorance, deletion and anergy) or extrinsic, namely due to the activity of suppressive cells (Figure 2).

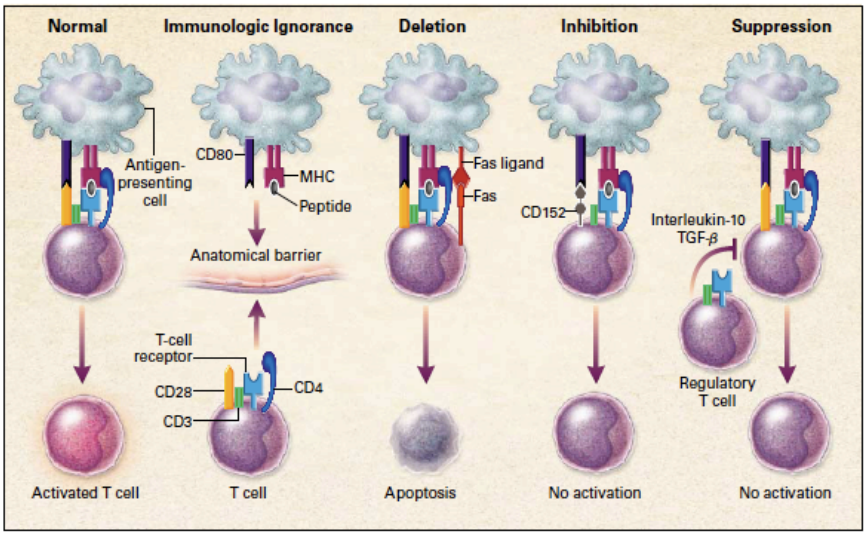


Figure 2: Peripheral tolerance mechanisms. T cells that are physically separated from their specific Ag cannot become activated a circumstance referred as immunological ignorance. Fas-expressing T cells can undergo apoptosis by clonal deletion upon signals from cells that expressed FasL. Activation of T cells can be inhibited by antigen presenting cells (APCs) *via*, for instance, CD152/CD80 interaction. Regulatory T cells can suppress other cells, through different mechanisms, including production of inhibitory cytokines *(Modified from Mackay I.R. & Rosen F.S., The New England Journal of Medicine, 2001)*

1.2.1 Ignorance

The concept of ignorance deals with the fact that potentially self-reactive T cells are not activated since the specific Ag is expressed in low concentration by APCs or at immune privileged sites, such as eyes, Central Nervous System (CNS), and testis. In the latter situations, anatomical barriers separate the potentially autoreactive T cells from the cells that express the tissue-specific Ag [13] are not activate and do not mediate immune reactions.

1.2.2 Deletion

When self-reactive T cells are chronically activated by self-peptide MHC (pMHC) complexes, activation-induced cell death (AICD) occurs. The interaction between Fas on the T cells with Fas ligand on APCs leads to T cell apoptosis [14]. This is a mechanism of protection adopted by several tissues, such as the anterior chamber of the eye where the parenchymal cells constitutively express Fas ligand. In this case, when Fas^+ T cells enter the tissue undergo programmed cell death without damaging the surrounding tissue [15]. The importance of this pathway has been demonstrated in patients with defective Fas signaling, who develop severe lympho-proliferative diseases [16].

1.2.3 Anergy

Anergy is a state of unresponsiveness to an immunogenic stimulus and inability of T cells to synthesize interleukin(IL)-2, the major T cell growth factor, and other inflammatory cytokines, such as interferon(IFN)-γ and tumor necrosis factor(TNF)-α [17]. Anergic cells are blocked at the G1/S phase of the cell cycle and are in a metabolically anergic state characterized by a failure to up-regulate nutrient transporters, such as CD71, CD98 and Glut1, and to switch to anabolic state of metabolism. Several situations lead to establishment of an anergic phenotype and can be sub-divided into two major categories: the absence of co-stimulation during Ag priming and the altered and/or chronic TCR-mediated activation of T cells [18]. T cells to be properly activated and undergo proliferative response require the interaction of their TCR to a specific peptide/MHC complex in the presence of co-stimulation provided by CD28 [19]. CD28 co-stimulation is required for IL-2 expression and the consequent progression of lymphocytes through cell cycle. Addition of exogenous IL-2 can indeed compensate the absence of a proper CD28 activation and reverts anergy [20]. Conversely, inhibition of IL-2 receptor (IL-2R) signal and of cell cycle progression by rapamycin (RAPA) induces T-cell anergy, even in the presence of CD28 co-stimulation [21] [22]. Thus, anergy induction is primarily dependent on the lack of IL-2.
Engagement of the inhibitory receptors Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), an analog of CD28 that interact with CD80 and CD86 on APCs [23], and Programmed Cell Death 1 (PD-1),

expressed on activated T cells that binds to PDL-1 and PDL-2 on macrophages, DCs and parenchymal cells [24], are also required to maintain T-cell anergy.

1.2.4 Dendritic cells

A critical role in determining the outcome of T cell responses is mediated by DCs. Depending on their lineage, stage of maturation, cytokines to which are exposed, and on pathogen-derived products, DCs either activate T cells or promote T-cell anergy. Microbial products, necrosis and pro-infammatory cytokines induce DC maturation. Mature DCs migrate from the periphery to the lymph node, and promote activation of naïve T cells [25] (Figure 3). In steady state, immature (i)DCs constitutively uptake apoptotic cells that, unlike necrotic ones, interfere with DC activation [26,27] (Figure 3). Both myeloid iDCs and immature plasmacytoid DC (pDC)s can induce anergy [28]. Moreover, DCs could become tolerogenic by expressing a variety of immune modulatory molecules and cytokines, such as IL-10 [29–32], inducible T-cell co-stimulator (ICOS) [33], PDL-1 [34,35], Immunoglobulin-like transcript (ILT)3 and ILT4 [36], and indoleamine 2,3-dioxygenase (IDO) [37,38].

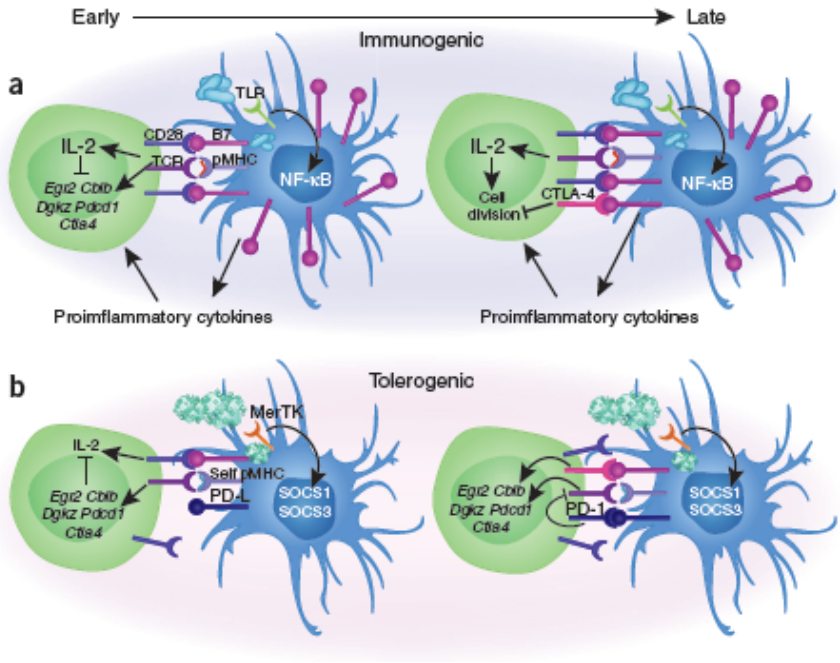


Figure 3: Control of immune response by DC. a) Mature DCs trigger activation of T cells, with induction of autocrine growth factors, such as IL-2, promoting cell cycle progression and inhibiting the transcription of anergy factors (*Egr2, Cblb, Dgkz, Pdcd1* and *Ctla4*). b) Tolerogenic DCs induce the transcription of anergic factors in T cells, such as PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA)-4. *(Modified from Gregersen P.K. & Behrens T.W., Nature Reviews Genetics, 2006)*

1.2.5 Active suppression by T regulatory cells

Anergy may have widespread consequences, since certain subsets of anergic T cells can secrete immune-suppressive cytokines, such as IL-10, which suppress Teff cells. When anergic T cells actively suppress immune responses are referred as T regulatory cells (Tregs) [39]. $CD4^+$ Tregs can be divided into thymic-derived ($FOXP3^+$tTregs) and peripherally induced Tregs. Moreover a

number of induced Tregs can be generated in the periphery from naïve $CD4^+$ T cells count several different subtypes (i.e. $FOXP3^+$pTregs, Th3, iTr35 and Treg type 1 (Tr1) cells) (Figure 4). The latter cells will be extensively discussed in the next chapter.

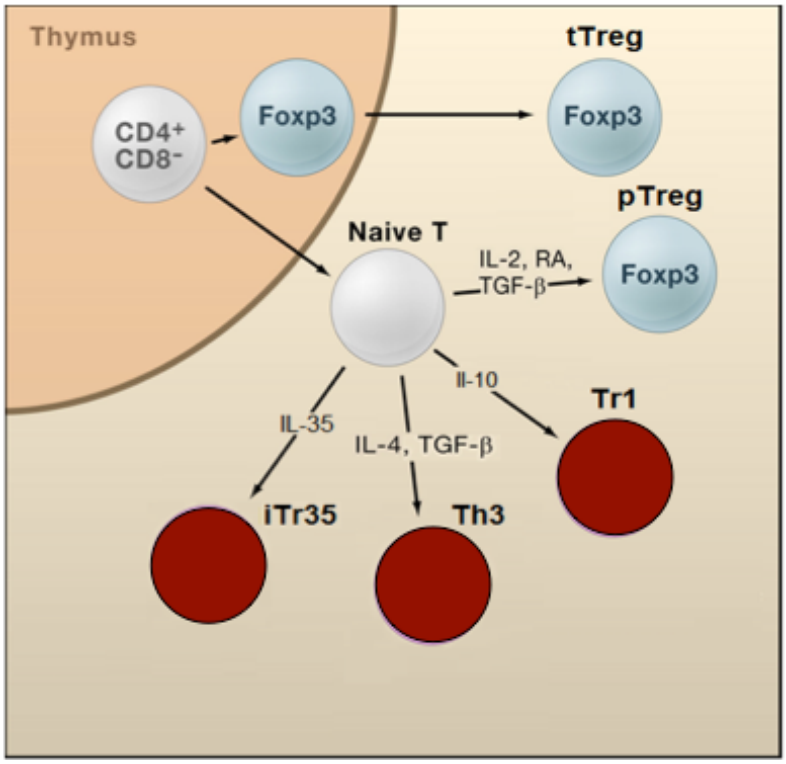


Figure 4. Natural and inducible regulatory T cells in humans. $FOXP3^+CD4^+CD25^+$ Treg (tTreg) cells mature and migrate from the thymus; they express the cell-surface marker CD25 and the transcription factor FOXP3. Adaptive regulatory T cells can be induced from naïve $CD4^+CD25^-$ or $CD8^+CD25^-$ T cells in the periphery upon encounter with their specific Ag in a tolerogenic milieu, such as in the presence of transforming growth factor (TGF)-β and IL-10. The inducible populations of regulatory T cells include: inducible $FOXP3^+CD4^+CD25^+$ Treg (pTreg) and IL-10-producing Type 1 T regulatory (Tr1) cells that can secrete IL-10 (*Modified from Sakaguchi et al. Cell 2008*).

Thymic-derived Treg cells

Thymic-derived Tregs, also called naturally-occurring Tregs, are generated in the thymus following high-avidity recognition of self-peptide/MHC complexes as discussed above. The importance of tTregs in physiological conditions has been demonstrated by adoptive transfer experiments showing that Treg-depleted cells induced multi-organ autoimmunity in recipient mice [40]. tTregs represent 1-2% of total peripheral blood cells [41], and can be distinguished by the constitutive surface expression of CD25, CTLA-4, the glucocorticoid-induced TNF receptor family-related protein (GITR), and CD39 [42], and low levels of IL-7 receptor α (CD127). The main hallmark of tTregs is the constitutive expression of the transcription factor FOXP3, which is a master regulator of tTreg cell development and function [12]. However, tTregs, once committed to their lineage, do not depend anymore on FOXP3 expression for survival [43]. In humans, mutations in *foxp3* gene result in a severe autoimmune syndrome named Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX), characterized by the lack of functional tTregs [44,45] and which clinical conditions depend on the functionality and on the amounts of FOXP3 protein expression [44,46]. *Scurfy* mice, a murine model carrying a loss-of-function mutation in the *foxp3* gene, lack tTregs, and develop a lethal lymphoprolipherative disease similar to IPEX [47]. Similarly to humans, mutations in *foxp3* gene that lead to a decrease of FOXP3 expression cause *scurfy*-like symptoms in mice [48]. To date, the most reliable feature unambiguously

identifying tTregs is the epigenetic remodeling of a specific region in the *foxp3* locus, indicated as Treg-specific-demethylated-region (TSDR) [49].

tTregs mediate suppression *via* a number of different mechanisms that can be grouped into suppression by: the secretion of inhibitory cytokines, cytolysis, metabolic disruption and modulation of DC maturation or function (Figure 5). tTregs require direct interaction with Teff cells to dispatch their function, which seem not to be Ag-specific [50]. CTLA-4 plays a major role in this mechanism since it: i) outcompetes the activating receptor CD28 and keeps tTregs in an hyporesponsive state [51]; ii) binds to CD80 and CD86 on activated Teff cells, inhibiting T-cell proliferation [52]; iii) interacts with CD86 on DCs and modulates their function, inducing the immune-regulatory enzyme indoleamine-2,3-deoxigenase (IDO) [53]. Other surface molecules contribute to the suppressive function of tTregs, such as lymphocyte activation gene (LAG)-3 and CD83 [54,55], and soluble factors, and one of the most characterized is IL-10. Survival and functional competences of tTregs rely on the presence of IL-2 in the environment, which promotes the expression of FOXP3 and other functional molecules (like CTLA-4). However, tTregs do not produce IL-2 by themselves, instead they utilize the cytokine produced during conventional immune responses by Teff cells, and in turn induce hyporesponsiveness also in Teff cells, which undergo apoptosis after 72 hours of co-culture *in vitro* because deprived of the surviving cytokine [56].

Even if cytolysis mediated through the secretion of granzymes had long been considered the specialty of natural killer (NK) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs), many human $CD4^+$ T cells exhibit cytotoxic activity. Consistent with this, activated human Tregs have been shown to express granzyme A (GzA). Furthermore, Treg-cell-mediated target-cell killing was found to be mediated by granzyme A and perforin through the adhesion of CD18 [57]. The notion that Tregs might possess cytolytic activity was supported by studies showing that Tregs can kill B cells in a GzB dependent and partially perforin-dependent manner that results in the suppression of B−cell function [58]. Moreover a recent study has suggested that activated Tregs induce apoptosis of Teff cells through a TRAIL–DR5 (tumour-necrosis−factor-relatedapoptosis-inducing ligand–death receptor 5) pathway [59].
Overall, Tregs possess multiple mechanisms to mediate their suppressive activity. The different suppressor mechanisms might be integrated and used predominantly, depending on the nature of the immune response, the eliciting agent, the effector cell subset to suppress and the tissue compartment.

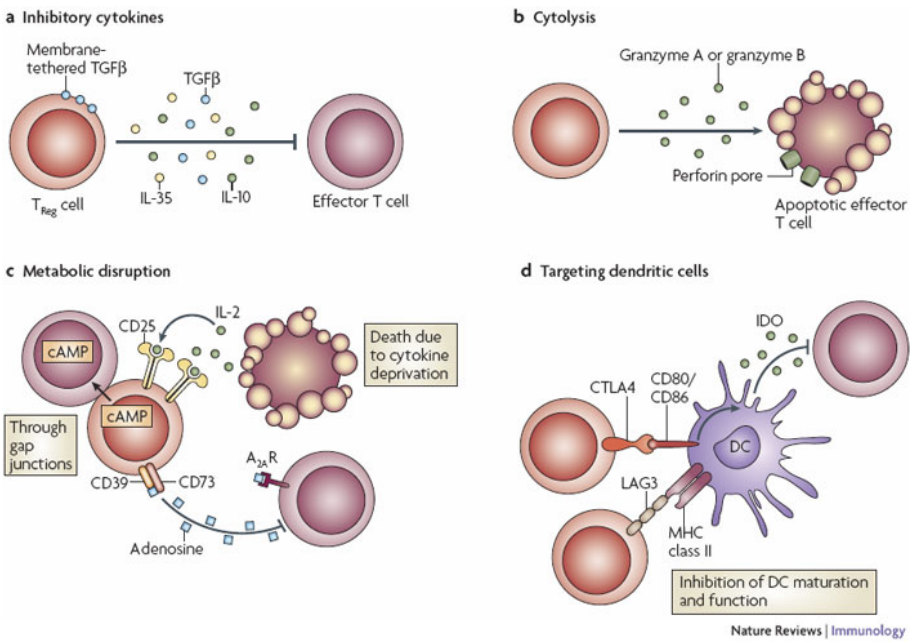


Figure 5: Suppression mechanisms of Treg cells. $CD4^+$ $FOXP3^+CD4^+CD25^+$ Tregs suppress immune-response by distinct mechanisms here grouped in four basic mode of action. Metabolic disruption by IL-2-deprivation-mediated apoptosis, by cAMP- mediated inhibition, and by adenosine-mediated suppression. Targeting DC by inhibiting their maturation via LAG3-MHC-class-II. GZA-, GZB- and PRF- dependent killing. Inhibitory cytokines such as IL-10, TGF-β, and IL-35 *(Modified from Vignali D. A., Nature reviews Immunology, 2008).*

Peripheral-induced Treg cells

These cells derive from $CD4^+CD25^-$ naïve T cells, reside mainly in the peripheral lymphoid tissue, and their existence has been demonstrated in transgenic mice adoptively transferred with $CD4^+CD25^-$ T cells in which $CD4^+CD25^+$ cells were induced [60]. pTregs are phenotypically superimposable to tTregs, since they constitutively express CD25 in the absence of CD127 and are $FOXP3^+$; however, as indicated above, the two sub-populations

can be distinguished by the methylation pattern of TSDR, which is consistently de-methylated in tTregs while not in pTregs. The conditions favoring pTregs induction include suboptimal DCs activation, sub-immunogenic doses of high-affinity peptide, and the presence of appropriate cytokines as TGF-β and IL-2 [61–63]. Similarly to tTregs, pTregs are able to suppress the expansion of $CD4^+$ Teff cells and to inhibit secretion of T helper (Th)1, Th2 and Th17 cytokines. So far, tTregs and pTregs do not show distinct functional differences but, it was suggested that, while thymic derived cells play a central role in preventing autoimmunity, pTregs are mainly involved in establishment of oral tolerance in response to dietary Ags [64,65].

Adaptive Tregs induced in the periphery distincted from pTreg

Inducible Tr35 cells

Collison and colleagues showed that high concentration of IL-35 in the milieu induces the differentiation of a subtype of Tregs able to suppress Teff cell proliferation in an IL-35-dependent manner [66]. These Tregs, named iTr35 cells, are hyporesponsive to re-stimulation, express CTLA-4 and CD25, but not FOXP3. It is still elusive the role of these cells in the immune physiology. The inhibitory function of iTr35 cells is cell-cell contact independent, and requires the secretion of IL-35, which is a member of IL-12 cytokine family with suppressive properties. Ectopic expression of IL-35 on pancreatic β-cells can protect against experimental autoimmune diabetes [67].

Th3

Th3 cells represent another subset of adaptive Tregs, firstly described in orally tolerized animals by mucosal stimulation with Ag [68]. Although these cells do not constitutively express FOXP3, Th3 definitely resemble pTregs because of their reliance on TGF-β. These cells derive from naïve $CD4^+$ T cells receiving Ag stimulation and CD80/86 co-stimulus in the presence of TGF-β and IL-4 [69,70]. Th3 cell growth and division depends on the presence of IL-4 and TGF-β, rather than IL-2, in the environment, and they produce TGF-β themselves. Th3 cells can mediate suppression of Teff cell via TGF-β secretion in response to CTLA-4-mediated ligation [68]. Th3 and pTregs cannot be unequivocally distinguished, because of the lack of specific markers.

2. T regulatory Type 1 (Tr1) cells

2.1 Biological features

Tr1 cells originally reported by Groux and colleagues [71] are characterized by their specific cytokine profile, which is distinct from the others Th cells: upon activation, Tr1 cells secrete high levels of IL-10, and minimal amounts of IL-4 and IL-17. Tr1 cells secrete also TGF-β, variable amounts of IL-5, GM-CSF and IFN-γ, but low levels of IL-2 [71–74]. In contrast to other T-cell subsets that secrete IL-10, including Th1, Th2 [75–77], Th9 [78], Th17 cells [79], and FOXP3^{+} Tregs [80] Tr1 cells show a distinct kinetic of IL-10 production, since they secrete IL-10 early after activation (Bacchetta et al., 1994). Tr1 cells are characterized by low proliferative response upon polyclonal T Cell Receptor (TCR)-mediated or Ag-specific activation *in vitro* (Bacchetta et al., 1994; Bacchetta et al., 2002; Groux et al., 1997). Tr1 cells up-regulate activation markers, such as HLA-DR, CD25, CD69 and CD40L upon TCR stimulation (Bacchetta et al., 2002). The anergic phenotype of Tr1 cells is mainly due to the autocrine production of IL-10, indeed addition of neutralizing anti-IL-10 antibodies partially restores their proliferative responses (Bacchetta et al., 1994; Bejarano et al., 1992; Groux et al., 1997). Despite the low proliferative capacity *in vitro*, Tr1 clones and lines can be expanded in the presence of exogenous IL-15 and low amounts of IL-2, due to the expression of high levels of CD122 (IL-2/IL-15Rb) and CD132 (IL-2R common g chain) (Bacchetta et al., 2002;

Magnani et al., 2011).

Tr1 cells do not constitutively express FOXP3 [84], but it can be transiently up-regulated upon activation (Brun et al., 2011; Brun, Bastian, Neveu, & Foussat, 2009; Gregori, Goudy, & Roncarolo, 2012; Megan K. Levings et al., 2005), but at lower levels compared to Tregs and pTregs. Tr1 cells express inhibitory receptors: CTLA-4 [81,89,90], PD-1 (Akdis 2008), and inducible co-stimulatory molecule (ICOS) [91]. Tr1 cells express also the ectoenzymes CD39 and CD73 that generate adenosine via the enzymatic hydrolysis of extracellular ATP, increasing cytosolic cAMP levels that disrupt the metabolic state of Teff cells [92,93].

The chief mechanism by which Tr1 cells control immune responses is through the secretion of IL-10 and TGF-β [71,73,85,86,94,95]. To exert their suppressive function Tr1 cells need to be activated *via* their TCR, but, once activated, Tr1 cells can mediate bystander suppressive activity against other Ags *via* IL-10 and TGF-β [71]. IL-10 directly suppresses T cell responses by inhibiting IL-2, IFN-γ, and GM-CSF production by T cells [96]. Similarly, TGF-β has potent immune-modulatory effects that directly inhibit T cell responses [97,98]. Tr1 cells also suppress Teff cells indirectly by inhibiting inflammatory fators and activating tolerogenic pathways of APCs [99]. Many of the molecules hindered by IL-10 on APC, like HLA class II, co stimulatory molecules [100], and pro-inflammatory cytokines [101,102] are important mediators of adaptive immune responses. IL-10 treated APCs, which up-regulate a number of tolerogenic molecules, including ILT-3 and 4

[32,103], and the non-classical HLA-G [32,104], become regulatory cells capable of dampening immune responses and inducing Tregs [32,105].

Suppressive effects of Tr1 cells can be partially reversed by addition of anti-IL10 and anti-TGF-β neutralizing mAbs [88], indicating that additional mechanisms may also contribute to Tr1 cell-mediated suppression, such as cytolysis [57,83], up-regulation of inhibitory receptors (Akdis 2008), and expression of ectoenzymes [93]. Human Tr1 cells express and release high levels of Granzyme B (GzB), and specifically lyse cells of myeloid origin, but not other APCs or T and B lymphocytes (Magnani et al. 2011). Tr1-cell–mediated cytotoxicity of myeloid APCs is Ag-independent and requires recognition and activation *via* HLA class I molecules expressed on target cells. This differs from NK cells, which kill target cells lacking HLA class I molecules. Specific killing of myeloid APCs by Tr1 cells depends also on high expression levels of CD54, CD58, CD155, and CD112 on target myeloid cells, which, upon interaction with their ligands (LFA-1, CD2, and CD226, respectively) on Tr1 cells, mediate stable Tr1-cell/myeloid target adhesion and Tr1-cell activation (Magnani et al. 2011). Killing of myeloid cells by Tr1 cells represents an additional indirect mechanism of suppression that may contribute to bystander suppression mediated by Tr1 cells. It is tempting to speculate that upon encounter with their cognate Ag Tr1 cells up-regulate GzB expression and kill myeloid cells in an Ag-non-specific manner. This indirect effect could result in a reduction of allo-reactive APC in the case of transplantation, or APC that present self-Ags in the

case of autoimmune diseases, limiting priming and expansion of effector T cells.

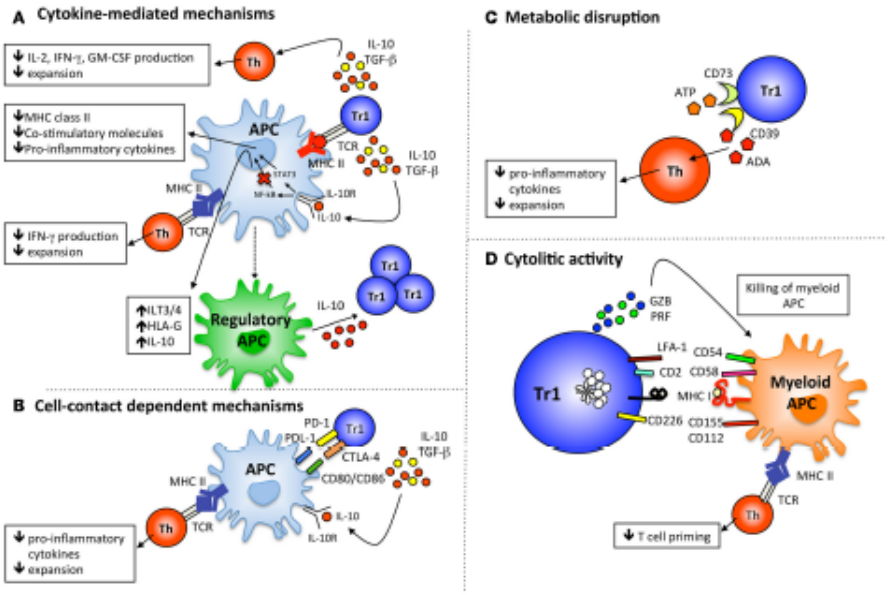


Figure 6. Mechanisms of suppression mediated by Tr1 cells. (A) Tr1 cells upon Ag-specific activation secrete IL-10 and TGF-β that directly inhibit the function of Teff cells and indirectly modulate APCs, which in turn limit Teff cell proliferation and cytokine production. IL-10 produced by Tr1 cells up-regulates the expression of tolerogenic molecules (ILT3, ILT4 and HLA-G) on APCs and their ability to produce IL-10, and become regulatory APCs able to promote de novo induction of Tr1 cells. **(B)** CTLA-4 andPD-1onTr1cells down-regulate or prevent the up-regulation of MHC class II and co-stimulatory molecules on APC upon interaction with their ligand, which limits effector T cell activation. **(C)** Catalytic inactivation of extracellular ATP by the ectoenzymes CD39 and CD73 represents an anti-inflammatory mechanism used by Tr1 cells to prevent effector T cell proliferation and cytokine production. **(D)** IL-10 produced by Tr1 cells upon activation promotes autocrine granzyme B (Gz-B) that in association with perforin (PRF) allows an Ag-nonspecific Tr1-mediated killing of myeloid APCs. Myeloid-specific killing by Tr1 cells requires HLA-class I activation and the expression of CD54, CD58, CD112 and CD155 on myeloid cells. *(Modified from Gregori S., Frontiers in Immunology, 2012).*

2.2 Biomarkers of Tr1

From their discovery, much efforts have been made to identify surface molecule or transcription factors specifically expressed by Tr1 cells, in order to distinguish them from other subtypes of T cells. it was suggested that mouse Tr1 cell clones expressed the transcriptional repressor of GATA-3 (ROG), nevertheless ROG is not specific for Tr1 cells since it is expressed also by activated T cells [106]. Afterwards the early response gene 2 (*Erg-2*) was proposed to be specifically expressed by Tr1 cells [107]. In murine $CD4^+$ T cells IL-27 promotes the Egr-2 binding to the promoter of Prdm1 and induces expression of B lymphocyte induced maturation protein-1 (Blimp-1), which in turn binds *il-10* locus and promotes its expression [108]. Moreover, Erg-2 expression strongly correlates with LAG-3 expression on $CD4^+$ IL-10-producing suppressor T cells. Additionally, Erg-2 has recently been shown to play a key role in humoral immune responses by inducing $CD4^+CD25^-LAG3^+$ Treg cells [109]. Based on these findings, Erg-2 has been proposed to be a regulator of IL-10 production in Tr1 cells [110].However, we found that Tr1 cells *ex vivo* isolated from the small intestine of anti-CD3 mAb treated mice or *in vitro* induced with IL-27 and TGF-β express Erg-2 at levels comparable to that observed in other Teff cells [74]. Thus, it still controversial if Erg-2 expression is specific for Tr1 cells.

C-Maf has been shown to activate the *il-10* promoter in Tr1 cells, partnering with aryl hydrocarbon receptor (AhR) [111,112]. However, the expression of these factors is not specific for Tr1 cells since

both human and murine Th17 cells also express cMaf and AhR at comparable, or even higher, levels, than those observed in IL-27-induced Tr1 cells [112–115].

Several groups, including us, aimed at identifying markers specific for Tr1 cells. However, most of the surface molecules proposed, such as ICOS [91], CTLA-4 [81,89,90] or PD-1 [89] have been associated not only with human IL-10- producing T cells, but they are also expressed on other T-cell subsets. Our group firstly showed that CD226 (DNAX accessory molecule-1; DNAM-1), an adhesion/signalling molecule that contributes to the NK-mediated lysis, is expressed at high levels on human Tr1 cells and it is critically involved in the specific killing of myeloid APCs by Tr1 cells (Magnani et al. 2011). Subsequently, in 2013 our group identified CD49b and LAG-3 as specific markers of Tr1 cells [74]. Okamura initially proposed LAG-3 as phenotypic marker of IL-10-producing FOXP3-independent induced Treg cells. This was based on the observation that the expression of this molecule seemed to be restricted to $CD4^+$ $CD25^-$ $CD45RB^{low}$ T cells, which include IL-10 producing $FOXP3^-$ cells [107]. Moreover, it has been showed that IL-27 induces the expression of both IL-10 and LAG-3 on murine $CD4^+$ T cells [108]. However, even if LAG-3 expression is associated with IL-10 production, this surface marker can be found also on $FOXP3^+$ Tregs [116,117] and plays a central role in controlling expansion and homeostasis of activated Teff cells [118,119]. CD49b, the α 2 integrin subunit of the very-late-activation antigen (VLA)-2, has been proposed as a marker for IL-10-producing T cells [120]. CD49b is expressed by a population of $CD4^+$ T cells induced by

repetitive injections of immature bone marrow-derived DC [120]. A significant proportion of CD49b$^+$ cells produce IL-10 upon *in vitro* re-stimulation and dampen immune response *in vivo* when adoptively transferred in a mouse model of contact hypersensitivity [121]. CD49b is associated with IL-10 production also in humans [122], however, even it can not be considered as an exclusive maker of Tr1 cells, since both murine and human Teff cells can express CD49b[123]. Moreover, VLA-2 (CD49b/CD29) is the major integrin expressed by human Th17 cells [124]. Thus, nor the single expression of CD49b neither of LAG-3 is specific for Tr1 cells, while their co-expression allows the identification and isolation of both human and murine Tr1 cells [74].

2.3 *In vitro* induction of Tr1 cells

Several experimental protocols have been established to generate Tr1 cells *in vitro* from naïve precursors, but IL-10 remains the main driving force. Groux et al. [71] demonstrated that mouse T cells repeatedly stimulated with splenic APCs and ovalbumin (OVA) peptide in the presence of IL-10 proliferate poorly in response to antigenic stimulation. Transfer of these OVA-specific cells *in vivo* prevented colitis in SCID mice reconstituted with CD4$^+$CD45RB^{high} splenic T cells. Human Tr1 cells were first generated by addition of exogenous IL-10 in cultures of human peripheral blood mononuclear cells (PBMC) or purified CD4$^+$ T cells stimulated with allogeneic monocytes (mixed lymphocytes reactions MLR/IL-10) [125]. The resulting T cells were anergic and contained precursors of IL-10-producing Tr1 cells [71]. Anergy induced by IL-

10 was Ag-specific, since IL-10-anergized CD4+ and CD8+ T cells did not proliferate upon re-challenge with the same Ag used during cell priming. Notably, after IL-10 anergization, the bulk culture contained also non-anergic T cells that were non-alloAg-specific and maintained their ability to respond to other Ags, such as pathogens or third party allo Ags (Bejarano et al., 1992; Rosa Bacchetta et al., 2010b).

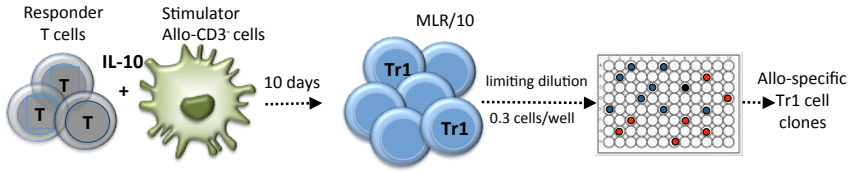


Figure 7. *In vitro* differentiation of Tr1 cells using monocytes or DC. Purified CD4+ T cells are stimulated in vitro with irradiated allogeneic monocytes in the presence of recombinant human IL-10 (primary MLR). Cloning of the obtained IL-10-anergized cultures in the presence of feeder mixture consisting of allogeneic PBMC, Epstein-Barr virus lymphoblastoid cells (EBV-LC), and anti-CD3 mAb demonstrated that the original population is enriched of Tr1 cells. modified from *(Adapted from Maria Grazia Roncarolo et al., 2006).*

The molecular mechanisms underlying Tr1 cell induction *via* IL-10 are still elusive. Nevertheless, STAT3-dependent activation by the IL-10/IL-10R interaction is required for anergy induction and IL-10 secretion by CD4+ T cells [127]. The importance of the STAT3 pathway to induce Tr1 cells is underlined by the observation that other STAT3 activating cytokines such as IFN-α (Levings et al. 2001), IL-6 (Jin et al. 2013) or IL-27 [128–131] promote IL-10-producing Tr1 cells. It has been reported that the over-expression of STAT3 in human CD4+T cells *per se* induces them to acquire a tolerogenic program [132].

AhR was shown to be involved in Tr1 cell differentiation [111,133,134]. In mouse $CD4^+$ T cells STAT3 activation by IL-27 leads to the induction of c-Maf and of AhR that hetero-dimerize and trans-activate *il-10* and *il-21* promoters, sustaining Tr1 cell induction *in vitro* [111,135]. Like in the mouse, AhR was shown to be involved in human Tr1 cell differentiation. Stimulation of human $CD4^+$ T cells in the presence of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) or FICZ (6-formylidolo[3,2-b]canbazole) selectively activates AhR that co-operates with c-Maf to trans-activate the *il10* promoter and to enhance IL-10 production (Apetoh et al. 2010; Gandhi et al. 2010). It was recently showed that the AhR, in cooperation with STAT3, controls the expression of the transcription factor Aiolos, which is expressed by IL-27-induced Tr1 cells [136]. More recently, IL-6 was also shown to promote murine IL-10-producing Tr1 cells (Jin et al. 2013). IL-6-mediated activation of STAT3 promotes IL-21 production, which in turn co-operates with IL-2 to induce the expression of c-Maf and AhR, thereby leading to IL-10 production in CD4+ T cells. This mechanism is independent of both IL-27 and TGF-β [137].

Although IL-10 has been shown to be indispensable for Tr1 cell induction, it is not *per se* sufficient for the differentiation of Tr1 cells in the absence of APCs *in vitro* [138]. We developed an *in vitro* system using artificial APCs expressing high levels of CD58 and CD80 to generate Tr1 cells. Addition of exogenous IL-10 and of IFN-α, a cytokine crucial for the clearance of viral infections, results in efficient differentiation of human Tr1 cells *in vitro* [127]. Activation of CD4+ T cells with artificial APCs bearing CD2 has

been shown to promote IL-10-producing Tr1 cells. Signaling *via* CD2, the ligand for CD58, inhibits CD4+ T cell proliferation in an IL-10-dependent way, and enhances the induction of Ag-specific Tr1 cells [139]. Cross-linking of CD4+ T cells with anti-CD3 and anti-CD46 mAbs, a complement regulator protein, in the presence of IL-2 induces IL-10-producing Tr1-like cells. However, it is still unclear whether these CD3/CD46-stimulated T cells are *bona fide* Tr1 cells, since they proliferate strongly, and do not suppress T-cell responses when directly co-cultured with responder cells [140].
Repetitive stimulation of $CD4^+$ T cells in the presence of vitamin D3 and dexamethasone induces a population of Ag-specific Tr1 cells in an IL-10-dependent manner [94]. Finally, an alternative co-stimulatory pathway, involving CD97/CD55 interaction has been shown to promote the differentiation of a discrete population of Tr1 cells [141].
Tr1 cells can be induced by immature DC, as well as by a specialized subsets of DC, termed tolerogenic DC (Morelli & Thomson, 2007). Repetitive stimulation of human naïve $CD4^+$ T cells [88,143] with allogeneic iDC induces differentiation of human Tr1 cells *in vitro*. Several biological or pharmacological agents, such as IL-10 alone (Jonuleit et al. 2000) or in combination with IFN-α [103,144], G-CSF [145,146], or vitamin D3 alone [147] or in combination with dexamethasone [148] can induce tolerogenic DC with the ability to generate Tr1 cells. These agents impair DC maturation and inhibit up-regulation of costimulatory molecules, secretion of proinflammatory cytokines, in particular IL-12, and allostimulatory capacity. A population of human tolerogenic DCs,

termed DC-10, that is present *in vivo* in peripheral blood and in secondary lymphoid tissues and can be differentiated *in vitro* from peripheral blood monocytes in the presence of IL-10 have been identified (Figure 8). DC-10 mature myeloid cells, express high levels of the tolerogenic molecules ILT2, ILT3, ILT4, and membrane-bound HLA-G, secrete high levels of IL-10 but low amounts of IL-12, display a low stimulatory capacity, and induce the differentiation of a population of cells containing a significant proportion of anergic allo-specific IL-10-producing Tr1 cells (Figure 8) [32,126].

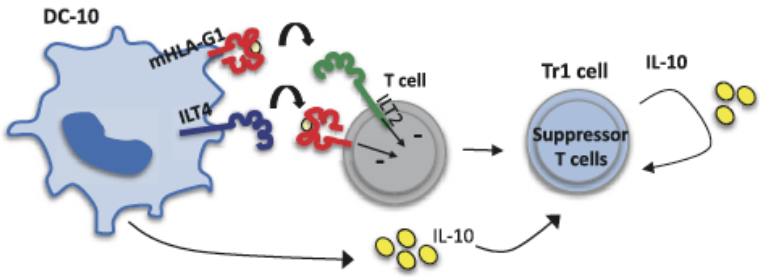


Figure 8: Molecular mechanism underneath Tr1 cell induction by DC-10. DC-10 promote the induction of Tr1 cell lines from naïve $CD4^{+}CD45RO^{-}$ T cells, via HLA-G1/ILT4 pathway. Interaction between ILT4 and HLA-G1 drives signals on DC that sustains the tolerogenic phenotype on T cells and promote Tr1 cell induction *(modified from Gregori S., Human Immunology, 2009).*

Differentiation of Tr1 cells by DC-10 is dependent on IL-10, HLA-G1 and ILT4, but not ILT2, since neutralizing anti-IL-10R, anti-ILT4, or anti-HLA-G mAbs reverts the anergic status and the suppressive ability of the differentiated T cells [149]. Moreover, we recently reported that HLA-G expression level on DC-10 are genetically imprinted and influence the tolerogenic activity of human DC-10 [150].

2.4 IL-10 gene transfer

The different strategies described above are rather efficient in promoting the induction of functional Tr1 cells, however the resulting population is not homogeneous and includes a significant fraction of contaminating non-Tr1 cells, which may limit the efficacy of Tr1 cells *in vivo*.

IL-10 is the major driving force for Tr1 cell differentiation and is a key player in Tr1-mediated tolerance [87], thus several groups have modified T cells to over-express IL-10, by means of viral vectors, with the goal to convert conventional $CD4^+$ T cells into Tr1-like cells. Murine or viral IL-10 gene transfer has been applied [151–154]. Setoguchi et al. transduced splenic DO11.10 T cells with murine IL-10 using a replication-defective retro-viral vector (RV), and showed that adoptive transfer of these IL-10–transduced T cells ameliorated OVA-induced arthritis. Transferred T cells preferentially accumulate in the inflamed joint and amelioration of the disease was not accompanied by impairment of the systemic immune response to the Ags [151]. Similar results were obtained using ovalbumin-specific CD4 T-helper cells engineered to express IL-10, in the following years, in murine models of allergen-induced hyperreactivity and inflammation [152] and in $CD45RB^{high}$-induced colitis [153].

More recently, Brandt et al. transduced rat T cells with a bicistronic RV, encoding for viral IL-10 and a marker gene [154]. IL-10-engineered T cells (T_{VIL-10}) acquire all the functional features of Tr1 cells: display a memory-like phenotype, secrete, in addition to

vIL-10, rat IL-10 and IFN-γ but no IL-4, and inhibit proliferation and IFN-γ production of naïve T cells activated in allo-specific manner using irradiate lymph nodes stimulator cells. Despite this *in vitro* immune-modulatory capacity, T_{VIL-10} cells were not able to prolong allograft survival in a high-responder strain combination, meaning that their regulatory capacity *in vitro* did not predict their *in vivo* suppressive function [154].

Guichelaar et al. transduced murine proteoglycan-specific $CD4^+$ T cells with the murine *IL-10* gene using replication-defective RV to obtain Ag-specific Tr1 cells (T_{IL-10})[155]. Proteoglycan-specific T_{IL-10} cells are suppressive *in vitro*, reduce the proteoglycan-specific inflammatory immune response *in vivo*, and promote the endogenous IL-10 response when adoptively transferred in a murine model of chronic arthritis [155]. Notably, in these studies, retroviral vectors were used to overexpress IL-10 in murine $CD4^+$ T cells, but no extensive characterization of the resulting murine IL-10-transduced T cells were performed.

In recent years, lentiviral vectors (LVs) have strongly appeared in biomedicine as an alternative to γ-RV. As RVs, LVs are devoid of viral proteins, stably incorporate their genome into the host cell, and lead to long-term transgene expression. In addition, unlike the simple RV, they can transduce nondividing cells. Indeed, LVs have been shown to guarantee higher transduction efficiency in T cells even in the absence of TCR triggering, resulting in preservation of an intact T-cell repertoire and of immune competence *in vitro* [156,157]. On the contrary, it has been demonstrated that the sustained proliferation required for the

efficient transduction by RV that impair the half-life, repertoire and immune-competence of transduced cells [158]. Moreover, RV transduction often results in an enrichment in memory T cells, with loss of the naïve T cells subset [157], a skew in the TCR [159] and a reduced alloreactivity [160].

We recently developed a method to generate IL-10-producing Tr1 cells using a bidirectional LV encoding for human IL-10 and green flurescent protein (GFP), as marker gene (Andolfi et al., 2012). We demonstrated that human $CD4^+$ T cells transduced with LV-IL-10 ($CD4^{IL10}$) acquired the typical Tr1 cytokine profile: IL-10^{+++}, TGF-β^+, IL-5^+, IFN-γ^+, and IL-$4^{low/neg}$; up-regulated IL-10-related surface markers such as PD-1 [161], ICOS-L, ICOS [91], IL-10R and the non-classical MHC class I molecule HLA-G [149]. Like Tr1 cells, $CD4^{IL-10}$ cells are anergic, suppress allogeneic $CD4^+$ and $CD8^+$ T cell responses mainly *via* IL-10 and TGF-β, express high levels of GzB, and acquired the ability to specifically lyse cells of myeloid origin. $CD4^{IL-10}$ cells showed suppressive activities not only *in vitro*, but also *in vivo* in NOD.scid mice in which xeno graft-versus-host was induced [162]. Thus, constitutive over-expression of IL-10 in human $CD4^+$ T cells leads to a stable cell population that recapitulates the phenotype and function of Tr1 cells.

2.5 Tr1 cells as therapeutic tool

Induction of Tr1 cells *in vivo* or *ex vivo* has been exploited as therapeutic strategy in immune-mediated diseases [138,163]. The benefits of the use of Tr1 cell therapy over conventional treatments are the lack of general immune suppression, and the

possible induction of a long-term and stable tolerance In pre-clinical murine model the infusion of polyclonal Tr1 cells promoted graft tolerance only in the non-stringent model of islet transplantation. Conversely, injection of allo-specific Tr1 cells was required to induce tolerance in the stringent model [164], thus suggesting that Ag-specificity may be required for a more efficient and safe result.

Pre-clinical studies demonstrated also that *in vivo* transfer of *in vitro* induced IL-10-producing Tregs controlled graft versus Host Disease (GvHD) following bone-marrow transplantation [165]. Based on these data our group performed a clinical trial in which adoptive cell therapy with Tr1 cells was administered into patients affected with advanced haematological malignancies undergoing haploidentical hematopoietic stem cell transplantation (HSCT) therapy with the aim to provide fast immune-reconstitution and prevent GvHD [166]. Accordingly to the need of immune reconstitution within a high degree of HLA disparity, these patients were provided with donor T cells, which have been primed for a short time (10-days) in the presence of the host APCs and IL-10 to generate Ag-specific Tr1 cells (Figure 9). The method to obtain the infused cells was validated in good-manufacturing-practice (GMP), to generate allo-specific IL-10-anergized T cells by using host monocytes in the presence of IL-10 (IL-10 DLI) [126,142,167]. In this small proof-of-concept clinical trial (ALT-TEN trial), 12 patients received T cell depleted (TCD) haplo-HSCT and were treated with IL- 10-DLI. These cells were infused 1-month after treatment with a mega-dose of $CD34^+$ cells and

when the patients showed myeloid engraftment. Overall, the outcome of TCD haplo- HSCT combined with IL-10-DLI cell therapy was positive in four patients with high-risk/advanced stage hematologic malignancies. These patients achieved full donor chimerism, immune reconstitution, and long-term persistent disease remission. Cells from all four patients expressed biomarkers consistent with tolerance and Tr1 cell enrichment. This study indicates the feasibility of cell therapy with IL-10-DLI to promote fast immune reconstitution and tolerance after haplo-HSCT [166].

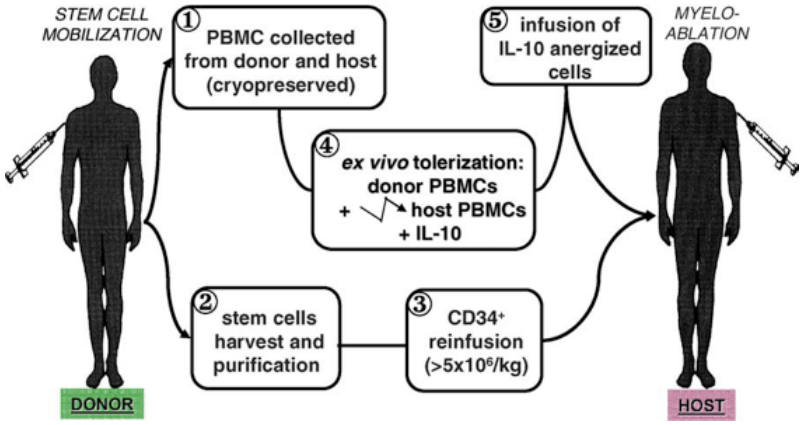


Figure 9: Clinical protocol with IL-10-anergized cells. 1) PBMC are collected and cryopreserved from the HLA-haploidentical donor prior to mobilization and from the host prior conditioning. 2) Stem cells are harvested after donor mobilization and (3) purified $CD34^+$ cells are infused into the recipient who underwent myelo-ablation. 4) After the first signs of neutrophil reconstitution, donor PBMC are cultured with irradiated host PBMC or DC-10 in the presence of IL-10. 5) After 10 days of culture, the IL-10 anergized donor cells are infused in the patients (*Adapted from Battaglia et al. Seminar in Immunology, 2006*).

Clinical grade production of Ag-specific Tr1 cells has been developed by the group of Foussat [85] and a phase I/II trial in patients displaying severe Crohn's disease has been completed proving the safety of the cell-therapy. This clinical trial of Tr1 cell therapy consisted of OVA-specific Tr1 cells clones in single injection to patients affected with refractory Chron's disease (CD) [90]. To ensure activation of OVA-specific Tr1 cells migrating to the gut, patients ingested an OVA-enriched diet. Results showed a clinical significant improvement in 8 of the 20 patients (40%), with a stronger effect in the group of patients who received 10^6 cells. Overall, the study was safe and also showed some clinical benefit; however, the effect was time limited, reaching the maximum 5-weeks after treatment and declining thereafter. This observation suggests that the infusion of multiple doses of Tr1 cells may be required for long-term control of the disease. The same group has planned a similar trial to treat rheumatoid arthritis (http://www.txcell.com) It was previuosly demonstrated in two preclinical models of arthritis the clinical benefit of adoptively transferred *in vitro* differentiated Tr1 cells specific for a locally expressed Ag [168]. In addition, a phase I/II proof of principle clinical trial has been planned to treat the rare disease Autoimmune Uveitis by the same group (http://www.txcell.com).

A cell-therapy protocol is now planned using DC-10 as APC to produce IL-10-anergized T cells *in vitro* to prevent graft reject after kidney transplantation. In this case, DC-10 *in vitro* differentiated from donor monocytes will be used to anergize recipient T cells. This Tr1 containing T cells will be injected into

patients transplanted with kidney from living donors in combination with standard immunosuppressive drugs. This study will evaluate the benefit of the cell therapy in terms of preventing graft rejection even long after the transplant, for which standard immunosuppression has shown not to be efficient. This clinical trial is part of "The ONE Study", an integrated European Union-funded project, that aims at developing and testing different subsets of regulatory cell products in kidney transplanted recipients allowing a direct comparison of the safety, clinical practicality and therapeutic efficacy of different cell types (http://www.onestudy.org/).

Since Tr1 cells have the advantage of being quickly generated *in vitro* in an Ag-specific manner, their potential application is very broad. Optimization of the best modality to obtain Tr1 cells suitable for cell therapy will contribute to facilitate their use in diseases different from those selected for the initial proof of concept trials. In allogeneic HSCT, Tr1 cells could be used in transplant from unrelated matched donors to prevent chronic GvHD; in organ transplantation, their use could be extended to liver and pancreatic islet transplant possibly to prevent or limit the need of long-term immunosuppressive therapy. Furthermore, autologous Tr1 cell therapy can be indicated in food allergy and in celiac disease, in which the patients could be fed with low doses of the specific antigens to maintain the peripheral Tr1 cell pool operational and to favour the gut homing. Ultimately, a variety of autoimmune diseases, especially those with recurrent course and high inflammatory component such as rheumatoid arthritis, could

also be a good indication for Tr1 cell therapy, which could be performed by local administration to further avoid systemic side effect of anti-inflammatory drugs.

3. Hematopoietic Stem Cell Transplantation

Hematopoietic Stem Cell Transplantation (HSCT) is life saving, curative treatment for a range of diseases, including hematological disorders, malignancies, immunodeficiencies and metabolic storage disorders. This chapter will be focused mainly on HSCT for the cure of hematological malignancies, such as cancer.

Table1.

Autologous Transplantation *

Cancers

- Multiple myeloma
- Non-Hodgkin's lymphoma
- Hodgkin's disease
- Acute myeloid leukemia
- Neuroblastoma
- Ovarian cancer
- Germ-cell tumors

Other diseases

- Autoimmune disorders
- Amyloidosis

Autologous Transplantation §

Cancers

- Acute myeloid leukemia
- Acute lymphoblastic leukemia
- Chronic myeloid leukemia
- Myelodysplastic syndromes
- Myeloproliferative disorders
- Non-Hodgkin's lymphoma
- Hodgkin's disease
- Chronic lymphocytic leukemia
- Multiple myeloma
- Juvenile chronic myeloid leukemia

Other diseases

- Aplastic anemia
- Paroxysmal nocturnal hemoglobinuria
- Franconi's anemia
- Blackfan-Diamond anemia
- Thalassemia major
- Sickle cell anemia
- Severe combined immunodeficiency
- Wiskott-Aldrich syndrome
- Inborn errors of metabolism

Table 1. Diseases commonly treated with HSCT

* more than 30,000 autologous transplantations are performed annually wordwide, two thirds for multiple myeloma or non-Hodgkin's lymphoma.
§ more than 15,000 allogeneic transplantations are performed annually wordwide, nearly half for acute leukemias. The vast majority are performed to treat lymphoid and hematologic cancers. (*Adapted from Copelan EA, NEJM 2006*)

The principal aim of an effective HSCT protocol for the cure of cancer is (*a*) to eliminate or suppress host immunity, through the administration of a conditioning regimen including cytotoxic drugs and whole body irradiation, (*b*) to eliminate malignant cells, (*c*) to minimize the adverse event of GvHD without affecting the beneficial graft-*versus*-leukemia effect, and (*d*) to minimize toxicity to other tissues [169,170]

Different types of HSCT can be classified based on the source of stem cells, the choice of the donor and the conditioning regimen used to prepare the recipient.

3.1 HSCT donor

The choice of the type of transplantation autologous (from the patient itself), allogeneic (from an HLA matched unrelated donor), or haploidentical (from a parent of the patient with only one identical HLA haplotype) depends on the type of malignancy, age of the recipient, availability of a suitable donor, the ability to collect a tumor-free graft, the stage and status of disease. Autologous transplantation is readily available and has a lower risk of life-threatening complications: no risk of GvHD and no need for immunosuppressive therapy. Immune reconstitution is

more rapid than an allogeneic transplant, the risk of opportunistic infections is lower and graft failure occurs rarely. Treatment-related mortality is lower than 2% in most studies [169], and elderly patients can tolerate treatment relatively well [171,172].

The donor of an allogeneic HSCT can be a sibling, a family member or a non-family member. Allogeneic transplants may be associated with several complications such as regimen-related organ toxicity, graft failure, and GvHD. Immune reconstitution is slower and opportunistic infections are more frequent. Treatment related mortality is significantly higher compared to autologous transplantation and it increases in case of mismatched or unrelated donors compared to an HLA-identical sibling donor [173].

The transplantation of stem cells from a parent, sibling or child of a patient with only one identical HLA haplotype (haploidentical) was initially associated with high rate of engraftment failure and GvHD. In the past decade, technical advances have improved the outcome of this approach [174]. The success of this type of transplantation depends on the activity of alloreactive natural killer cells (NK), which express combination of activating and inhibitory killer-cell immunoglobulin-like receptors that interact with class I HLA epitopes. The balance of signals determines the cytolitic activity of the natural killer cells. Alloreactivity improves the chances of engraftment and reduces the risk of GvHD [175].

Mismatched donors offer several advantages: a) donor availability for virtually all transplant candidates; b) ability to select the best among different relatives on the basis of age, infectious disease status and, NK cell alloreactivity [176]; c) controlled graft

composition; and d) immediate access to donor-derived cellular therapies if required after transplantation. Furthermore, for nearly all patients who reject the graft, the haploidentical transplant offers the advantage of either another family member who is immediately available as an alternative donor or even a second graft from the original donor.

3.2 Source of HSCT

The source of hematopoietic stem cells for transplant can be: bone marrow, peripheral blood, and cord blood.

Bone marrow was the first source of hematopoietic stem cells. It can be obtained from the puncture of the posterior iliac crest while the donor is under a local anesthesia [169]. Since hematopoietic stem cells are able to migrate from the bone marrow to the periphery, they can also be obtained also from peripheral blood. When using peripheral blood as the source of transplant, the reconstitution is more rapid compared to bone marrow. On the other side, the use of peripheral blood for transplantation increases the incidence of GvHD [177]. The number of hematopoietic stem cells ($CD34^+$) can be increased in the peripheral blood by mobilizing them from the bone marrow with the administration of granulocyte colony stimulating factors (G-CSF) in combination with AMD3100, a small molecule, which is a reversible inhibitor of the CXC chemokine receptor 4 (CXCR4) [178].

Cord blood has been identified as a good source of hematopoietic stem cells because umbilical cord and placenta are rich of hematopoietic progenitors. They can be easily and safely collected

but they are limited in volume. Because hematologic and immunologic reconstitution is slow, patients transplanted with cord blood are more susceptible to infections. Cord blood transplantation require less stringent HLA matching than does the transplantation of adult peripheral blood or marrow, because mismatched cord blood cells are less likely to cause GvHD, without loosing the graft versus leukemia effect [179].

3.3 Graft-*versus*-Host Disease

Graft-*versus*-Host Disease represents a major cause of mortality and morbidity after allogeneic HSCT. This is the second leading cause of death in patients undergoing allogeneic HSCT, with a fatality rate of nearly 20% (http://www.cibmtr.org). GvHD is a complex disease resulting from immunological attack on target recipient organs, such as skin, liver, lung, mucosae and gut, by donor allogeneic T cells within the stem cell graft. The development and severity of GvHD in transplant recipients depend on different factors such as recipient age, toxicity of the preparative regimen, hematopoietic graft source and GvHD prophylaxis schedule [180].

Clinical GvHD has an acute and a chronic forms. Acute GvHD has a strong inflammatory component, with robust T cell activation and proliferation causing the damage of skin, liver and the gastrointestinal tract [170]. Chronic GvHD has more diverse manifestations and display more autoimmune and fibrotic features (Figure 10-11) [180].

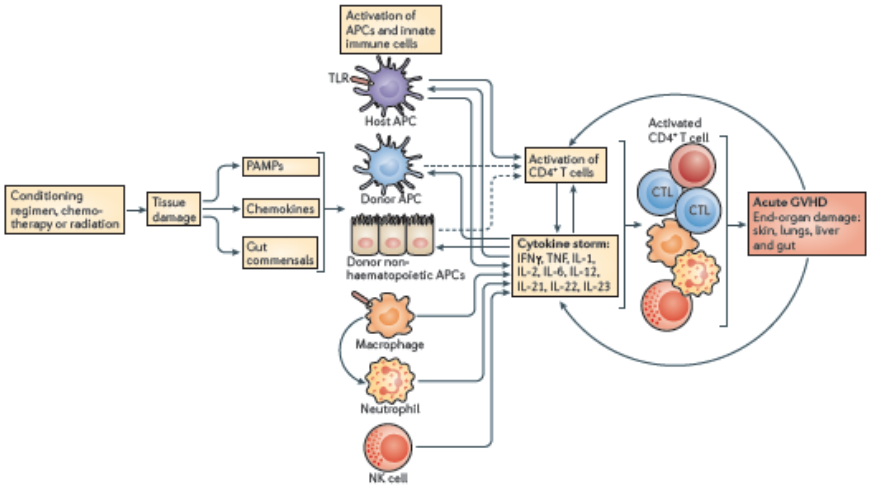


Figure 10. The overall acute GvHD cascade. Although the conditioning phase is not absolutely necessary for the induction of acute GVHD, in many of the models it activates antigen-presenting cells (APCs), via tissue destruction, and increases APC function. Through the release of gut bacteria, pathogen-associated molecular patterns (PAMPs) and chemokines, the conditioning phase can also lead to the activation of innate immune cells that participate in direct tissue damage and contribute to the cytokine storm. Following the presentation of antigens to T cells, a strong cytokine response is initiated. These cytokines further promote antigen presentation and the recruitment of effector T cells and innate immune cells, which further augment the pro-inflammatory cytokine milieu. Finally, the effector T cells, natural killer (NK) cells, macrophages and pro-inflammatory cytokines result in end-organ damage, which is clinically recognized as acute GVHD in the skin, lungs, gut and liver. CTL, cytotoxic T lymphocyte; IFNγ, interferon-γ; TLR, Toll-like receptor (*Adapted from Blazar RB, Nature Review 2012*).

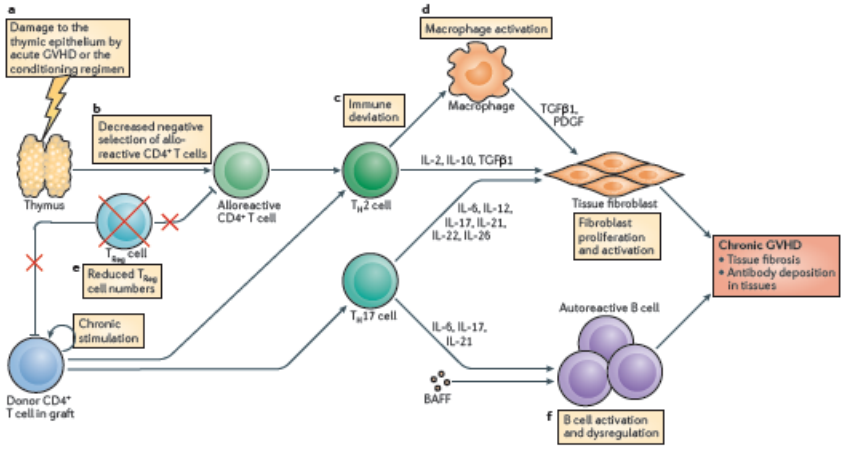


Figure 11. Crucial factors in the developement of chronic GvHD. The pathophysiology of chronic GvHD mainly depends on the polarization of $CD4^+$ T cells into T helper 2 (T_H2) cells, but there are six hallmarks that are unique to this syndrome. The first feature is damage to the thymus (a), which can be caused by the conditioning regimen or, more importantly, by prior occurrence of acute GvHD. This damage results in decreased negative selection of alloreactive $CD4^+$ T cells (b). There is immune deviation to a T_H2-type cytokine response (c), which includes the production of interleukin-4 (IL-4), IL-5 and IL-11. This response leads to the release of fibrogenic cytokines - such as IL-2, IL-10 and transforming growth factor-β1 (TGFβ1) - and the activation of macrophages that produce platelet-derived growth factor (PDGF) and TGFβ1 (d). These molecules induce the proliferation and activation of tissue fibroblasts. Low numbers of regulatory T (TReg) cells are the fifth hallmark (e), and finally there is B cell dysregulation (f), which leads to the emergence of autoreactive B cells and the production of autoreactive antibodies. It has been suggested that autoreactive B cell activation may be due to the presence of high levels of B cell-activating factor (BAFF) in the lymphoid microenvironment. All these events contribute to an autoimmune-like systemic syndrome that is associated with fibroproliferative changes. (*Adapted from Blazar RB, Nature Review 2012*).

Acute GvHD occurs within first 100 days after transplantation, and chronic GvHD occurs after day 100 post-transplantation. However, many investigators currently believe that a pathological classification is more useful, because histological analysis

demonstrated that acute GvHD could occur after day +100, particularly when immunosuppressive drugs are withdrawn. In 2005 the National Institute oh Health (NIH) proposed a consensus criteria for diagnosis and classification of chronic GvHD that rely on clinical manifestation rather than on timing after transplantation [181]. A retrospective study support the consensus recommendations that, with appropriate stratification, clinical trials can include patients with late acute GvHD as well as those with NIH chronic GvHD [182].

Table 2. Categories of acute and chronic GvHD

Category	Time of Symptoms after HCT or DLI	Presence of Acute GVHD Features*	Presence of Chronic GVHD Features*
Acute GVHD			
Classic acute GVHD	≤100 d	Yes	No
Persistent, recurrent, or late-onset acute GVHD	>100 d	Yes	No
Chronic GVHD			
Classic chronic GVHD	No time limit	No	Yes
Overlap syndrome	No time limit	Yes	Yes

HCT indicates hematopoietic cell transplantation, DLI, donor lymphocyte infusion. (*see Table 1 of *Filipovich A.H. et al., Biol of Blood Marrow Transpl, 2005*)

GvHD is primarily a T cell-mediated event. Researchers have considered acute GvHD to be a process driven mainly by Th1- and Th17-type immune responses. On the contrary, chronic GvHD has been described as a Th2-type mediated disease. However, this paradigm has been challenged by recently human and mouse studies and is not absolute [183–187].

Acute GvHD begins with a conditioning regimen, such as chemo- and/or radio-therapy, which induces tissue damage that initiates a cascade of pro-inflammatory cytokines release. This “cytokine storm” activates APCs, which present Ag to alloreactive T cells.

Tissue damage caused by the cytotoxic T cells leads to the recruitment of other effector cells (including macrophages, neutrophils, and NK cells), which further amplify the tissue injury, creating a positive feedback lop that is difficult to control even with immunosuppressive drug treatment.

Manifestation of chronic GvHD is typically of autoimmune nature, with donor T cells interacting with bone marrow B cells, recipient macrophages and fibroblasts to cause antibody deposition and tissue fibrosis; however its pathophysiology is less understood than that of acute GvHD, in part due to a lack of good animal models, able to represent that represent the full pathological spectrum for this disease [180].

To diminish the risk of GvHD, all patients receive immunosuppressive agents that impair T cell functions. Alternatively, recipients receive allografts that have been depleted of T cells, which have negative consequences: it greatly impairs immune reconstitution, which increases the risk of infections; it diminishes or, in the case of rigorous T-cell depletion, completely abrogates T cell-mediated GvL.

3.3.1 Genetics of GvHD

Major incompatibility antigens have a major effect on the biology and occurrence of GvHD in the HSCT setting. MHC, is located on the short arm of chromosome 6 (p21): class I HLA-A, -B and -C are expressed on all nucleated cells, sibling donors and recipients who share HLA antigen have better engraftment and reduced rates and severity of GvHD [188]; HLA Class II (DR, DQ and DP) are

more selectively expressed on cells of the immune system, and are abundantly in skin and gastro-intestinal tract epithelium and may contribute to the specific organ sites of acute GvHD [189,190].

The optimization of high resolution typing and donor-recipient HLA matching at allele-level has improved HSCT outcome [191]. Single mismatch of HLA-A, -B, -C, DRB1 or DQB1 was associated with significant decrement in survival, although did not increase the risk of acute GvHD. The presence of multiple mismatches was worse for survival and severe acute GvHD (grade III-IV)[192]. Since HLA class I molecules are crucial in both T cell and NK-mediated immune responses, there is a great interest in understanding the molecular mechanisms of GvHD. Different data about the role of KIR mismatching and missing ligands on transplant outcome has been produced [174,175,193]. KIR receptors can have inhibitory or activated potential. HLA-C serves as ligand for both inhibitory KIR receptors (KIR2DL1, KIR2DL2, KIR2DL3) and selected activating receptors (KIR2DS1, KIR2DS4). The HLA-Bw4 motif is encoded by select HLA-A and HLA-B molecules, and is a ligand for inhibitory KIR (KIR3DL1). Therefore, when HLA-A, B and C polymorphisms are evaluated together, is it possible to have a number of information for evaluating the clinical importance of ligand mismatching and missing ligands on transplant outcome.

Minor histocompatibility molecules (HAs) play also a role in the pathogenesis of GvHD. Minor HAs are peptides derived from intracellular proteins presented by specific MHC molecules to donor T cells [194]. These minor Ags express genetic polymorphisms encoded by a wide range of genes and are important in the

initiation of GvHD in the identical sibling and sex-mismatched allogeneic transplant setting [195]. Human minor HAs are mostly, but not exclusively, restricted to class I HLA. Tissue expression of some minor HAs is limited to the hematopoietic system (HA-1 and HA-2), whereas other minor antigens are more widely expressed (HA-Y and HA-3). Mismatches between donor and recipient for HA-1, HA-2 and HA-5 are associated with an increased risk of GvHD [196].

Although the risk of GvHD occurrence is higher in case of HLA mismatching, clinically significant GvHD can arise also in the case of HLA identical transplants, suggesting that other genes should be involved in the process of graft compatibility [197,198](**Tab 3)**.

Gene	*Polymorphism*	*Recipient/donor*	*Donor type*	*aGvHD outcome*
TNFα	d3/d3	Recipient	Identical sibling	↑ grade II–IV, GvHD, increased mortality
	TNF-863, TNF-857	Donor and/or recipient	MUD	Increased
	TNFα-238, TNFβ-252	Donor and/or recipient	Unrelated donor	Increased grade II–IV, increased mortality
	TNFd4, TNFα-1031C and TNFa5	Donor and/or recipient	Unrelated donor	Increased mortality
	TNFd4	Recipient	Identical sibling	↑ moderate aGvHD
	TNFRII-196R	Donor	MUD	↑ grade severe aGvHD
	TNFRII-196M	Homozygous donor	MUD	Reduced risk aGvHD
	TNFRII-196R	Recipient	Identical sibling	↑ grade severe aGvHD
IL-10	Low ACC producer	Recipient	Identical sibling	↑ grade severe aGvHD
	Intermediate ATA	Recipient	Identical sibling	↑ grade severe aGvHD, increased mortality
	R3-GCC	Recipient	MUD	Reduced aGvHD and mortality
IL-6	Il-6-174	Recipient	MUD	↑ grade severe aGvHD
INFγ	INFγ 2/2	Recipient	Identical sibling	Reduced aGvHD
	INFγ 3/3	Recipient	Identical sibling	↑ aGvHD
IL-1 family	IL-Ra	Donor	Identical sibling	Reduced aGvHD
		Recipient	Identical sibling	↑ Chronic GvHD
	IL-1α 889 (pediatrics)	Donor and recipient	MUD	Improved survival, less TRM
TGFβ	TGFβ-509	Donor and recipient	Identical sibling	No effects
	TGFβ codon 10 (pediatric)	Donor	Identical sibling	↑ aGvHD
	TGFβ codon receptor II (pediatric)	Recipient	Identical sibling	↑ aGvHD

Tab. 3 GvHD risk correlating with cytokine genes donor/recipient polimorphisms.

Abbreviations: MUD= matched unrelated donor; TGF= trasforming growth factor (*Adapted from Ball L.M. and Egeler R.M. Bone Marrow Transplantation 2008*).

3.3.2 Treatment of Graft-versus-Host Disease

Immunosuppression has been the primary pharmacological strategy to prevent GvHD. The introduction in the 1980s of two immunosuppressive agents, cyclosporine and tacrolimus, has dramatically improved allograft survival rates. Cyclosporine and tacrolimus inhibit of the cytoplasmic enzyme calcineurin, which is important for T cell activation [199].

Calcineurin inhibitors are usually administrated in combination with other immunosuppressive drugs, such as methotrexate, a cytotoxic drug, which at low dose, exert anti-inflammatory effects. The combination of cyclosporine and methotrexate has demonstrated superiority over single agent use [200], making the combination therapy the most commonly used GvHD prophylaxis regimen [201]. Mycophenolate mofetil (MMF) is an anti-metabolite and the pro-drug of mycophenolic acid, which selectively inhibits inosine monophosphate dehydrogenase in T cells, has been used in alternative to methotrexate. The combination of MMF and any calcineurin inhibitors has shown synergistic activity for GvHD prophylaxis [202]. In a prospective randomised trial, patients who received MMF as part of GvHD prophylaxis had significantly less severe mucositis and more rapid neutrophil engraftment than did those who received methotrexate. Frequency and severity of acute GvHD was similar between the two groups [203]. Due to the fast neutrophil engraftment, MMF is used for umbilical-cord blood transplantation for which graft failure is a major concern [204]. For the same reason, MMF is sometimes administrated after reduce-

intensity conditioning regimen [205,206].

Sirolimus (also known as Rapamycin) is an immunosuppressive drug that is structurally similar to tacrolimus that inhibits the mammalian target of the rapamycin (mTOR) pathway, which blocks IL-2 mediated signal transduction and prevents cell-cycle progression in naive T cells [207]. A phase I–II trial, in which patients received sirolimus combined with tacrolimus and methotrexate showed a lower incidence of grade 2–4 acute GvHD in patients conditioned with a myeloablative regimen followed by unrelated and mismatched donor grafts compared with historical controls [208]. An other clinical study investigating the feasibility of unmanipulated haploidentical peripheral blood stem cell (PBSC) transplantation in patients affected by high-risk hematological malignancies showed that a calcineurin inhibitor-free sirolimus-based GvHD prophylaxis promotes the in vivo expansion of Tregs, and permits PBSC transplantation from haploidentical donors [209]. In an effort to omit methotrexate, and thereby minimize potential complications, the sole combination of tacrolimus and sirolimus was tested in a related donor setting. In this phase II study, feasibility and encouraging incidences of grade 2–4 acute GvHD, neutrophil recovery, and overall survival at 1-year follow up were reported [210]. However, the drug damages endothelial cells and it might increase transplant associated thrombotic microangiopathy, which is associated with calcineurin inhibitors [210,211]. To date, there is no clear consensus on optimal drug combination, dosing, or timing [212]. One of the most critical steps in GvHD pathogenesis is the recruitment of activated T cells to target

organs. Chemokine-chemokine receptor interactions mediate this migration, and CCR5 has been shown to be a key mediator of T cell trafficking to GvHD target organs, especially the gastro intestinal tract [213]. Exciting new success has been reported with maraviroc, a CCR5 antagonist that blocks T-cell chemotaxis and dramatically decreased the incidence of gastrointestinal and liver GvHD [214]. At the same time, despite the central role of cytokines IL-1 and TNF-α, drugs that block these pathways (etanercept, infliximab) failed to improve rates of acute GvHD [215,216].

Alemtuzumab is a humanized monoclonal antibody directed against the CD52 receptor that depletes B and T lymphocytes by complement fixation and antibody-dependent cell-mediated cytotoxicity mechanisms. Early phase II studies of treatment with alemtuzumab before allogeneic HSCT have shown decreased incidence and severity of GvHD, and reduced mortality. However, the perceived benefit was offset by the increased graft failure, disease recurrence, and delayed immune reconstitution [217]. Several studies focused their attention on antithymocyte globulin (ATG) or anti-lymphocyte globulin preparation as T cell depleting agent. ATG are polyclonal immunoglobulins produced by immunizing rabbits (rabbit ATG or thymoglobulin) with the T-lymphoblastic Jurkat cell line (ATG-Fresenius, ATG-F), or immunizing horses (equine ATGAM) with human thymus lymphocytes [218]. The effect of ATG is variable, since it was observed that even different preparations show different biological effects [219].

Alternatively a single-arm trial of soluble CTLA4-Ig (also known

as abatacept) targeting the CD28:B7 costimulatory pathway successfully induced anergy and reported a low rate of GvHD [220]. The feasibility of adding abatacept to cyclosporine and methotrexate therapy for GvHD prevention following unrelated donor HSCT has recently been investigated in a pilot trial involving 10 patients [221]. Only two patients developed grade 2–4 acute GvHD, but seven patients showed cytomegalovirus (CMV) or EBV reactivation [221].

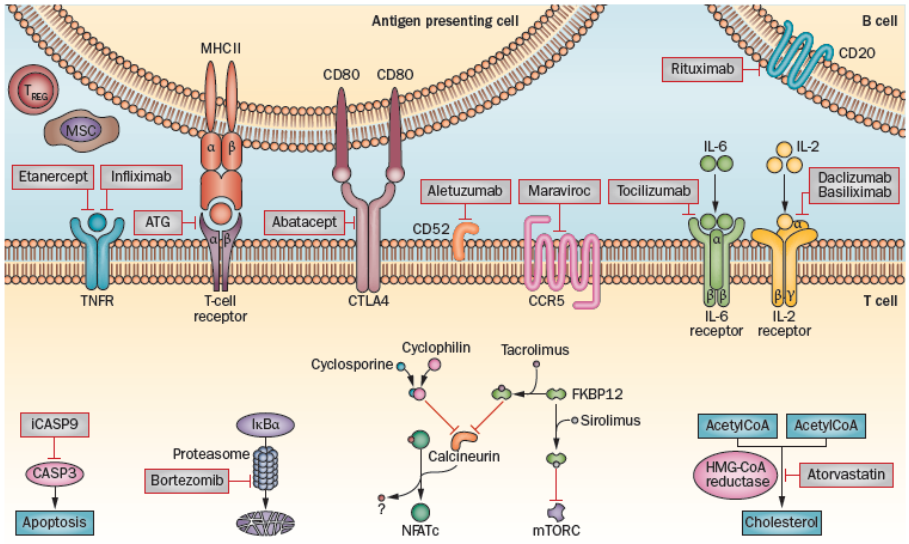


Figure 11. Standard and emerging therapies for the prevention of acute GvHD. The different medications and their targets against B cells and T cells are illustrated in this figure. Mesenchymal stem cell (MSC) and regulatory T-cell (TREG) infusions are depicted extracellularly. Abbreviations: Acetyl CoA, acetyl coenzyme A; ATG, anti-thymocyte globulin; CCR5, C-C chemokine receptor 5; CLTA4, cytotoxic T lymphocyte antigen 4; FKBP12, FK506 binding protein 12; GVHD, graft-versus-host disease; HMG CoA reductase, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase; iCasp9, inducible caspase 9; IkB, nuclear factor of kappa light polypeptide gene enhance in B cells inhibitor; IL, interleukin; MHC II, major histocompatibility class II; mTORC, mammalian target of rapamycin complex; NFATc, nuclear factor of activated T cells cytoplasmic; TNFR, tumour necrosis factor receptor (*Adapted from Choi S.W and Reddy P., Nature 2014*).

In addition to targeted drugs, cell-based therapies represent another potentially successful intervention for preventing GvHD. Several types of immunosuppressive cells are currently under investigation in clinical trials.

Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are one of the cell types with immunomodutolary properties that have been studied. MSCs are a group of heterogeneous plastic-adherent cells with the capacity to differentiate *in vitro* into a variety of supportive cells. MSCs exert a wide range of immunosuppressive and immunomodulatory effect on innate and adaptive immune cells [222]. MSCs may have a protective effect against GvHD [223]. Results of clinical trials are also confusing, as earlier trials showed substantial benefits, whereas two recent Phase III trials with at least one source of MSCs did not show any benefit [224,225]. Differences in manufacturing and defining MSCs, in the expression of homing receptors and in the type of GvHD injury may all contribute to the difficulty in comparing results between laboratories and clinical outcomes.

Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs are a heterogeneous cell population of myeloid origin that consist of progenitors and mature macrophages, granulocytes and DCs. MDSCs are defined as $CD11b^{+}Gr1^{+}$ cells in mice, as $LIN^{-}HLA\text{-}DR^{-}CD33^{+}$ or $CD11b^{+}CD14^{-}CD33^{+}$ cells in humans, although they have also been defined within $CD15^{+}$ peripheral blood cell population [226,227]. MDSCs can be expanded *in vitro* and can

suppress T cell function by expressing enzyme that regulate essential amino acid metabolism, such as arginase 1 and IDO, by releasing soluble mediators, such as IL-10, reactive oxygen species or nitric oxide [228]. Animal models have shown that MDSCs can suppress acute GvHD [229]. Moreover, in one study, *in vivo* arginine depletion could also be accomplished through the use of a drug, pegylated arginase 1, suggesting a new pharmacological approach to acute GvHD prevention [230].

NK cells and NKT cells

Donor NK cells has been shown to be able to inhibit acute GvHD. Clinical studies showed that infusion of NK cells within the graft was associated with a decreased GvHD occurrence and severity, compared to HSCT alone [231]. Morover, it has been demonstrated that signalling by the KIR family is important in determining the activity of NK cells and the outcome after allogeneic HSCT for acute myeloid leukaemia (AML) [175,232]. Patients with AML were also significantly protected against leukemia relapse when they received a transplant from NK alloreactive donors [175,232–234]. This has stimulated interest in optimizing NK cell use not only to suppress GvHD but also to promote GvT responses, as haploidentical NK cell transfer can increase GvT responses in patients with AML [235]. Furthermore, it has been demonstrated that enriched NK cells can be infused safely as adoptive immunotherapy in patients with leukemia and with cancer after nonmyeloablative and myeloablative immunosuppressive chemotherapy with some cases achieving significant clinical

responses [236,237]. Moreover, in another study it has been better investigated the feasibility and safety of infusing highly purified NK cells from haploidentical KIR ligand–mismatched donors into a cohort of elderly patients with high-risk AML and it was shown the donor NK-cell repertoires, trafficking, and function in the peripheral blood (PB), BM, or both of recipients at different time points [238].

NKT cells, a cell subset co-expressing both NK and T cell markers, has also been shown to control GvHD in mice in an IFNγ- and IL-4-dependent manner [239]. In mouse models, the *in vivo* activation of NKT cells with glycosphingolipids such as α-galactosylceramide has been shown to be able to inhibit GvHD. However, a recent murine study demonstrated that an early administration of a syntetic form of α-galactosylceramide (KRN7000) result in hyperacute GvHD [240]. Thus, it remains to be determined whether NKT cell-based therapy will be useful.

Tolerogeneic DCs

DCs are a heterogeneous group of professional, APCs that regulate immune responses, maintaining the balance between tolerance and immunity. Special subsets of DCs, which have tolerogenic functions, are able to inhibit GvHD in mice [241–243]. Tolerogenic or regulatory DCs can be generated *in vitro* from murine bone marrow precursors [244] or human blood monocytes [245], by culturing them with granulocyte-macrophage colony-stimulating factor (GM-CSF), combined with IL-4 and pharmacological agents or anti-inflammatory biological, for example IL-10, TGF-β or

vitD3.

Infusion of tolerogenic DCs has been shown to rescue animals from lethal acute GvHD, and this was associated with the generation of induced Treg cells [246].

T regulatory cells

Tregs have been shown to suppress the early expansion of alloreactive donor T cells and limit the capacity to induce GvHD without minimizing the graft-versus-leukemia (GvL) effect [247]. Given these pre-clinical data, human Tregs infusion has being tested in clinical trials for GvHD prevention. Treg-based therapy has been used for the first time to prevent GvHD in patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT). Six independent trials, using either $FOXP3^+$ Tregs or Tr1 cells, have been concluded, and all of them showed the feasibility and safety of Treg-based approaches [248–253]. In five of these trials either freshly isolated [251–253] or *ex-vivo* expanded $FOXP3^+$ Tregs [249,250] were infused in patients undergoing allo-HSCT for onco-haematological diseases. Three of these trials also indicate the potential efficacy of the treatment. Brunstein et al. [250] reported a decreased incidence of grade II-IV GvHD as compared to historical controls, without increased risk of infections. Similarly, Di Ianni et al. [251] described few cases of low grade GvHD (2 out of 26 patients) and no development of chronic (c)GvHD. More recently, it has been reported that in Treg-treated patients the cumulative incidence of relapse was significantly lower than in historical controls [253] .As a result of these phase I and II

successes, a variety of trials are now investigating ex vivo expanded or freshly purified Treg infusions as a means to prevent both acute and chronic GvHD. Despite the challenges associated with Tregs purity and manufacturing on a large scale, these two trials showed feasibility, with the goal of understanding which populations are most efficacious and easier to generate in large numbers. Another way to enhance Treg expansion might be through administration of low-dose of IL-2, as has been studied in the context of chronic GvHD [254,255]. Daily IL-2 therapy for 8 weeks increased the proliferation of peripheral Treg and increased the generation of thymic Treg, which correlated with clinical improvement in manifestations of chronic GvHD and reduction of glucocorticosteroid dose. Clinical trials exploring the efficacy of Treg for the treatment of GVHD are much more challenging than prevention trials, and therefore it is not surprising that evidence is still sparse. In a recent report [249] it was suggested that the transfer of *in vitro* expanded donor Treg contributed to the amelioration of chronic GvHD in a single patient and allowed MMF withdrawal and a reduction in steroids. A second patient treated with higher Treg numbers for treatment-resistant acute GvHD had no benefit. The last Treg infusion for that patient contained only 40% Foxp3+ cells, and it is questionable whether such a cell product should have been administered in a life-threatening disease caused by donor T con. Tr1 cells have only recently been investigated as a therapeutic: our group performed a clinical trial in which adoptive cell therapy with Tr1 cells was administered into patients affected with advanced hematological malignancies

undergoing haploidentical HSCT therapy with the aim to provide immune reconstitution and prevent GvHD [256]. As discussed above, in four haploidentical HSCT patients treated with infusions of Tr1 cells shortly after transplant, immune reconstitution was enhanced compared to historical controls (who had undergone haploidentical HSCT) with no GvHD and no long-term immunosuppression required [256]. Studies to examine these cells in solid organ transplantation are just being initiated. New methodologies to generate antigen specific Treg cells will be tested in future trials, in order to restricting the immunosuppressive activity of these cells to acute GvHD, while maintain a GvT response.

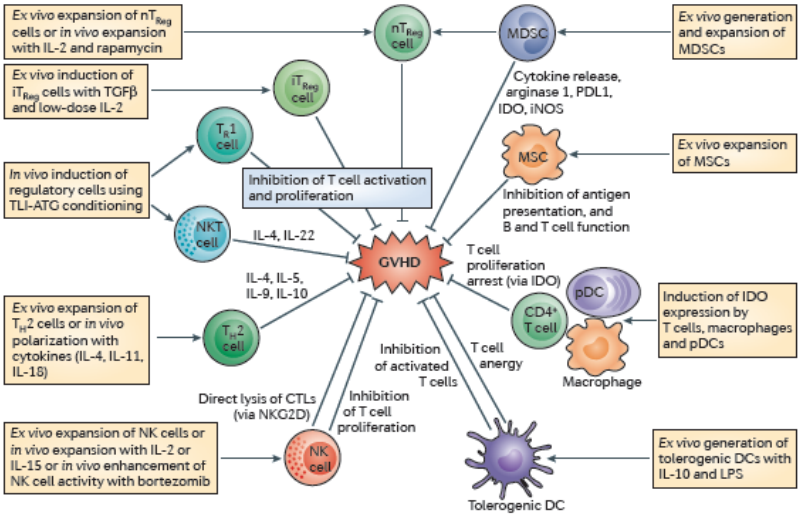


Figure 13. Potential targets for cellular immunotherapies in GvHD. Regulatory T (Treg) cells either develop in the thymus or are induced in the periphery from naive T cells .The inducible subset of TR1 cells produces interleukin-10 (IL-10) and has shown potent suppressive effects in graft-versus-host disease (GvHD) in the context of a total lymphoid irradiation and anti-thymocyte globulin (TLI-ATG) conditioning regimen, which also induces the generation of IL-4-producing natural killer T (NKT) cells. *Ex vivo*-expanded T helper 2 (TH2) cell populations are in clinical trials for the treatment of acute GvHD. NK cell clinical trials are also underway using NK cell infusion or the *in vivo* activation of NK cells to promote the deletion of alloreactive T cells. Substantial data indicating the suppressive effects of T cell, macrophage and plasmacytoid dendritic cell (pDC) populations that produce indoleamine 2,3-dioxygenase (IDO) suggest that these cells are prime candidates for future clinical trials. The infusion of mesenchymal stem cells (MSCs) for the treatment of GvHD has had mixed results. However, the transfer of donor-derived TReg cell populations that are expanded *ex vivo* has been more promising. Furthermore, in preclinical models, the infusion of myeloid-derived suppressor cell (MDSC) populations that are expanded *ex vivo* using G-CSF (granulocyte colony-stimulating factor) and GM-CSF (granulocyte–macrophage colony-stimulating factor) with or without IL-13 has been shown to be a feasible approach with anti-GVHD effects. Injection of pegylated arginase 1 may have the same benefit and be more practical. CTL, cytotoxic T lymphocyte; IFNγ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; PDL1, PD1 ligand 1 (*Adapted from Blazar B.R., Nature Review 2012*).

3.4 Graft-*versus*-Leukemia

While severe GvHD is associated with increased morbidity and mortality after allogeneic HSCT, it also associated with beneficial anti-leukemic immune effect known as Graft-versus Leukemia (GvL) mediated by donor T and NK cells. GvL effect may induce long-term remission of leukemia, even in cases in which the disease is resistant to conventional treatment [257]. Notably, complete removal of T cells of donor origin from the graft do not result in full abrogation of the GvL effect, since alloreactive NK cells of donor origin present in the graft or developing from donor stem cells following transplantation were found to be capable of mediating an anti-leukemic effect [175]. The major challenge in allogeneic HSCT for onco-hematological disorders is to limit GvHD without compromising the beneficial GvL effect of donor T cells, making it the main focus of current HSCT research.

Adoptive immunotherapy is one approach towards this goal and has been tested extensively in pre-clinical models and clinical settings. Donor lymphocyte infusions (DLI) consist of transfusing T lymphocytes from the donor to recipient in an un-manipulated form. DLI as a treatment for recurrence of the malignant disease has resulted in 20-90% complete remissions depending on the malignancy (NC1 1st international workshop). However, while allo-specific stimulation of donor T cells is required for the GvL effect, activated T cells generate a cytokine storm through the release of pro-inflammatory cytokines that can lead to GvHD effects [169]. A series of approaches have been explored to improve

the anti-leukemic efficacy of DLI and lessen the associated risk of GvHD. Selective depletion of $CD8^+$ T cells from the DLI product has shown promise in separating a GvL effect from the associated GvHD in a small pilot study [258]. Alternatively, the use of anergic T cells reported encouraging disease responses without an excess of GvHD [259].

The transfer of so-called ‘‘suicide genes’’ into donor lymphocytes has also been studied as a means of curtailing donor T-cell expansion in the event of severe GvHD. Herpes simplex virus thymidine kinase (HSV-TK) is the most widely studied suicide gene, which confers selective sensitivity to the prodrug ganciclovir (GCV). T cells modified with TK have been infused in more than 120 patients after HLA-identical or HLA-haploidentical HSCT [260]. In these trials, every case of GvHD has been controlled after GCV-mediated elimination of TK-modified T cells. One limitation of the TK suicide gene therapy approach is the development of an immune response against the transgene product, which results in rapid clearance of the infused lymphocytes [261]. The clinical exploitation of TK-DLIs after HLA-identical HSCT showed that a neutralizing immune response against TK epitopes occurs only into relatively immunocompetent patients [262]. Therefore, when TK cells were infused in highly immunosuppressed patients, as it happens early after a T-cell depleted allo-HSCT, no immune response against the transgene was detected. Given this limitation, several non immunogenic suicide genes have been described which may be more useful in this setting [263]. Among these alternative suicide genes, an

inducible form of the late pro-apoptotic molecule caspase 9 (iC9), and therefore humanized, has finally reached the clinical application [264]. Four children developing GvHD after the infusion of iC9-modified T cells have been successfully rescued by a single dose of the small molecule AP1903, which mediates iC9 activation [264,265].

Alternatively to DLI, donor T lymphocytes can be modified to redirect their T cell-specificity toward cancer cells through the genetic transfer of tumor-specific TCR (Figure 14). TCRs are typically cloned from patient tumor-reative T cell clones [266], from humanized murine models [266,267], or through the use of phage display technology [268,269]. In the TCR gene transfer approach the transgenic TCR is expressed in T lymphocytes that still bear their endogenous ones. The α and β chains of the endogenous TCR might mispair with the respective α and β chains chains of the transgenic TCR to produce new hybrid TCRs. Hybrid TCRs lose the specificity for the tumor antigen, diluting the anti-tumor TCR and possibly resulting in a reduced anti-tumor activity. To overcome these limitations, the TCR gene editing approach couples the transfer of the tumor-specific TCR to the somatic disruption, accomplished by the use of zinc-finger nucleases, of the α and β chains of the endogenous TCR. By expressing only the tumor-specific TCR at high levels, TCR-edited cells display improved efficacy and safety profiles [270]. The first clinical trial using TCR transferred autologous T cells was reported by the group of Rosemberg and demonstrated the feasibility and safety of this therapeutic approach. Clinical responses were observed,

indicating that redirected T cells might be effective against cancer [271]. Although initially tested in the autologous setting, TCR gene transfer might also increase the efficacy of DLI in the context of allo-HSCT [272].

Chimeric antigen receptors (CAR) are monomeric receptors constructed by fusing the single-chain fragment of a monoclonal antibody (scFv) with one or more signaling endodomains derived from immune cells [273] that are specific for antigen expressed by tumor cells. At present, retro- or lentiviral, or electroporational transfer of CARs whose target recognition is dependent on a scFv of a monoclonal antibody (mAb) is employed for stable production of sufficient numbers of therapeutic CAR-T cells uniformly retaining specificity for their defined targets, generally in 2-week culture [274].

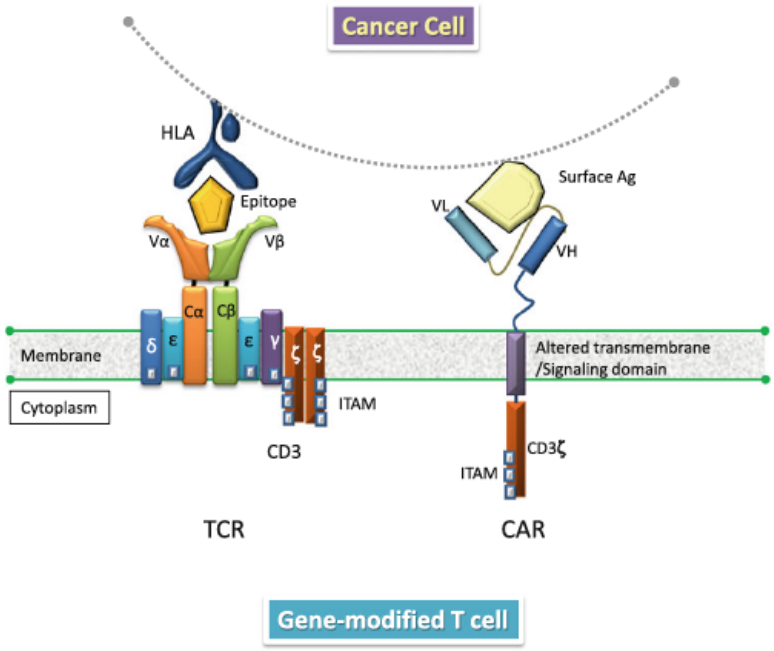


Figure 14. Introduced TCR or CAR in a gene-modified T cell. Each construct of TCR (left) or CAR (right) in a gene-modified T cell is illustrated. TCR recognizes the epitope in the context of the specific HLA (left), whereas CAR recognizes the cell surface antigen based on the recognition machinery of a monoclonal antibody, independently of HLA (right). TCR T-cell receptor, CAR chimeric antigen receptor, HLA human leukocyte antigen, Va variable region of the TCR a-chain, Vb variable region of the TCR b-chain, Ca constant region of the TCR a-chain, Cb constant region of the TCR b-chain, e, c d, f each subunit of the CD3 molecule, Ag antigen, VL variable region of the immunoglobulin light chain, VH variable region of the immunoglobulin heavy chain, ITAM immunoreceptor tyrosine-based activation motif (*Adapted from Fujiwara H., Int J Hematol 2014*).

Unlike TCR-mediated antigen recognition, CARs function independently of HLA and can therefore be used in any genetic background. Second-generation CARs [275] not only mediate antigen recognition and initiate T cell activation but also harness costimulation to enhance T cell function and prolong T cell

persistence [276]. Third-generation CARs, which contain two costimulatory domains along with an activation domain, may provide superior T cell function [277–279], although their effectiveness remains to be evaluated in clinical trials. Promising outcomes have been achieved in clinical trials using CD19-specific CAR constructs for patients with refractory chronic lymphocytic leukemia [280–283], B-cell [283–286], and acute lymphoblastic leukemia [282,287]. Interestingly, more impressive clinical benefits seemed to be achieved using the CD19-CAR construct containing a costimulatory domain from CD 137 (4.1BB) in the signaling motif [280,281,287] than with the use of CD28 [282,284], although the mechanism underlying this difference in antitumor efficacy is not fully understood. Other tumor antigens like CD20, CD44v6, and CD123 have also been used as targets in clinical and preclinical settings [279,288,289]. Future clinical CAR therapies might also involve NK cells instead of T-cells for certain applications. There are several advantages to using NK cells instead of T-cells for CAR therapy. Chiefly, NK cells have a limited lifespan in patients, addressing concerns about persistent CAR-associated side effects, and eliminating the need for including an inducible suicide gene on the construct.

5. References

1. Delves, P. J. & Roitt, I. M. The immune system. First of two parts. *N. Engl. J. Med.* **343,** 37–49 (2000).

2. Tonegawa, S. Somatic generation of antibody diversity. *Nature* **302,** 575–581 (1983).

3. Ignatowicz, L., Kappler, J. & Marrack, P. The repertoire of T cells shaped by a single MHC/peptide ligand. *Cell* **84,** 521–529 (1996).

4. Laufer, T. M., DeKoning, J., Markowitz, J. S., Lo, D. & Glimcher, L. H. Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex. *Nature* **383,** 81–85 (1996).

5. Wardemann, H. *et al.* Predominant autoantibody production by early human B cell precursors. *Science* **301,** 1374–1377 (2003).

6. Jacobson, D. L., Gange, S. J., Rose, N. R. & Graham, N. M. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin. Immunol. Immunopathol.* **84,** 223–243 (1997).

7. Gregersen, P. K. & Behrens, T. W. Genetics of autoimmune diseases--disorders of immune homeostasis. *Nat. Rev. Genet.* **7,** 917–928 (2006).

8. Lind, E. F., Prockop, S. E., Porritt, H. E. & Petrie, H. T. Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early

lymphoid development. *J. Exp. Med.* **194,** 127–134 (2001).

9. Spits, H. Development of alphabeta T cells in the human thymus. *Nat. Rev. Immunol.* **2,** 760–772 (2002).

10. Derbinski, J., Schulte, A., Kyewski, B. & Klein, L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat. Immunol.* **2,** 1032–1039 (2001).

11. Gao, J.-X. *et al.* Perinatal blockade of b7-1 and b7-2 inhibits clonal deletion of highly pathogenic autoreactive T cells. *J. Exp. Med.* **195,** 959–971 (2002).

12. Fontenot, J. D., Gavin, M. a & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* **4,** 330–336 (2003).

13. Mueller, D. L. Mechanisms maintaining peripheral tolerance. *Nat. Immunol.* **11,** 21–27 (2010).

14. Dhein, J. *et al.* Molecular mechanisms of APO-1/Fas(CD95)-mediated apoptosis in tolerance and AIDS. *Behring Inst. Mitt.* 13–20 (1995).

15. Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R. & Ferguson, T. A. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* **270,** 1189–1192 (1995).

16. Rieux-Laucat, F. *et al.* Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* **268,** 1347–1349 (1995).

17. Schwartz, R. H. T cell anergy. *Annu. Rev. Immunol.* **21,** 305–334 (2003).

18. Choi, S. & Schwartz, R. H. Molecular mechanisms for adaptive tolerance and other T cell anergy models. *Seminars in Immunology* **19,** 140–152 (2007).

19. Thompson, C. B. *et al.* CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1333–1337 (1989).

20. Beverly, B., Kang, S. M., Lenardo, M. J. & Schwartz, R. H. Reversal of in vitro T cell clonal anergy by IL-2 stimulation. *Int. Immunol.* **4,** 661–671 (1992).

21. Powell, J. D., Lerner, C. G. & Schwartz, R. H. Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. *J. Immunol.* **162,** 2775–2784 (1999).

22. Wells, A. D. Cyclin-dependent kinases: Molecular switches controlling anergy and potential therapeutic targets for tolerance. *Seminars in Immunology* **19,** 173–179 (2007).

23. Greenwald, R. J., Boussiotis, V. A., Lorsbach, R. B., Abbas, A. K. & Sharpe, A. H. CTLA-4 regulates induction of anergy in vivo. *Immunity* **14,** 145–155 (2001).

24. Keir, M. E. *et al.* Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J. Exp. Med.* **203,** 883–895 (2006).

25. Banchereau, J. & Steinman, R. M. Dendritic cells and the control of immunity. *Nature* **392,** 245–252 (1998).

26. Gallucci, S., Lolkema, M. & Matzinger, P. Natural adjuvants: endogenous activators of dendritic cells. *Nat. Med.* **5,** 1249–1255 (1999).

27. Rothlin, C. V., Ghosh, S., Zuniga, E. I., Oldstone, M. B. A. & Lemke, G. TAM Receptors Are Pleiotropic Inhibitors of the Innate Immune Response. *Cell* **131,** 1124–1136 (2007).

28. Kuwana, M., Kaburaki, J., Wright, T. M., Kawakami, Y. & Ikeda, Y. Induction of antigen-specific human CD4+ T cell anergy by peripheral blood DC2 precursors. *Eur. J. Immunol.* **31,** 2547–2557 (2001).

29. Steinbrink, K., Wölfl, M., Jonuleit, H., Knop, J. & Enk, A. H. Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol.* **159,** 4772–4780 (1997).

30. Kubsch, S., Graulich, E., Knop, J. & Steinbrink, K. Suppressor activity of anergic T cells induced by IL-10-treated human dendritic cells: Association with IL-2- and CTLA-4-dependent G1 arrest of the cell cycle regulated by p27Kip1. *Eur. J. Immunol.* **33,** 1988–1997 (2003).

31. Bacchetta, R. *et al.* Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy. *Haematologica* **95,** 2134–2143 (2010).

32. Gregori, S. *et al.* Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* **116,** 935–944 (2010).

33. Tuettenberg, A. *et al.* The role of ICOS in directing T cell responses: ICOS-dependent induction of T cell anergy by tolerogenic dendritic cells. *J. Immunol.* **182,** 3349–3356 (2009).

34. Selenko-Gebauer, N. *et al.* B7-H1 (programmed death-1 ligand) on dendritic cells is involved in the induction and maintenance of T cell anergy. *J. Immunol.* **170,** 3637–3644 (2003).

35. Brown, J. A. *et al.* Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J. Immunol.* **170,** 1257–1266 (2003).

36. Chang, C. C. *et al.* Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4. *Nat. Immunol.* **3,** 237–243 (2002).

37. Mellor, A. L. & Munn, D. H. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat. Rev. Immunol.* **4,** 762–774 (2004).

38. Munn, D. H. *et al.* GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. *Immunity* **22,** 633–642 (2005).

39. Wing, K. & Sakaguchi, S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat. Immunol.* **11,** 7–13 (2010).

40. Asano, M., Toda, M., Sakaguchi, N. & Sakaguchi, S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med.* **184,** 387–396 (1996).

41. Ng, W. F. *et al.* Human CD4+CD25+ cells: A naturally occurring population of regulatory T cells. *Blood* **98,** 2736–2744 (2001).

42. Salomon, B. *et al.* B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* **12,** 431–440 (2000).

43. Gavin, M. A. *et al.* Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* **445,** 771–775 (2007).

44. Bacchetta, R. *et al.* Defective regulatory and effector T cell functions in patients with FOXP3 mutations. *J. Clin. Invest.* **116,** 1713–1722 (2006).

45. Bennett, C. L. *et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* **27,** 20–21 (2001).

46. Mcmurchy, A. N. *et al.* Point mutants of forkhead box P3 that cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked have diverse abilities to reprogram T cells into regulatory T cells. *J. Allergy Clin. Immunol.* **126,** 1242–1251 (2010).

47. Brunkow, M. E. *et al.* Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* **27,** 68–73 (2001).

48. Wan, Y. Y. & Flavell, R. A. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* **445,** 766–770 (2007).

49. Wieczorek, G. *et al.* Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. *Cancer Res* **69,** 599–608 (2009).

50. Thornton, A. M. & Shevach, E. M. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J. Immunol.* **164,** 183–190 (2000).

51. Buonavista, N., Balzano, C., Pontarotti, P., Le Paslier, D. & Golstein, P. Molecular linkage of the human CTLA4 and CD28 Ig-superfamily genes in yeast artificial chromosomes. *Genomics* **13,** 856–861 (1992).

52. Paust, S., Lu, L., McCarty, N. & Cantor, H. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 10398–10403 (2004).

53. Grohmann, U. *et al.* CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat. Immunol.* **3,** 1097–1101 (2002).

54. Liang, B. *et al.* Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J. Immunol.* **180,** 5916–5926 (2008).

55. Reinwald, S. *et al.* CD83 expression in CD4+ T cells modulates inflammation and autoimmunity. *J. Immunol.* **180,** 5890–5897 (2008).

56. Pandiyan, P., Zheng, L., Ishihara, S., Reed, J. & Lenardo, M. J. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat. Immunol.* **8,** 1353–1362 (2007).

57. Grossman, W. J. *et al.* Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* **104,** 2840–2848 (2004).

58. Zhao, D.-M., Thornton, A. M., DiPaolo, R. J. & Shevach, E. M. Activated CD4+CD25+ T cells selectively kill B lymphocytes. *Blood* **107,** 3925–3932 (2006).

59. Ren, X. *et al.* Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells. *Cell Death Differ.* **14,** 2076–2084 (2007).

60. Apostolou, I., Sarukhan, A., Klein, L. & von Boehmer, H. Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* **3,** 756–763 (2002).

61. Chen, W., Jin, W., Hardegen, N. & Lei, K. Conversion of peripheral CD4+ CD25â˜ naive T cells to CD4+ CD25+ regulatory T cells by TGF-Î² induction of transcription factor Foxp3. *J. Exp. Med.* **198,** 1875–86. (2003).

62. Kretschmer, K. *et al.* Inducing and expanding regulatory T cell populations by foreign antigen. *Nat. Immunol.* **6,** 1219–1227 (2005).

63. Selvaraj, R. K. & Geiger, T. L. A kinetic and dynamic analysis of Foxp3 induced in T cells by TGF-beta. *J. Immunol.* **179,** 11 p following 1390 (2007).

64. Curotto de Lafaille, M. A. *et al.* Adaptive Foxp3+ Regulatory T Cell-Dependent and -Independent Control of Allergic Inflammation. *Immunity* **29,** 114–126 (2008).

65. Mucida, D. *et al.* Oral tolerance in the absence of naturally occurring Tregs. *J. Clin. Invest.* **115,** 1923–1933 (2005).

66. Collison, L. W. *et al.* IL-35-mediated induction of a potent regulatory T cell population. *Nat. Immunol.* **11,** 1093–1101 (2010).

67. Bettini, M., Castellaw, A. H., Lennon, G. P., Burton, A. R. & Vignali, D. A. A. Prevention of autoimmune diabetes by ectopic pancreatic ??-cell expression of interleukin-35. *Diabetes* **61,** 1519–1526 (2012).

68. Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A. & Weiner, H. L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science (80-.).* **265,** 1237–1240 (1994).

69. Inobe, J. I. *et al.* IL-4 is a differentiation factor for transforming growth factor-?? secreting Th3 cells and oral administration of IL-4 enhances oral tolerance in experimental allergic encephalomyelitis. *Eur. J. Immunol.* **28,** 2780–2790 (1998).

70. Weiner, H. L. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol. Rev.* **182,** 207–214 (2001).

71. Groux, H. *et al.* A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389,** 737–742 (1997).

72. Bacchetta, R. *et al.* Host-reactive CD4+ and CD8+ T cell clones isolated from a human chimera produce IL-5, IL-2, IFN-gamma and granulocyte/macrophage-colony-stimulating factor but not IL-4. *J. Immunol.* **144,** 902–908 (1990).

73. Bacchetta, R. *et al.* High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *J Exp Med* **179,** 493–502 (1994).

74. Gagliani, N. *et al.* Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat. Med.* **19,** 739–46 (2013).

75. Yssel, H. *et al.* IL-10 is produced by subsets of human CD4+ T cell clones and peripheral blood T cells. *J. Immunol.* **149,** 2378–2384 (1992).

76. Chang, H. D. *et al.* Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the IL-10 gene is imprinted by GATA-3. *Eur. J. Immunol.* **37,** 807–817 (2007).

77. Saraiva, M. *et al.* Interleukin-10 Production by Th1 Cells Requires Interleukin-12-Induced STAT4 Transcription Factor and ERK MAP Kinase Activation by High Antigen Dose. *Immunity* **31,** 209–219 (2009).

78. Veldhoen, M. *et al.* Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat. Immunol.* **9,** 1341–1346 (2008).

79. McGeachy, M. J. *et al.* TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat. Immunol.* **8,** 1390–1397 (2007).

80. Ito, T. *et al.* Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. *Immunity* **28,** 870–880 (2008).

81. Bacchetta, R. *et al.* Growth and expansion of human T regulatory type 1 cells are independent from TCR activation but require exogenous cytokines. *Eur. J. Immunol.* **32,** 2237–2245 (2002).

82. Bejarano, M. T. *et al.* Interleukin 10 inhibits allogeneic proliferative and cytotoxic T cell responses generated in primary mixed lymphocyte cultures. *Int. Immunol.* **4,** 1389–1397 (1992).

83. Magnani, C. F. *et al.* Killing of myeloid APCs via HLA class I, CD2 and CD226 defines a novel mechanism of suppression by human Tr1 cells. *Eur. J. Immunol.* **41,** 1652–1662 (2011).

84. Vieira, P. L. *et al.* IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *J. Immunol.* **172,** 5986–5993 (2004).

85. Brun, V., Bastian, H., Neveu, V. & Foussat, A. Clinical grade production of IL-10 producing regulatory Tr1 lymphocytes for cell therapy of chronic inflammatory diseases. *Int. Immunopharmacol.* **9,** 609–613 (2009).

86. Brun, V. *et al.* Isolation of functional autologous collagen-II specific IL-10 producing Tr1 cell clones from rheumatoid arthritis blood. *Int. Immunopharmacol.* **11,** 1074–1078 (2011).

87. Gregori, S., Goudy, K. S. & Roncarolo, M. G. The cellular and molecular mechanisms of immuno-suppression by human type 1 regulatory T cells. *Frontiers in Immunology* **3,** (2012).

88. Levings, M. K. *et al.* Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. *Blood* **105,** 1162–1169 (2005).

89. Akdis, M. T-cell tolerance to inhaled allergens: mechanisms and therapeutic approaches. *Expert Opin. Biol. Ther.* **8,** 769–777 (2008).

90. Desreumaux, P. *et al.* Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease. *Gastroenterology* **143,** 1207–1217.e2 (2012).

91. Haringer, B., Lozza, L., Steckel, B. & Geginat, J. Identification and characterization of IL-10/IFN-gamma-producing effector-like T cells with regulatory function in human blood. *J Exp Med* **206,** 1009–1017 (2009).

92. Bergmann, C., Strauss, L., Zeidler, R., Lang, S. & Whiteside, T. L. Expansion and characteristics of human T regulatory type 1 cells in co-cultures simulating tumor microenvironment. *Cancer Immunol. Immunother.* **56,** 1429–1442 (2007).

93. Mandapathil, M. *et al.* Adenosine and prostaglandin e2 cooperate in the suppression of immune responses mediated by adaptive regulatory T cells. *J. Biol. Chem.* **285,** 27571–27580 (2010).

94. Barrat, F. J. *et al.* In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J. Exp. Med.* **195,** 603–616 (2002).

95. Veldman, C., Höhne, A., Dieckmann, D., Schuler, G. & Hertl, M. Type I regulatory T cells specific for desmoglein 3 are more frequently detected in healthy individuals than in patients with pemphigus vulgaris. *J. Immunol.* **172,** 6468–6475 (2004).

96. Vieira, P. *et al.* Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 1172–1176 (1991).

97. Gorelik, L. & Flavell, R. A. Transforming growth factor-beta in T-cell biology. *Nat. Rev. Immunol.* **2,** 46–53 (2002).

98. Gorelik, L. & Flavell, R. A. Transforming growth factor-beta in T-cell biology. *Nat. Rev. Immunol.* **2,** 46–53 (2002).

99. Roncarolo, M. G. *et al.* Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological Reviews* **212,** 28–50 (2006).

100. De Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G. & de Vries, J. E. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* **174,** 1209–1220 (1991).

101. Fiorentino, D. F. *et al.* IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol.* **146,** 3444–3451 (1991).

102. Mosser, D. M. & Zhang, X. Interleukin-10: New perspectives on an old cytokine. *Immunological Reviews* **226,** 205–218 (2008).

103. Manavalan, J. S. *et al.* High expression of ILT3 and ILT4 is a general feature of tolerogenic dendritic cells. *Transpl. Immunol.* **11,** 245–258 (2003).

104. Moreau, P. *et al.* IL-10 selectively induces HLA-G expression in human trophoblasts and monocytes. *Int. Immunol.* **11,** 803–811 (1999).

105. Morelli, A. E. & Thomson, A. W. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat. Rev. Immunol.* **7,** 610–621 (2007).

106. Miaw, S. C., Choi, A., Yu, E., Kishikawa, H. & Ho, I. C. ROG, repressor of GATA, regulates the expression of cytokine genes. *Immunity* **12,** 323–333 (2000).

107. Okamura, T. *et al.* by the transcription factor Egr-2. 1–6 (2009).

108. Iwasaki, Y. *et al.* Egr-2 transcription factor is required for Blimp-1-mediated IL-10 production in IL-27-stimulated CD4+ T cells. *Eur J Immunol* **43,** 1063–1073 (2013).

109. Okamura, T. *et al.* TGF-β3-expressing CD4+CD25−LAG3+ regulatory T cells control humoral immune responses. *Nat. Commun.* **6,** 6329 (2015).

110. Okamura, T., Fujio, K., Sumitomo, S. & Yamamoto, K. Roles of LAG3 and EGR2 in regulatory T cells. *Ann. Rheum. Dis.* **71 Suppl 2,** i96–100 (2012).

111. Apetoh, L. *et al.* The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat Immunol* **11,** 854–861 (2010).

112. Pot, C. *et al.* Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *J. Immunol.* **183,** 797–801 (2009).

113. Xu, J. *et al.* c-Maf regulates IL-10 expression during Th17 polarization. *J. Immunol.* **182,** 6226–6236 (2009).

114. Bauquet, A. T. *et al.* The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat. Immunol.* **10,** 167–175 (2009).

115. Quintana, F. *et al.* Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* **453,** 65–71 (2008).

116. Huang, C. T. *et al.* Role of LAG-3 in regulatory T cells. *Immunity* **21,** 503–513 (2004).

117. Camisaschi, C. *et al.* LAG-3 expression defines a subset of CD4(+)CD25(high)Foxp3(+) regulatory T cells that are expanded at tumor sites. *J. Immunol.* **184,** 6545–6551 (2010).

118. Workman, C. J. & Vignali, D. A. A. Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). *J Immunol* **174,** 688–695 (2005).

119. Bettini, M. *et al.* Cutting edge: accelerated autoimmune diabetes in the absence of LAG-3. *J. Immunol.* **187,** 3493–8 (2011).

120. Charbonnier, L. M. *et al.* Immature dendritic cells suppress collagen-induced arthritis by in vivo expansion of CD49b+ regulatory T cells. *J Immunol.* **177,** 3806–3813 (2006).

121. Charbonnier, L. M. *et al.* Adoptive transfer of IL-10-secreting CD4+CD49b+ regulatory T cells suppresses ongoing arthritis. *J. Autoimmun.* **34,** 390–399 (2010).

122. Rahmoun, M. *et al.* Enhanced frequency of CD18- and CD49b-expressing T cells in peripheral blood of asthmatic patients correlates with disease severity. *Int. Arch. Allergy Immunol.* **140,** 139–149 (2006).

123. Kassiotis, G., Gray, D., Kiafard, Z., Zwirner, J. & Stockinger, B. Functional specialization of memory Th cells revealed by expression of integrin CD49b. *J. Immunol.* **177,** 968–975 (2006).

124. Boisvert, M., Chetoui, N., Gendron, S. & Aoudjit, F. Alpha2beta1 integrin is the major collagen-binding integrin expressed on human Th17 cells. *Eur. J. Immunol.* **40,** 2710–9 (2010).

125. Groux, H., Bigler, M., de Vries, J. E. & Roncarolo, M. G. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J. Exp. Med.* **184,** 19–29 (1996).

126. Bacchetta, R. *et al.* Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy. *Haematologica* **95,** 2134–2143 (2010).

127. Levings, M. K. *et al.* IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *J. Immunol.* **166,** 5530–5539 (2001).

128. Awasthi, A. *et al.* A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol* **8,** 1380–1389 (2007).

129. Fitzgerald, D. C. *et al.* Suppressive effect of IL-27 on encephalitogenic Th17 cells and the effector phase of experimental autoimmune encephalomyelitis. *J Immunol* **179,** 3268–3275 (2007).

130. Stumhofer, J. S. *et al.* Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat. Immunol.* **8,** 1363–1371 (2007).

131. Murugaiyan, G. *et al.* IL-27 is a key regulator of IL-10 and IL-17 production by human CD4+ T cells. *J. Immunol.* **183,** 2435–43 (2009).

132. Schmetterer, K. G. *et al.* STAT3 governs hyporesponsiveness and granzyme B-dependent suppressive capacity in human CD4+ T cells. *FASEB J.* 1–13 (2014). doi:10.1096/fj.14-257584

133. Wu, H. Y. *et al.* In vivo induction of Tr1 cells via mucosal dendritic cells and AHR signaling. *PLoS One* **6,** e23618 (2011).

134. Gandhi, R. *et al.* Activation of the aryl hydrocarbon receptor induces human type 1 regulatory T cell-like and Foxp3(+) regulatory T cells. *Nat Immunol* **11,** 846–853 (2010).

135. Pot, C., Apetoh, L. & Kuchroo, V. K. Type 1 regulatory T cells (Tr1) in autoimmunity. *Seminars in Immunology* **23,** 202–208 (2011).

136. Quintana, F. J. *et al.* Aiolos promotes TH17 differentiation by directly silencing Il2 expression. *Nature Immunology* **13,** 770–777 (2012).

137. Jin, J. O., Han, X. & Yu, Q. Interleukin-6 induces the generation of IL-10-producing Tr1 cells and suppresses autoimmune tissue inflammation. *J. Autoimmun.* **40,** 28–44 (2013).

138. Roncarolo, M.-G. & Battaglia, M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat. Rev. Immunol.* **7,** 585–598 (2007).

139. Wakkach, A., Cottrez, F. & Groux, H. Differentiation of regulatory T cells 1 is induced by CD2 costimulation. *J. Immunol.* **167,** 3107–3113 (2001).

140. Kemper, C. *et al.* Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature* **421,** 388–392 (2003).

141. Sutavani, R. V *et al.* CD55 costimulation induces differentiation of a discrete T regulatory type 1 cell population with a stable phenotype. *J. Immunol.* **191,** 5895–903 (2013).

142. Gregori, S., Roncarolo, M. G. & Bacchetta, R. Methods for in vitro generation of human type 1 regulatory T cells. *Methods Mol Biol* **677,** 31–46 (2011).

143. Jonuleit, H., Schmitt, E., Schuler, G., Knop, J. & Enk, A. H. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J. Exp. Med.* **192,** 1213–1222 (2000).

144. Corre, B. *et al.* Type I interferon potentiates T-cell receptor mediated induction of IL-10-producing $CD4^+$ T cells. *Eur. J. Immunol.* **43,** 2730–40 (2013).

145. Rutella, S. *et al.* Granulocyte colony-stimulating factor promotes the generation of regulatory DC through induction of IL-10 and IFN-?? *Eur. J. Immunol.* **34,** 1291–1302 (2004).

146. Rossetti, M., Gregori, S. & Roncarolo, M. G. Granulocyte-colony stimulating factor drives the in vitro differentiation of human dendritic cells that induce anergy in na??ve T cells. *Eur. J. Immunol.* **40,** 3097–3106 (2010).

147. Penna, G. & Adorini, L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J. Immunol.* **164,** 2405–2411 (2000).

148. Pedersen, A. E., Gad, M., Walter, M. R. & Claesson, M. H. Induction of regulatory dendritic cells by dexamethasone and 1??,25-Dihydroxyvitamin D3. *Immunol. Lett.* **91,** 63–69 (2004).

149. Gregori, S. *et al.* Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* **116,** 935–944 (2010).

150. Amodio, G. *et al.* HLA-G expression levels influence the tolerogenic activity of human DC-10. *Haematologica* (2015). doi:10.3324/haematol.2014.113803

151. Setoguchi, K. *et al.* Antigen-specific T cells transduced with IL-10 ameliorate experimentally induced arthritis without impairing the systemic immune response to the antigen. *J. Immunol.* **165,** 5980–5986 (2000).

152. Oh, J. W. *et al.* CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. *J. Allergy Clin. Immunol.* **110,** 460–468 (2002).

153. Van Montfrans, C. *et al.* Prevention of colitis by interleukin 10-transduced T lymphocytes in the SCID mice transfer model. *Gastroenterology* **123,** 1865–1876 (2002).

154. Brandt, C. *et al.* Allo-specific T-cells encoding for viral IL-10 exert strong immunomodulatory effects in vitro but fail to prevent graft rejection. *Am. J. Transplant.* **5,** 268–281 (2005).

155. Guichelaar, T. *et al.* Autoantigen-specific IL-10-transduced T cells suppress chronic arthritis by promoting the endogenous regulatory IL-10 response. *J. Immunol.* **180,** 1373–1381 (2008).

156. Cavalieri, S. *et al.* Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. *Blood* **102,** 497–505 (2003).

157. Marktel, S. *et al.* Immunologic potential of donor lymphocytes expressing a suicide gene for early immune

reconstitution after hematopoietic T-cell-depleted stem cell transplantation. *Blood* **101,** 1290–1298 (2003).

158. Sauce, D. *et al.* Retrovirus-mediated gene transfer in primary T lymphocytes impairs their anti-Epstein-Barr virus potential through both culture-dependent and selection process-dependent mechanisms. *Blood* **99,** 1165–1173 (2002).

159. Ferrand, C. *et al.* Retrovirus-mediated gene transfer in primary T lymphocytes: influence of the transduction/selection process and of ex vivo expansion on the T cell receptor beta chain hypervariable region repertoire. *Hum. Gene Ther.* **11,** 1151–1164 (2000).

160. Drobyski, W. R., Majewski, D., Ozker, K. & Hanson, G. Ex vivo anti-CD3 antibody-activated donor T cells have a reduced ability to cause lethal murine graft-versus-host disease but retain their ability to facilitate alloengraftment. *J. Immunol.* **161,** 2610–2619 (1998).

161. Akdis, M. *et al.* Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J. Exp. Med.* **199,** 1567–1575 (2004).

162. Andolfi, G. *et al.* Enforced IL-10 Expression Confers Type 1 Regulatory T Cell (Tr1) Phenotype and Function to Human CD4+ T Cells. *Molecular Therapy* **20,** 1778–1790 (2012).

163. Allan, S. E. *et al.* CD4+ T-regulatory cells: Toward therapy for human diseases. *Immunol. Rev.* **223,** 391–421 (2008).

164. Gagliani, N. *et al.* Antigen-specific dependence of Tr1-cell therapy in preclinical models of islet transplant. *Diabetes* **59,** 433–439 (2010).

165. Zeller, J. C. *et al.* Induction of CD4+ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-beta. *J. Immunol.* **163,** 3684–3691 (1999).

166. Bacchetta, R. *et al.* Immunological Outcome in Haploidentical-HSC Transplanted Patients Treated with IL-10-Anergized Donor T Cells. *Front Immunol* **5,** 16 (2014).

167. Roncarolo, M. G., Gregori, S., Lucarelli, B., Ciceri, F. & Bacchetta, R. Clinical tolerance in allogeneic hematopoietic stem cell transplantation. *Immunol Rev* **241,** 145–163 (2011).

168. Asnagli, H. *et al.* Type 1 regulatory T cells specific for collagen-type II as an efficient cell-based therapy in arthritis. *Arthritis Res. Ther.* **16,** R115 (2014).

169. Copelan, E. A. Hematopoietic Stem-Cell Transplantation. 1813–1826 (2015).

170. Welniak, L. a, Blazar, B. R. & Murphy, W. J. Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu. Rev. Immunol.* **25,** 139–170 (2007).

171. Miller, C. B. *et al.* Impact of age on outcome of patients with cancer undergoing autologous bone marrow transplant. *J. Clin. Oncol.* **14,** 1327–1332 (1996).

172. Kusnierz-Glaz, C. R. *et al.* Influence of age on the outcome of 500 autologous bone marrow transplant procedures for hematologic malignancies. *J. Clin. Oncol.* **15,** 18–25 (1997).

173. Szydlo, R. *et al.* Results of allogeneic bone marrow transplants for leukemia using donors other than HLA-identical siblings. *J. Clin. Oncol.* **15,** 1767–1777 (1997).

174. Aversa, F. *et al.* Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. *N. Engl. J. Med.* **339,** 1186–1193 (1998).

175. Ruggeri, L. *et al.* Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* **295,** 2097–2100 (2002).

176. Aversa, F., Reisner, Y. & Martelli, M. F. The haploidentical option for high-risk haematological malignancies. *Blood Cells, Mol. Dis.* **40,** 8–12 (2008).

177. Cutler, C. *et al.* Acute and chronic graft-versus-host disease after allogeneic peripheral-blood stem-cell and bone marrow transplantation: a meta-analysis. *J. Clin. Oncol.* **19,** 3685–91 (2001).

178. Flomenberg, N. *et al.* The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. *Blood* **106,** 1867–1874 (2005).

179. Wagner, J. E. *et al.* Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: Influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood* **100,** 1611–1618 (2002).

180. Blazar, B. R., Murphy, W. J. & Abedi, M. Advances in graft-versus-host disease biology and therapy. *Nat. Rev. Immunol.* **12,** 443–458 (2012).

181. Filipovich, A. H. *et al.* National Institutes of Health Consensus Development Project on criteria for clinical trials in chronic graft-versus-host disease: I. diagnosis and staging working group report. *Biol. Blood Marrow Transplant.* **11,** 945–956 (2005).

182. Vigorito, A. C. *et al.* Evaluation of NIH consensus criteria for classification of late acute and chronic GVHD Evaluation of NIH consensus criteria for classification of late acute and chronic GVHD. **114,** 702–708 (2009).

183. Broady, R. *et al.* Cutaneous GVHD is associated with the expansion of tissue-localized Th1 and not Th17 cells. *Blood* **116,** 5748–5751 (2010).

184. Imanguli, M. M. *et al.* Increased T-bet + cytotoxic effectors and type i interferon-mediated processes in chronic graft-versus-host disease of the oral mucosa. *Blood* **113,** 3620–3630 (2009).

185. Murphy, W. J. *et al.* Differential effects of the absence of interferon-gamma and IL-4 in acute graft-versus-host disease after allogeneic bone marrow transplantation in mice. *J. Clin. Invest.* **102,** 1742–8 (1998).

186. Nikolic, B., Lee, S., Bronson, R. T., Grusby, M. J. & Sykes, M. Th1 and Th2 mediate acute graft-versus-host disease, each with distinct end-organ targets. *J. Clin. Invest.* **105,** 1289–1298 (2000).

187. Ratajczak, P. *et al.* Th17/Treg ratio in human graft-versus-host disease. *Blood* **116,** 1165–1171 (2010).

188. Martin, P. J., Gooley, T., Anasetti, C., Petersdorf, E. W. & Hansen, J. A. HLAs and risk of acute graft-vs.-host disease after marrow transplantation from an HLA-identical sibling. *Biol. Blood Marrow Transplant.* **4,** 128–133 (1998).

189. Goker, H., Haznedaroglu, I. C. & Chao, N. J. Acute graft-vs-host disease: Pathobiology and management. *Experimental Hematology* **29,** 259–277 (2001).

190. Bland, P. W. & Whiting, C. V. Induction of MHC class II gene products in rat intestinal epithelium during graft-versus-host disease and effects on the immune function of the epithelium. *Immunology* **75,** 366–371 (1992).

191. Lee, S. J. *et al.* High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood* **110,** 4576–4583 (2007).

192. Loiseau, P. *et al.* HLA Association with Hematopoietic Stem Cell Transplantation Outcome: The Number of Mismatches at HLA-A, -B, -C, -DRB1, or -DQB1 Is Strongly Associated with Overall Survival. *Biol. Blood Marrow Transplant.* **13,** 965–974 (2007).

193. Cook, M. A. *et al.* The impact of donor KIR and patient HLA-C genotypes on outcome following HLA-identical sibling hematopoietic stem cell transplantation for myeloid leukemia. *Blood* **103,** 1521–1526 (2004).

194. Den Haan, J. M. *et al.* The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. *Science* **279,** 1054–1057 (1998).

195. Goulmy, E. *et al.* Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the

development of graft-versus-host disease after bone marrow transplantation. *N. Engl. J. Med.* **334,** 281–285 (1996).

196. Dickinson, A. M. & Charron, D. Non-HLA immunogenetics in hematopoietic stem cell transplantation. *Current Opinion in Immunology* **17,** 517–525 (2005).

197. Perreault, C. *et al.* Minor histocompatibility antigens. *Blood* **76,** 1269–1280 (1990).

198. Takami, A. The role of non-HLA gene polymorphisms in graft-versus-host disease. *Int. J. Hematol.* **98,** 309–318 (2013).

199. Liu, J. *et al.* Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66,** 807–815 (1991).

200. Storb, R. *et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. The New England journal of medicine* **314,** (1986).

201. Ruutu, T. *et al.* Prophylaxis and treatment of GVHD: EBMT-ELN working group recommendations for a standardized practice. *Bone Marrow Transplant.* **49,** 168–73 (2014).

202. Brunstein, C. G. *et al.* Umbilical cord blood transplantation after nonmyeloablative conditioning: Impact on transplantation outcomes in 110 adults with hematologic disease. *Blood* **110,** 3064–3070 (2007).

203. Bolwell, B. *et al. A prospective randomized trial comparing cyclosporine and short course methotrexate with cyclosporine and mycophenolate mofetil for GVHD prophylaxis in myeloablative*

allogeneic bone marrow transplantation. Bone marrow transplantation **34,** (2004).

204. Barker, J. N. *et al.* Rapid and complete donor chimerism in adult recipients of unrelated donor umbilical cord blood transplantation after reduced-intensity conditioning. *Blood* **102,** 1915–1919 (2003).

205. Baron, F. *et al.* Kinetics of engraftment in patients with hematologic malignancies given allogeneic hematopoietic cell transplantation after nonmyeloablative conditioning. *Blood* **104,** 2254–2262 (2004).

206. Burroughs, L. *et al.* Extending postgrafting cyclosporine decreases the risk of severe graft-versus-host disease after nonmyeloablative hematopoietic cell transplantation. *Transplantation* **81,** 818–825 (2006).

207. Terada, N. *et al.* Rapamycin blocks cell cycle progression of activated T cells prior to events characteristic of the middle to late G1 phase of the cycle. *J. Cell. Physiol.* **154,** 7–15 (1993).

208. Antin, J. H. *et al.* Sirolimus, tacrolimus, and low-dose methotrexate for gaft-versus-host disease prophylaxis in mismatched related donor or unrelated donor transplantation. *Blood* **102,** 1601–1605 (2003).

209. Peccatori, J. *et al.* Sirolimus-based graft-versus-host disease prophylaxis promotes the in vivo expansion of regulatory T cells and permits peripheral blood stem cell transplantation from haploidentical donors. *Leukemia* 1–10 (2014). doi:10.1038/leu.2014.180

210. Cutler, C. *et al.* Sirolimus and tacrolimus without methotrexate as graft-versus-host disease prophylaxis after matched related donor peripheral blood stem cell transplantation. *Biol. Blood Marrow Transplant.* **10,** 328–336 (2004).

211. Alyea, E. P. *et al.* Sirolimus, Tacrolimus, and Low-Dose Methotrexate as Graft-versus-Host Disease Prophylaxis in Related and Unrelated Donor Reduced-Intensity Conditioning Allogeneic Peripheral Blood Stem Cell Transplantation. *Biol. Blood Marrow Transplant.* **14,** 920–926 (2008).

212. Sung, A. D. & Chao, N. J. Concise review: acute graft-versus-host disease: immunobiology, prevention, and treatment. *Stem Cells Transl. Med.* **2,** 25–32 (2013).

213. Murai, M. *et al.* Peyer's patch is the essential site in initiating murine acute and lethal graft-versus-host reaction. *Nat. Immunol.* **4,** 154–160 (2003).

214. Reshef, R. *et al.* Blockade of Lymphocyte Chemotaxis in Visceral Graft-versus-Host Disease. *New England Journal of Medicine* **367,** 135–145 (2012).

215. Antin, J. H. *et al.* Interleukin-1 blockade does not prevent acute graft-versus-host disease: Results of a randomized, double-blind, placebo-controlled trial of interleukin-1 receptor antagonist in allogeneic bone marrow transplantation. *Blood* **100,** 3479–3483 (2002).

216. Hamadani, M. *et al. Addition of infliximab to standard acute graft-versus-host disease prophylaxis following allogeneic peripheral blood cell transplantation. Biology of blood and marrow*

transplantation : journal of the American Society for Blood and Marrow Transplantation **14,** (2008).

217. Chakraverty, R. *et al.* Impact of in vivo alemtuzumab dose before reduced intensity conditioning and HLA-identical sibling stem cell transplantation: Pharmacokinetics, GVHD, and immune reconstitution. *Blood* **116,** 3080–3088 (2010).

218. Raefsky, E. L., Gascon, P., Gratwohl, A., Speck, B. & Young, N. S. Biological and immunological characterization of ATG and ALG. *Blood* **68,** 712–719 (1986).

219. Bacigalupo, A. Antilymphocyte/thymocyte globulin for graft versus host disease prophylaxis: efficacy and side effects. *Bone Marrow Transplant.* **35,** 225–231 (2005).

220. Guinan, E. C. *et al. Transplantation of anergic histoincompatible bone marrow allografts. The New England journal of medicine* **340,** (1999).

221. Koura, D. T. *et al.* In vivo T cell costimulation blockade with abatacept for acute graft-versus-host disease prevention: a first-in-disease trial. *Biol. Blood Marrow Transplant.* **19,** 1638–49 (2013).

222. Aggarwal, S. & Pittenger, M. F. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* **105,** 1815–1822 (2005).

223. Polchert, D. *et al.* IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. *Eur. J. Immunol.* **38,** 1745–1755 (2008).

224. Tolar, J., Le Blanc, K., Keating, A. & Blazar, B. R. Concise review: Hitting the right spot with mesenchymal stromal cells. *Stem Cells* **28,** 1446–1455 (2010).

225. Kebriaei, P. & Robinson, S. Treatment of graft-versus-host-disease with mesenchymal stromal cells. *Cytotherapy* **13,** 262–268 (2011).

226. Lechner, M. G., Liebertz, D. J. & Epstein, A. L. Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. *J. Immunol.* **185,** 2273–2284 (2010).

227. Peranzoni, E. *et al.* Myeloid-derived suppressor cell heterogeneity and subset definition. *Current Opinion in Immunology* **22,** 238–244 (2010).

228. Serafini, P., Mgebroff, S., Noonan, K. & Borrello, I. Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. *Cancer Res.* **68,** 5439–5449 (2008).

229. Zhou, Z. *et al.* Development and function of myeloid-derived suppressor cells generated from mouse embryonic and hematopoietic stem cells. *Stem Cells* **28,** 620–32 (2010).

230. Highfill, S. L. *et al.* Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. *Blood* **116,** 5738–5747 (2010).

231. Tanaka, M. *et al.* The impact of the dose of natural killer cells in the graft on severe acute graft-versus-host disease after

unrelated bone marrow transplantation. *Leuk. Res.* **36,** 699–703 (2012).

232. Ruggeri, L. *et al.* Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood* **94,** 333–339 (1999).

233. Ruggeri, L. *et al.* Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: Challenging its predictive value. *Blood* **110,** 433–440 (2007).

234. Pende, D. *et al.* Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: Evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity. *Blood* **113,** 3119–3129 (2009).

235. Miller, J. S. *et al.* Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. *Blood* **109,** 5058–5061 (2007).

236. Miller, J. S. *et al.* Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood* **105,** 3051–3057 (2005).

237. Shi, J. *et al.* Infusion of haplo-identical killer immunoglobulin-like receptor ligand mismatched NK cells for relapsed myeloma in the setting of autologous stem cell transplantation. *Br. J. Haematol.* **143,** 641–653 (2008).

238. Curti, A. *et al.* Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after

infusion in elderly high risk acute myeloid leukemia patients. *Blood* **118,** 3273–3279 (2011).

239. Leveson-Gower, D. B. *et al.* Low doses of natural killer T cells provide protection from acute graft-versus-host disease via an IL-4-dependent mechanism. *Blood* **117,** 3220–3229 (2011).

240. Kuns, R. D. *et al.* Invariant natural killer T cell-natural killer cell interactions dictate transplantation outcome after α-galactosylceramide administration. *Blood* **113,** 5999–6010 (2009).

241. Hippen, K. L. *et al.* Generation and large-scale expansion of human inducible regulatory T cells that suppress graft-versus-host disease. *Am. J. Transplant.* **11,** 1148–1157 (2011).

242. Sela, U., Olds, P., Park, A., Schlesinger, S. J. & Steinman, R. M. Dendritic cells induce antigen-specific regulatory T cells that prevent graft versus host disease and persist in mice. *J. Exp. Med.* **208,** 2489–96 (2011).

243. Semple, K., Yu, Y., Wang, D., Anasetti, C. & Yu, X. Z. Efficient and Selective Prevention of GVHD by Antigen-Specific Induced Tregs via Linked-Suppression in Mice. *Biol. Blood Marrow Transplant.* **17,** 309–318 (2011).

244. Inaba, K. *et al.* Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **176,** 1693–1702 (1992).

245. Goxe, B., Latour, N., Chokri, M., Abastado, J. P. & Salcedo, M. Simplified method to generate large quantities of dendritic cells

suitable for clinical applications. *Immunol.Invest* **29,** 319–336 (2000).

246. Sato, K., Yamashita, N., Yamashita, N., Baba, M. & Matsuyama, T. Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse. *Immunity* **18,** 367–379 (2003).

247. Edinger, M. *et al.* CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat. Med.* **9,** 1144–1150 (2003).

248. Bacchetta, R. *et al.* Interleukin-10 anergized donor T cell infusion improves immune reconstitution without severe graft-versus-host-disease after haploidentical hematopoietic stem cell transplantation. *Blood* **114,** 3448–9 (2009).

249. Trzonkowski, P. *et al.* First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. *Clin. Immunol.* **133,** 22–26 (2009).

250. Brunstein, C. G. *et al.* Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood : safety profile and detection kinetics Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood : safety profile and . **117,** 1061–1070 (2011).

251. Di Ianni, M. *et al.* Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* **117,** 3921–3928 (2011).

252. Edinger, M. & Hoffmann, P. Regulatory T cells in stem cell transplantation: Strategies and first clinical experiences. *Curr. Opin. Immunol.* **23,** 679–684 (2011).

253. Martelli, M. F. *et al.* HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. *Blood* **124,** 638–644 (2014).

254. Koreth, J. *et al.* Interleukin-2 and regulatory T cells in graft-versus-host disease. *N. Engl. J. Med.* **365,** 2055–66 (2011).

255. Matsuoka, K. *et al.* Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease. *Sci. Transl. Med.* **5,** 179ra43 (2013).

256. Bacchetta, R. *et al.* Immunological outcome in haploidentical-HSC transplanted patients treated with IL-10-anergized donor T Cells. *Front. Immunol.* **5,** (2014).

257. Storb, R. *et al.* Graft-versus-host disease and graft-versus-tumor effects after allogeneic hematopoietic cell transplantation. *J. Clin. Oncol.* **31,** 1530–8 (2013).

258. Alyea, E. P. *et al. CD8+ cell depletion of donor lymphocyte infusions using cd8 monoclonal antibody-coated high-density microparticles (CD8-HDM) after allogeneic hematopoietic stem cell transplantation: a pilot study. Bone marrow transplantation* **34,** (2004).

259. Porter, D. L. *et al.* A phase 1 trial of donor lymphocyte infusions expanded and activated ex vivo via CD3/CD28 costimulation. *Blood* **107,** 1325–1331 (2006).

260. Oliveira, G., Greco, R., Lupo-Stanghellini, M. T., Vago, L. & Bonini, C. Use of TK-cells in haploidentical hematopoietic stem cell

transplantation. *Current Opinion in Hematology* **19,** 427–433 (2012).

261. Berger, C., Flowers, M. E., Warren, E. H. & Riddell, S. R. Analysis of transgene-specific immune responses that limit the in vivo persistence of adoptively transferred HSV-TK-modified donor T cells after allogeneic hematopoietic cell transplantation. *Blood* **107,** 2294–2302 (2006).

262. Traversari, C. *et al.* The potential immunogenicity of the TK suicide gene does not prevent full clinical benefit associated with the use of TK-transduced donor lymphocytes in HSCT for hematologic malignancies. *Blood* **109,** 4708–4715 (2007).

263. Straathof, K. C. *et al.* An inducible caspase 9 safety switch for T-cell therapy. *Blood* **105,** 4247–4254 (2005).

264. Zhou, X. *et al.* Long-term outcome after haploidentical stem cell transplant and infusion of T cells expressing the inducible caspase 9 safety transgene. *Blood* **123,** 3895–3905 (2014).

265. Di Stasi, A. *et al.* Inducible Apoptosis as a Safety Switch for Adoptive Cell Therapy. *New England Journal of Medicine* **365,** 1673–1683 (2011).

266. Johnson, L. A. *et al.* Gene transfer of tumor-reactive TCR confers both high avidity and tumor reactivity to nonreactive peripheral blood mononuclear cells and tumor-infiltrating lymphocytes. *J. Immunol.* **177,** 6548–6559 (2006).

267. Parkhurst, M. R. *et al.* Characterization of genetically modified T-cell receptors that recognize the CEA:691-699 peptide in the context of HLA-A2.1 on human colorectal cancer cells. *Clin. Cancer Res.* **15,** 169–180 (2009).

268. Li, Y. *et al.* Directed evolution of human T-cell receptors with picomolar affinities by phage display. *Nature biotechnology* **23,** 349–354 (2005).

269. Varela-Rohena, A. *et al.* Genetic engineering of T cells for adoptive immunotherapy. *Immunologic Research* **42,** 166–181 (2008).

270. Provasi, E. *et al.* Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. *Nature Medicine* **18,** 807–815 (2012).

271. Morgan, R. A. *et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science (New York, N.Y.)* **314,** (2006).

272. Bleakley, M., Turtle, C. J. & Riddell, S. R. Augmentation of anti-tumor immunity by adoptive T-cell transfer after allogeneic hematopoietic stem cell transplantation. *Expert Review of Hematology* **5,** 409–425 (2012).

273. Eshhar, Z., Waks, T., Gross, G. & Schindler, D. G. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 720–724 (1993).

274. Park, T. S., Rosenberg, S. a & Morgan, R. a. Treating cancer with genetically engineered T cells. *Trends Biotechnol.* **29,** 550–557 (2012).

275. Maher, J., Brentjens, R. J., Gunset, G., Rivière, I. & Sadelain, M. Human T-lymphocyte cytotoxicity and proliferation directed by

a single chimeric TCRzeta /CD28 receptor. *Nat. Biotechnol.* **20,** 70–75 (2002).

276. Sadelain, M. O NCOLOGY : R ECENT A DVANCES T-Cell Engineering for Cancer Immunotherapy. **15,** 451–455 (2009).

277. Carpenito, C. *et al.* Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 3360–3365 (2009).

278. Pulè, M. A. *et al.* A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. *Mol. Ther.* **12,** 933–941 (2005).

279. Till, B. G. *et al.* CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: Pilot clinical trial results. *Blood* **119,** 3940–3950 (2012).

280. Kalos, M. *et al.* T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci. Transl. Med.* **3,** 95ra73 (2011).

281. Porter, D. L., Levine, B. L., Kalos, M., Bagg, A. & June, C. H. *Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. The New England journal of medicine* **365,** 725–733 (2011).

282. Brentjens, R. J. *et al.* CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci. Transl. Med.* **5,** 177ra38 (2013).

283. Kochenderfer, J. N. *et al.* B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical

trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood* **119,** 2709–2720 (2012).

284. Kochenderfer, J. N. *et al.* Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood* **116,** 4099–4102 (2010).

285. Jensen, M. C. *et al.* Antitransgene rejection responses contribute to attenuated persistence of adoptively transferred CD20/CD19-specific chimeric antigen receptor redirected T cells in humans. *Biol. Blood Marrow Transplant.* **16,** 1245–1256 (2010).

286. Savoldo, B. *et al.* CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. *J. Clin. Invest.* **121,** 1822–1826 (2011).

287. Grupp, S. a *et al.* Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N. Engl. J. Med.* **368,** 1509–18 (2013).

288. Casucci, M. *et al.* CD44v6-targeted T cells mediate potent antitumor effects against acute myeloid leukemia and multiple myeloma. *Blood* **122,** 3461–72 (2013).

289. Mardiros, A. *et al.* T cells expressing CD123-specific chimeric antigen receptors exhibit specific cytolytic effector functions and antitumor effects against human acute myeloid leukemia. *Blood* **122,** 3138–3148 (2013).

4. Scope of the thesis

Although several protocols to generate human Tr1 cells *in vitro* have been developed, the resulting populations include a significant fraction of contaminating non-Tr1 cells, representing a major drawback for clinical application of Tr1 cells. We recently developed a protocol to generate Tr1 ($CD4^{IL\text{-}10}$) cells using a Lentiviral Vector (LV) encoding for human *IL-10* and *GFP*, as marker gene. We showed that enforced IL-10 expression confers Tr1 phenotype and functions to human $CD4^+$ T cells, including killing of myeloid cells. Moreover, adoptive transfer of $CD4^{IL\text{-}10}$ cells into immune-deficient mice suppresses xeno-GvHD (Andolfi et al. 2012). Despite these encouraging results, it is still unclear whether adoptive therapy with $CD4^{IL\text{-}10}$ cells could affect Graft versus Leukemia (GvL) activity mediated by allogeneic cells.

Two major lines of research have been investigated in this PhD thesis:

1. To characterize the molecular and cellular mechanisms underlying cytotoxicity mediated by $CD4^{IL\text{-}10}$ cells (Chapter 2): We investigated whether the killing mediated by $CD4^{IL\text{-}10}$ cells is super-imposable to that of classical Tr1 cells. We validated the use of polyclonal $CD4^{IL\text{-}10}$ cells as cell therapy to prevent GvHD while sparing GvL in humanized pre-clinical models of GvL and xeno-GvHD. Results from this study will provide a strong rationale for the use of $CD4^{IL\text{-}10}$ cells as cell products in allogeneic

hematopoietic stem cell transplantation for heamatological malignancies.

2. To generate allo-specific $CD4^{IL\text{-}10}$ cells (Chapter 3):

We established an *in vitro* protocol to generate a homogeneous population of allo-specific IL-10-producing Tr1 cells by LV-IL-10 gene transfer. This will represent the first step for the development of a pure population of allo-**$CD4^{IL\text{-}10}$ cells** for clinical use to prevent allograft rejection, while minimizing general immune suppression.

In **chapter 4**, all the results presented in this thesis are briefly discussed as well as the future perspectives of this research in molecular and translational medicine.

Chapter 2

IL-10 engineered human CD4+ Tr1 cells kill leukemic cells and protect from Graft-versus-Host Disease.

Grazia Locafaro[1,2*], Grazia Andolfi[1*], Fabio Russo[1], Barbara Camisa[3], Fabio Ciceri[4], Attilio Bondanza[3], Maria Grazia Roncarolo[1,5,*,§] and Silvia Gregori[1,*,§].

[1]San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Department of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute (HSR-DREaM-GENE), Milan, Italy; [2]Ph.D Program in Translational and Molecular Medicine (DIMET), University of Milan-Bicocca, Italy; [3]Experimental Hematology Unit, PIBIC, Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milano, Italy; [4]Hematology and Bone Marrow Transplantation Unit, Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute (HSR-DREaM-GENE), Milan, Italy; [5]Università Vita-Salute San Raffaele, Milan, Italy.

* These authors equally contributed to the study

§ Co-corresponding authors

Abstract

Regulatory T cells (Tregs) play a key role in preventing T cell-mediated diseases. Clinical trials showed that Tregs modulate Graft-versus Host Disease (GvHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, their impact on Graft versus Leukemia (GvL) remains to be determined. Enforced IL-10 expression converts human $CD4^+$ T cells into Tr1 ($CD4^{IL\text{-}10}$) cells that suppress effector T cells *in vitro* and *in vivo*. Here, we show that $CD4^{IL\text{-}10}$ cells also mediate anti-leukemic effects *in vitro* via a HLA-class I- and granzyme B-dependent mechanism. Cytotoxic activity of $CD4^{IL\text{-}10}$ cells is specific for $CD13^+$ target cells and requires CD54 and CD112 expression on target primary leukemic blasts. Adoptive transfer of $CD4^{IL\text{-}10}$ cells in humanized models prevents xeno-GvHD mediated by human allogeneic effector T cells, while sparing their GvL capacity. In addition, $CD4^{IL\text{-}10}$ cells have a direct non-antigen dependent anti-leukemia activity *in vivo*. These findings provide a strong rationale for the use of $CD4^{IL\text{-}10}$ cells to prevent GvHD while preserving GvL in allo-HSCT to cure myeloid malignancies.

Introduction

T regulatory cells (Tregs) promote and maintain immunological tolerance and are a fundamental component of the immune system. Several types of Tregs have been identified including the forkhead box P3 (FOXP3)-expressing $CD25^+$ Tregs [1] and the T regulatory type 1 (Tr1) cells [2]. Tr1 cells are induced in the periphery upon chronic antigen (Ag) stimulation in the presence of IL-10 [2]. Tr1 cells co-express CD49b and LAG-3 [3] and secrete IL-10, Transforming Growth Factor (TGF)-β, variable amounts of IFN-γ, low levels of IL-2, and minimal amounts of IL-4 and IL-17 [2–4]. Tr1 cells suppress T-cell responses primarily *via* the secretion of IL-10 and TGF-β [2,5] and the specific killing of myeloid cells mediated by the release of Granzyme B (GzB) and perforin [6]. Tr1 cells are induced *in vitro* in an Ag-specific manner in the presence of recombinant IL-10 or of tolerogenic DC-10 that produce high levels of IL-10 and express ILT4 and HLA-G [7,8].

In the past decade much effort has been devoted to develop suitable methods for the *in vitro* induction/expansion of $CD25^+$ Tregs and Tr1 cells to be used in Treg-based cell therapy to promote or restore tolerance in T-cell mediated diseases. To this aim, Treg-based cell therapy has been extensively tested in pre-clinical models of Graft-versus-Host-Disease (GvHD) [9–11] and in humanized mouse models of xeno-GvHD [12].

Proof-of-principle clinical trials in allogeneic hematopoietic stem cell transplantation (allo-HSCT) demonstrated the safety of $CD25^+$

Treg-based cell therapy [13–17]. Freshly isolated [14,15,17] or *in vitro* expanded polyclonal CD25+ Tregs [13,16] were infused after allo-HSCT or haploidentical HSCT for hematological malignancies to prevent GvHD. In these studies a reduction of GvHD was observed compared to historical controls. Furthermore, it has been recently reported that in CD25+ Treg-treated patients the cumulative incidence of relapse was significantly lower than in controls [17]. In a clinical trial aimed at promoting immune reconstitution in the absence of severe GvHD, we demonstrated the safety and feasibility of Tr1 cell-infusion in hematological cancer patients undergoing haploidentical HSCT [18]. We infused host-specific IL-10-anergized donor T cells (IL-10 DLI), containing Tr1 cells, in the absence of immuno-suppression. Although we treated a small cohort of patients, results demonstrated that after infusion of IL-10 DLI only mild GvHD (grade II or III, responsive to therapy) was observed and a tolerance signature was achieved. Furthermore, the treatment accelerated immune reconstitution after transplantation, and correlated with long-lasting disease remission [18]. A major difference between the CD25+ Treg-based trials and the IL-10 DLI trial is that, in the formers, a pool of polyclonal non-Ag-specific cells was administered, whereas we used a cell product containing *in vitro* primed donor-derived host-specific Tr1 cells.

Despite the significant progress in establishing *in vitro* protocols to induced allo-specific IL-10-anergized T cells for Tr1-based immunotherapy, the resulting population still contains a large proportion of effector T cells that could potentially limit the *in*

vivo efficacy of Tr1 cells. To overcome this limitation, we developed a method to generate a homogeneous population of IL-10-producing Tr1 cells by lentiviral vectors (LV)-mediated human IL-10 gene transfer. IL-10-engineered $CD4^+$ T ($CD4^{IL\text{-}10}$) cells displayed a cytokine profile and phenotype super-imposable to *bona fide* Tr1 cells, suppress T-cell responses and lyse myeloid cells *in vitro,* and prevent xeno-GvHD *in vivo* [19].

In the present study, we investigated the *in vitro* and *in vivo* anti-leukemic activity of $CD4^{IL\text{-}10}$ cells generated with a novel LV encoding for human IL-10 and ΔNGFR, as clinical grade marker gene. We demonstrated that $CD4^{IL\text{-}10}$ cells specifically kill primary myeloid blasts *in vitro* and we identified CD13, CD54, and CD112 as biomarkers of $CD4^{IL\text{-}10}$ cell-mediated anti-leukemic activity. $CD4^{IL\text{-}10}$ cells mediate potent anti-leukemic effect also *in vivo* and prevent xeno-GvHD without compromising the GvL effect mediated by allogeneic T cells. These data provide a strong rationale for the use of these Tr1 cells to prevent GvHD while preserving GvL after allo-HSCT for hematological myeloid malignancies.

Material and Methods

Plasmid construction. The coding sequence of human IL-10 was excised from pH15C (ATCC n° 68192). The resulting 549bp fragment was cloned into the multiple cloning site of pBluKSM (Invitrogen) to obtain pBluKSM-hIL-10. A fragment of 555bp was obtained by excision of hIL-10 from pBluKSM-hIL-10 and ligation to 1074.1071.hPGK.GFP.WPRE.mhCMV.dNGFR.SV40PA (here named LV-ΔNGFR), to obtain LV-IL-10/ΔNGFR. The presence of the bidirectional promoter (human PGK promoter plus minimal core element of the CMV promoter in opposite direction) allows co-expression of the two transgenes. The sequence of LV-IL-10/ ΔNGFR was verified by pyrosequencing (Primm).

Vector production and titration. VSV-G-pseudotyped third generation LVs were produced by Ca_3PO_4 transient four-plasmid co-transfection into 293T cells and concentrated by ultracentrifugation as described [20] with a small modification: 1 μM sodium butyrate was added to the cultures for vector collection. Titer was estimated on 293T cells by limiting dilution, and vector particles were measured by HIV-1 Gag p24 antigen immune capture (NEN Life Science Products; Waltham, MA). Vector infectivity was calculated as the ratio between titer and particle. For concentrated vectors, titers ranged from $5x10^8$ to $6x10^9$ transducing units/ml, and infectivity from $5x10^4$ to 10^5 transducing units/ng of p24.

Patients and donors. All protocols were approved by the Institutional Review Board and samples collected under written informed consent according to the Declaration of Helsinki. Patient characteristics are listed in Table 1. Peripheral blood mononuclear cells (PBMC) were prepared by centrifugation over Ficoll-Hypaque gradients. $CD4^+$ T cells were purified with the $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) with a resulting purity of >95%. $CD14^+$ and $CD3^+$ T cells were purified by positive selection with $CD14^+$ and $CD3^+$ Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) with a resulting purity of >95%.

Transduction of human $CD4^+$ T cells. Purified $CD4^+$ T cells were activated for 48 hours with soluble anti-CD3 monoclonal antibody (mAb, 30 ng/ml, OKT3, Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD28 mAb (1 μg/ml, BD, Biosciences) and rhIL-2 (50 U/ml, Proleukin, Novartis, Italy) and transduced with LV-GFP/ΔNGFR ($CD4^{GFP}$), LV-IL-10/ΔNGFR ($CD4^{IL\text{-}10}$) with MOI of 20 as previously described [19]. Transduced $CD4^+\Delta NGFR^+$ T cells were purified 14 days after transduction by FACS-sorting or using $CD271^+$ Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and expanded in X-VIVO 15 medium supplemented with 5% human serum (BioWhittaker-Lonza, Washington, DC), 100 U/ml penicillin–streptomycin (BioWhittaker), and 50 U/ml rhIL-2. $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells were stimulated every two weeks in the presence of an allogeneic feeder mixture containing 10^6 PBMC (irradiated at 6,000 rad) per ml, 10^5 JY cells (an Epstein–

Barr virus–transformed lymphoblastoid cell line expressing high levels of human leukocyte antigen and co-stimulatory molecules, irradiated at 10,000 rad) per ml, and soluble anti-CD3 mAb (1 μg/ml). Cultures were maintained in 50-100 U/ml rhIL-2. All FACS phenotypic analysis, *in vitro* and *in vivo* experiments were performed with cells obtained after at least 12 days feeder addition.

Cytokine determination. To measure cytokine production, $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells were stimulated with immobilized anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/ml) mAbs in a final volume of 200 μl of medium (96 well round-bottom plates, $2x10^5$/well). Supernatants were harvested after 48 hours of culture and levels of IL-4, IL-10, IFN-γ and IL-17 were determined by ELISA according to the manufacturer's instructions (BD Biosciences).

Suppression assays. To test the suppressive capacity of $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells, allogeneic PBMC were labeled with CFSE (Molecular Probes) or eFluor670 (Invitrogen) before stimulation with immobilized anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/ml) mAbs. Suppressor cells were added at 1:1 ratio. After 4–6 days of culture, proliferation of CFSE/eFluor670-labeled responder cells was determined by flow cytometry.

Cytotoxicity assays. T-cell degranulation was evaluated in a CD107a flow cytometric assay, according to the protocol

described in [6]. In some experiments anti-HLA-class I (clone W6/32, Biolegend, USA) and isotype control (IgG2a,k, BD Pharmigen, USA) mAbs were added at the indicated concentrations. The cytotoxic activity of $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells was analyzed in a standard ^{51}Cr-release assay as described in detail elsewhere. Briefly, 10^3 ^{51}Cr-labeled (NEN Dupont, Milan, Italy) target ALL-CM, BV-173, Daudi, U937 or K562 cells were incubated for 4 hours with $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells at various effector-target cell ratios, plated in duplicate. Subsequently, the supernatant was removed and counted on a g counter. Percentage of specific lysis was calculated according to the formula: 100x(^{51}Cr experimental release - spontaneous release)/(maximum release - spontaneous release). In some experiments Z-AAD-CMK (Sigma, CA, USA) was added at the indicated concentrations. Alternatively, cytotoxicity of $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells was analysed in co-culture experiments. Briefly, target and effector cells ($CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells) were plated in a ratio 1:1 for 3 days. At the end of co-culture, cells were harvested and surviving cells were counted and analysed by FACS. Elimination index (EI) was calculated as 1-(number of target remained in the co-culture with $CD4^{IL\text{-}10}$/number of target remained in the co-culture with $CD4^{GFP}$). In some experiments anti-HLA-class I (clone W6/32, Biolegend, USA) and isotype control (IgG2a,k, BD Pharmigen, USA) mAb were added at the indicated concentrations.

Flow cytometry analysis. For the detection of cell surface antigens $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells were stained with anti-CD4 (BD

Pharmingen, USA), anti-CD226 (Biolegend, USA), anti-CD2, anti-CD18, anti-CXCR4 (BD Pharmingen, USA) mAbs after a 2.4G2 blocking step. For the detection of cell surface antigens on target cells, leukemic cell lines and primary blasts were stained with anti-CD45, anti-HLA-class I, anti-CD112, anti-CD155 (Biolegend, USA), anti-CD13, anti-CD54, anti-CD58 (BD Pharmigen, USA). Cells were incubated with the aforementioned mAbs for 30 min at 4°C in PBS 2% FBS, washed twice and fixed with 0.25% formaldehyde. To evaluate human chimerism in peripheral blood of treated NSG mice, cells were co-stained with anti-human CD45 (Biolegend), anti-human CD3 (BD Bioscience), anti-human CD33 (Miltenyi Biotech), anti-CD271 (Miltenyi Biotech) and anti-murine CD45 (BD Bioscience) mAbs.

Samples were acquired using a FACS Canto II flow cytometer (Becton Dickinson, USA), and data were analyzed with FCS express (De Novo Software, CA, USA). We set quadrant markers to unstained controls.

Graft-versus-Leukemia model, ALL-CM leukemia model: 6- to 8-week-old female NSG mice were obtained from Charles-River Italia (Calco, Italy). The experimental protocol was approved by the internal committee for animal studies of our institution (Institutional Animal Care and Use Committee [IACUC #488]). On day 0 mice were infused with ALL-CM leukemia ($5x10^6$) and three days after with either allogeneic PBMC ($5x10^6$) or $CD4^{IL\text{-}10}$ or $CD4^{GFP}$ cells ($2.5x10^6$). Cells were re-suspended in 250 µl of PBS and infused intravenously. Survival and weight loss was

monitored at least 3 times per week as previously described [21,22] and moribund mice were humanely killed for ethical reasons. At weekly intervals, mice were bled and human chimerism was determined by calculating the frequency on human $CD45^+$ cells within the total lymphocyte population. In some experiments mice were euthanized at day 7 and 14 after ALL-CM injection to analyse human cells distribution in different organs.

Graft-versus-Leukemia model, THP-1 leukemia model: 6- to 8-week-old female NSG mice were obtained from Charles-River Italia (Calco, Italy). The experimental protocol was approved by the internal committee for animal studies of our institution (Institutional Animal Care and Use Committee [IACUC #488]). On day 0 mice were infused with THP-1 leukemia ($2x10^6$) and three days after with allogeneic PBMC ($2x10^6$) or fourteen days after with PBMC ($2x10^6$), $CD4^{IL\text{-}10}$ or $CD4^{GFP}$ cells ($1x10^6$). Cells were re-suspended in 250 µl of PBS and infused intravenously. Survival and weight loss was monitored at least 3 times per week as previously described [21] and moribund mice were humanely killed for ethical reasons. Mice were euthanized at week 4 after THP1 injection to analyse human cells in the liver.

Subcutaneous ALL-CM tumor model: 6- to 8-week-old female NSG mice were used. The experimental protocol was approved by the internal committee for animal studies of San Raffaele Scientific Institute (Institutional Animal Care and Use Committee [IACUC #488]). On day 0 mice were infused with ALL-CM ($2x10^6$) cells

and three days later with allogeneic PBMC ($2x10^6$) or with $CD4^{IL-10}$ cells ($1x10^6$) or $CD4^{GFP}$ cells ($1x10^6$). Cells were re-suspended in 1 ml of PBS and infused sub-cutaneously. Sarcoma growth was monitored by measurements at least 3 times per week and moribund mice were humanely killed for ethical reasons.

Graft-versus-Leukemia and Graft-versus Host Disease model: 6- to 8-week-old female NSG mice were used. The experimental protocol was approved by the internal committee for animal studies of San Raffaele Scientific Institute (Institutional Animal Care and Use Committee [IACUC #488]). At day 0, mice received total body irradiation with a single dose of 175-200 cGy irradiation from a linear accelerator according to the weight of mice, and were immediately infused with ALL-CM ($5x10^6$). On day 3 mice were then injected with allogeneic PBMC ($5x10^6$) alone or in the presence of with CD^{GFP} and $CD4^{IL-10}$ cells ($2.5x10^6$). Cells were re-suspended in 250 µl of PBS and infused intravenously. Survival and weight loss was monitored at least 3 times per week as previously described [21,22] and moribund mice were humanely killed for ethical reasons. At weekly intervals, mice were bled and human chimerism was determined by calculating the frequency on human $CD45^+$ cells within the total lymphocyte population. In some experiments mice were euthanized at day 7 and 14 after ALL-CM injection to analyse human cells distribution in different organs.

Statistical Analysis. Statistical analyses on the functional data were performed using a Mann-Whitney U test for non-parametric data and using a two-way analysis of variance test. ANOVA tests and Bonferroni's multiple comparisons were used to analyze the data from the *in vivo* experiments. P values less than 0.05 were considered significant. Statistic calculations were performed with the Prism program 5.0 (GraphPad Software).

Results

$CD4^{IL\text{-}10}$ cells mediated killing of myeloid cells is HLA-class I- and GzB-dependent. We generated $CD4^{IL\text{-}10}$ cells by transducing $CD4^+$ T cells with a novel bidirectional LV co-encoding for human IL-10 and ΔNGFR, as clinical grade marker gene (**Figure S1A**). As control, T cells transduced with a bidirectional LV co-encoding for GFP and ΔNGFR were used (**Figure S1A**). As previously described [19], enforced expression of human IL-10 into $CD4^+$ T cells allows the generation of cells ($CD4^{IL\text{-}10}$ cells) super imposable to Tr1 cells. $CD4^{IL\text{-}10}$ cells, in contrast to control LV-transduced $CD4^{GFP}$ cells, secreted significantly higher levels of IL-10 and IFN-g ($p \leq 0.0001$ and $p \leq 0.01$, respectively) and comparable low amounts of IL-4 and IL-17 (**Figure S1B**). $CD4^{IL\text{-}10}$ cells, but not $CD4^{GFP}$ cells, suppressed T-cell responses *in vitro* (**Figure S1C**). $CD4^{IL\text{-}10}$ cells expressed CD18, which in association with CD11a forms LFA-1, CD2 and CD226 at levels significantly higher than those detected on freshly isolated $CD4^+$ memory T cells ($p<0.05$ for CD18 and CD2, and $p \leq 0.001$ for CD226) and on $CD4^{GFP}$ cells ($p<0.05$ for all, **Figure 1A**).

To evaluate the ability of $CD4^{IL\text{-}10}$ cells to kill immortalized myeloid cells, we tested a panel of leukemic cell lines. Freshly isolated T lymphocytes (CD3) and monocytes (CD14) were used as negative and positive controls, respectively. We first evaluated the degranulation of $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells by analyzing the co-expression of GzB and CD107a. $CD4^{IL\text{-}10}$ cells co-cultured with CD14, U937, a monocytic cell lines, or ALL-CM, a cell line derived

from a patient suffering from a lymphoblastic crisis of chronic myelogenous leukemia [21,22] showed a significantly higher proportion of $GzB^+CD107a^+$ cells (a minimum of nine donors were tested, $CD4^{IL\text{-}10}$ versus $CD4^{GFP}$, $p \leq 0.0001$ for CD14 and $p \leq 0.001$ for U937 and ALL-CM **Figure 1B and S2**). The frequency of $GzB^+CD107a^+$ cells generated in co-culture with U937 and ALL-CM cells was also significantly higher compared to the spontaneous degranulation of $CD4^{IL\text{-}10}$ cells ($p \leq 0.001$ and $p \leq 0.0001$, paired T-test, respectively). Conversely, $CD4^{IL\text{-}10}$ cells did not degranulate when co-cultured with CD3, BV-173, a pre-B lymphoblastic leukemia [23], Daudi, a B lymphoblastic cell line, or K562, an erythroleukemic cell line (**Figure 1B and 2S**). We next assessed the cytotoxic activity of Tr1 cells. $CD4^{IL\text{-}10}$ cells killed U937 and ALL-CM cells at 100:1 (T^{eff}:target) ratio at significantly higher levels as compared to $CD4^{GFP}$ cells ($p<0.05$). On the contrary, $CD4^{IL\text{-}10}$ cells did not kill BV-173, Daudi, or K562 cells (**Figure 1C**). Similarly, in co-culture experiments $CD4^{IL\text{-}10}$ cells killed CD14, U932, ALL-CM, and THP-1, a prototypic monocytic cell line, but not BV-173 or K562 cells (**Figure 2A**).

Addition of a pan anti-HLA-class I (clone W6/32) mAb inhibited the degranulation of $CD4^{IL\text{-}10}$ cells (data not shown), and significantly prevented the killing of ALL-CM and U937 cells ($p<0.05$) (**Figure 2B**). Moreover, addition of the GzB inhibitor Z-AAD-CMK blocked $CD4^{IL\text{-}10}$-mediated killing of ALL-CM and U937 cells in a dose-dependent manner demonstrating that the killing is GzB-dependent, (**Figure 2C**). Overall, these data demonstrate that $CD4^{IL\text{-}10}$ cells kill immortalized myeloid cell lines, but not pre-B

lymphoblastic or erythroblastic cell lines, and that their cytotoxic activity requires HLA class I-mediated activation and is GzB-dependent.

$CD4^{IL\text{-}10}$ cells specifically kill $CD13^{+}$ leukemic cell lines and blasts that express CD54 and CD112. We next investigated whether the $CD4^{IL\text{-}10}$-mediated killing correlates with specific markers expressed by the leukemic cell lines. U937, THP-1, and ALL-CM cells, which were target of $CD4^{IL\text{-}10}$-mediated lysis, express CD13, CD54, HLA-class I, CD58, CD155, and CD112 (**Figures 3A**). BV-173 cells, which were not killed by $CD4^{IL\text{-}10}$ cells, were $CD13^{+}$HLA-class $I^{+}CD58^{+}$ and expressed CD54, CD155, and CD112 at low levels. K562 and Daudi cells expressed variable levels of CD54, CD58, CD155 or CD112 but were HLA-class I^{neg} and consequently were not killed by $CD4^{IL\text{-}10}$ (**Figures 3A**).

We then studied whether $CD4^{IL\text{-}10}$ cells kill primary AML blasts isolated from patients at diagnosis referred to the San Raffaele Hematology and Bone Marrow Transplantation Unit and classified according to French-American-British (FAB) subtypes and World Health Organization (WHO) categories (M0-M2, n= 8, **Table 1**). As negative controls, we used primary ALL blasts at diagnosis (n=3, **Table 1**). $CD4^{IL\text{-}10}$ cells, generated from four different healthy donors, killed four out of eight primary AML blasts tested (**Figure 3B**). $CD4^{IL\text{-}10}$-mediated lysis correlated with the expression of CD13, CD54, and CD112 on AML blasts, but not with CD58 or CD155 (**Figure 3C**). Notably, $CD4^{IL\text{-}10}$ cells did not eliminate blasts from Leu#7 donor that were $CD13^{+}CD54^{+}$ but $CD112^{neg}$ (**Figure**

3C). Surprisingly, $CD4^{IL\text{-}10}$ cells efficiently eliminated Leu#10 a primary ALL blasts that was $CD13^{+}CD54^{+}CD112^{+}$ (**Figure 3C**).
Based on these data, we conclude that $CD4^{IL\text{-}10}$ cells selectively eliminate $CD13^{+}$ leukemic cells and optimal $CD4^{IL\text{-}10}$-mediated killing requires stable CD54/LFA-1-mediated adhesion and CD112/CD226-mediated activation (**Figure S3**). Therefore, CD13, CD54, and CD112 can be used as biomarkers to identify target of the anti-leukemic effect mediated by $CD4^{IL\text{-}10}$ cells.

$CD4^{IL\text{-}10}$ cells mediate anti-leukemic effects *in vivo*. To test the anti-leukemic activity of $CD4^{IL\text{-}10}$ cells *in vivo*, we used four different clinically-relevant humanized models: the subcutaneous myeloid sarcoma, the ALL-CM leukemia model [21,22] the extramedullary THP-1 myeloid tumor [24], and the GvL/xeno-GvHD model. In the humanized model of subcutaneous myeloid sarcoma NSG mice were sub-cutaneously injected with ALL-CM cells and three weeks later myeloid sarcoma was quantified (13.9±1.16 mm, mean±SEM, n=9, **Figure 4A**). To test their anti-leukemic activity PBMC, $CD4^{IL\text{-}10}$ or $CD4^{GFP}$ cells were injected within the tumor at day three after ALL-CM infusion. Treatment with $CD4^{IL\text{-}10}$ cells significantly delayed myeloid sarcoma growth (**Figure 4A**). No signs of tumor development were observed in $CD4^{IL\text{-}10}$-injected mice within the first 14 days, and on day 21 the tumor size in $CD4^{IL\text{-}10}$ -treated mice was significantly lower than that in ALL-CM injected and un-treated mice (7.6±0.9 mm, mean ± SEM, n=8; $p \leq 0.001$; **Figure 4A**). As expected, mice injected with $CD4^{GFP}$ cells developed myeloid sarcoma similarly to control ALL-CM injected

and un-treated mice, whereas injection of allogeneic PBMC completely prevented tumor growth (**Figure 4A**).

We next evaluated whether $CD4^{IL\text{-}10}$ cells mediate anti-leukemic effects in a previously described ALL-CM leukemia model in NSG mice [21,22]. After intravenous infusion of ALL-CM cells, NSG mice developed leukemia in four weeks (**Figure 4B**). In this model, injection of allogeneic PBMC, three days after ALL-CM challenge, resulted in a significantly lower proportion of circulating human blasts (on average 4.8%, $p \leq 0.01$) as compared to that of mice injected with ALL-CM alone (on average 52.4%) and overall significantly delayed leukemia progression to 10 weeks (**Figure 4B**). Adoptive transfer of $CD4^{IL\text{-}10}$ cells or $CD4^{GFP}$ cells at day three after ALL-CM infusion did not delay leukemia development (**Figure 4B**), and at week four the percentage of circulating blasts was comparable to that observed in control ALL-CM injected and un-treated mice (data not shown).

These data show that $CD4^{IL\text{-}10}$ cells delay the development of subcutaneous myeloid sarcoma but do not inhibit systemic leukemia growth. We hypothesized that in the ALL-CM leukemia model, the absence of anti-leukemic effect by $CD4^{IL\text{-}10}$ cells could be due to their inability to co-localize with the leukemic cells in the bone marrow. To test this hypothesis, we first investigated the expression of CXCR4, which regulates the homing of human hematopoietic stem cells and myeloid leukemia in the bone marrow of humanized mice [25–27], on $CD4^{IL\text{-}10}$ cells. In contrast to ALL-CM cells and PBMC, resting $CD4^{IL\text{-}10}$ cells did not express significant levels of CXCR4 (**Figure S4**). We next investigated the

in vivo frequency of human cells in the peripheral blood, bone marrow, spleen, lung, and liver of NSG mice injected with ALL-CM and PBMC, $CD4^{IL\text{-}10}$ or $CD4^{GFP}$ cells. The frequency of circulating $CD4^{IL\text{-}10}$ cells at day seven after transfer was on average 16.3% (±15.7%, mean±StD, n=7), and declined to 6.7% (±4.2%, mean±StD, n=3) at day fourteen (**Figure 4C**). Similar results were obtained in mice injected with $CD4^{GFP}$ cells. In contrast, $CD4^{IL\text{-}10}$ cells were detected at very low frequency in the bone marrow at day seven (0.4±0.5% mean±StD, n=4), and were undetectable at day fourteen (**Figure 4C**). At day seven, $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells were detected also in the spleen, lung and liver but their frequencies declined at day fourteen (**Figure 4C**). In peripheral blood of mice treated with PBMC more than 70% and 30% human T cells were detected at day seven and fourteen, respectively (**Figure 4C**). T cells were also identified in the spleen, lung, liver, and in bone marrow of PBMC-injected mice at both time points (**Figure 4C**). These data show that that $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells display similar homing and survival properties upon *in vivo* transfer, indicating that the inability of $CD4^{IL\text{-}10}$ cells to home to the BM is not related to IL-10 production.

To further prove the anti-leukemic activity of $CD4^{IL\text{-}10}$ cells we tested their *in vivo* effect on a THP-1 myeloid tumor [24] (**Figure 4D**). In this model, mice injected with THP-1 cells develop cystis of myeloma cells in the liver after two weeks, as demonstrated by the increase of the liver weight from 1-1.1 gr to 1.7±0.12 gr (mean±StD, n=4). Four week after THP-1 injection the liver weight was 3.7±1.7 gr (mean±StD, n=12, **Figure 4D**). Injection of PBMC

either three days or two weeks after THP-1 cell infusion inhibited tumor growth (liver weight: 2.5±1.1 gr., mean±StD, n=10 and 2.7±0.3 gr., mean±StD, n=3, **Figure 4D**). Adoptive transfer of $CD4^{IL\text{-}10}$ cells two weeks after THP-1 injection significantly inhibited tumor growth (2.1±0.9 gr., mean±StD, n=8, $p \leq 0.01$, **Figure 4D**). No differences in tumor development after THP-1 injection were observed in $CD4^{GFP}$-treated versus un-treated mice (3.9±0.8 gr., mean±StD, n=7, and 3.7±1.7 gr., mean±StD, n=12, respectively, **Figure 4D**).

These findings indicate that despite the limited *in vivo* lifespan, $CD4^{IL\text{-}10}$ cells mediate potent and specific anti-leukemic effects in peripheral tissues in different humanized models.

Adoptive transfer of $CD4^{IL\text{-}10}$ cells prevents xeno-GvHD while spearing the GvL of allogeneic T cells.

We next investigated the effects of $CD4^{IL\text{-}10}$ cells on both GvL and xeno-GvHD mediated by allogeneic human T cells *in vivo*. To this end, we developed a humanized model of GvL/xeno-GvHD: NSG mice were sub-lethally irradiated to increase SDF-1 expression and secretion by stromal cells within murine bone marrow [28], and injected with ALL-CM cells. Three days later mice received allogeneic PBMC or $CD4^{IL\text{-}10}$ cells or $CD4^{GFP}$ cells alone or in combination (**Figure 5A and B**). We first analyzed the *in vivo* localization of human cells in sub-lethally irradiated NSG mice injected with ALL-CM and with PBMC, $CD4^{IL\text{-}10}$ or $CD4^{GFP}$ cells. The frequency of $CD4^{IL\text{-}10}$ cells in the bone marrow at day seven after transfer was on average 22.3% (±9.6%, mean±SEM, n=9), and

declined to 0.3% (±0.2%, mean±SEM, n=11) at day fourteen (**Figure 5A**). The peripheral blood tested in parallel showed comparable results, with up to 34.3% (±8.2%, mean±SEM, n=17) of circulating $CD4^{IL\text{-}10}$ cells at day seven, that declined to 3.0% (±1.6%, mean±SEM, n=14) at day fourteen (**Figure 5A)**. Similar results were obtained in mice injected with $CD4^{GFP}$ cells. In the bone marrow of PBMC-injected mice 39.4% (±7.0%, mean±SEM, n=8) and 23.5% (±5.0%, mean±SEM, n=14) of human T cells were detected at day seven and fourteen, respectively. In the peripheral blood of PBMC-injected mice, human T cells were detected at both time points analyzed (**Figure 5A**). These data indicate that in conditioned NSG mice $CD4^{IL\text{-}10}$ cells migrate to the bone marrow but have a limited lifespan.

We then tested the ability of $CD4^{IL\text{-}10}$ cells to mediate anti-leukemic effect in conditioned NSG mice. Results showed that adoptive transfer of $CD4^{IL\text{-}10}$ cells at day three after ALL-CM injection significantly delayed leukemia progression ($P<0.05$), whereas, treatment with $CD4^{GFP}$ cells did not (**Figure 5B**). It should be noted that leukemia progression in $CD4^{IL\text{-}10}$-injected mice was not significantly different to that observed with PBMC-injected mice ($P\leq0.0001$, **Figure 5B**). However, PBMC-treated mice developed severe xeno-GvHD within twenty days (**Figure 5B**). Remarkably, co-transfer of $CD4^{IL\text{-}10}$ cells and PBMC significantly delayed leukemia progression ($P<0.05$), with no sign of xeno-GvHD (**Figure 5B**). Overall, these findings demonstrate that adoptive transfer of $CD4^{IL\text{-}10}$ cells prevents xeno-GvHD, while spearing the GvL mediated by allogeneic T cells. In addition, $CD4^{IL\text{-}}$

10 cells have a direct non-antigen dependent anti-leukemia effects *in vivo*.

Discussion

We previously reported that enforced expression of IL-10 confers a Tr1-like phenotype and function, including killing of myeloid cells, to human $CD4^+$ T cells [19]. In this study, we show that Tr1 ($CD4^{IL\text{-}10}$) cells generated with a novel bidirectional LV encoding for human *IL-10* and *ΔNGFR*, as clinical grade marker gene, acquire the expression of CD18, CD2, and CD226, and the ability to express and secrete GzB. $CD4^{IL\text{-}10}$ cells display a cytotoxic activity restricted to myeloid cells and kill leukemic cell lines *in vitro*. Furthermore, we also establish for the first time that $CD4^{IL\text{-}10}$ cells eliminate primary leukemic blasts. The expression of HLA-class I on target myeloid cells is necessary but not sufficient to promote the $CD4^{IL\text{-}10}$-mediated cytotoxicity, which also requires stable CD54-mediated adhesion, and activation *via* CD226. Expression of CD13, CD54, and CD112 on primary blasts is also indispensable for $CD4^{IL\text{-}10}$-mediated killing. In humanized mouse models, $CD4^{IL\text{-}10}$ cells mediate potent non-antigen-dependent anti-leukemic effects and prevent xeno-GvHD without compromising the GvL effect mediated by allogeneic effector cells.

Previous studies indicated that Treg cells can kill effector cells through a granzyme-dependent mechanism and that this cytotoxic activity contributes to the suppression function [29,30]. In the present study we demonstrate that the cytotoxic activity of $CD4^{IL\text{-}10}$ cells is specifically directed against myeloid cells and myeloid leukemic blasts. This data confirmed previous studies demonstrating that IL-10-producing Tr1 cells eliminate myeloid

but not lymphoid cells, including activated T cells [6]. The correlation between the expression of the myeloid marker CD13 on target cells and the ability of $CD4^{IL\text{-}10}$ cells to eliminate primary blasts indicates that CD13 expression determines the myeloid specificity of $CD4^{IL\text{-}10}$ cells. Furthermore, killing of primary blasts by $CD4^{IL\text{-}10}$ cells correlates with CD54 expression, showing the importance of adhesion between target cells and $CD4^{IL\text{-}10}$ cells for effective cytolysis, as previously shown [6]. CD54 on myeloid blasts allows stable and prolonged interaction with LFA-1 (CD18-CD11a) on $CD4^{IL\text{-}10}$ cells, which is required to enhance the secretion of lytic granules containing GzB. In addition, the expression of CD112, one of the ligand of CD226 involved in Tr1-mediated cytolysis [6], is also required. Similar to the mechanism previously described for NK-mediated lysis of AML blasts, the interaction between CD112 on myeloid leukemic blasts and CD226 on $CD4^{IL\text{-}10}$ cells leads to their activation [31]. Since LFA-1 in critical importance in the downstream signaling cascade of CD226 [32], we can speculate that CD112/CD226 interaction promotes the stabilization of $CD4^{IL\text{-}10}$ -target cell conjugates that triggers $CD4^{IL\text{-}10}$ cell degranulation. CD226-mediated activation of T cells is inhibited by TIGIT (T cell Immunoreceptor with Ig and ITIM domains) [33], another receptor for CD112 and CD155 [34]. TIGIT binds to CD155 with higher affinity that CD226 and inhibits the cytotoxic activity and IFN-γ production by NK cells [35,36]. $CD4^{IL\text{-}10}$ cells express TIGIT (data not shown), whereas on primary blasts CD155 is expressed at lower levels than CD112 [31,37]. Furthermore, $CD4^{IL\text{-}10}$-mediated lysis does not correlate with the expression of

CD155, suggesting that activation of $CD4^{IL\text{-}10}$ cells *via* CD226/CD112 interaction is dominant over the inhibitory signal mediated by TIGIT/CD155. Based on these findings we propose that the selective expression of CD13, CD54 and CD112 on leukemic blasts could be used as a biomarker for the anti-leukemic effect mediated by $CD4^{IL\text{-}10}$ cells.

The anti-leukemic effect exerted by $CD4^{IL\text{-}10}$ cells is antigen-independent. $CD4^{IL\text{-}10}$ cells require recognition and activation *via* HLA-class I expressed on target cells, but not TCR engagement, to kill target cells. The inhibition of HLA class I recognition by neutralizing mAbs, or the lack of HLA class I expression on target blasts, prevent the lytic activity mediated by $CD4^{IL\text{-}10}$ cells. This mechanism of target recognition and lysis mediated by $CD4^{IL\text{-}10}$ cells resembles the non Ag-specific-mediated recognition and activation of NK cells. However, in contrast to NK cells that do not kill HLA-class I expressing target cells because of the expression of inhibitor killer Ig-like receptors (KIRs), $CD4^{IL\text{-}10}$ cells mediate lysis of HLA-class I^+ target cells. $CD4^{IL\text{-}10}$ cells do not express activating KIRs or CD94/NKG2D (data not shown) and further studies are warranted to identify the specific activating receptor expressed by $CD4^{IL\text{-}10}$ cells, and by Tr1 cells, that recognizes HLA class I molecules triggering their activation. The ability of $CD4^{IL\text{-}10}$ cells to eliminate myeloid leukemia in an alloAg-independent but HLA class I-dependent manner renders them an interesting tool for future clinical use to limit, and possibly to overcome, leukemia relapse caused by the lost of shared HLA alleles after haploidentical-HSCT, or matched unrelated HSCT [38,39].

$CD4^{IL\text{-}10}$ cells mediate anti-leukemic effect *in vivo* in humanized murine models of myeloid tumors. This anti-tumor effect is strictly related to the co-localization of $CD4^{IL\text{-}10}$ and leukemic cells. $CD4^{IL\text{-}10}$ cells display an effective anti-tumor activity when either locally injected within the myeloid sarcoma, or systemically injected in mice with liver-bearing myeloid tumors. Moreover, $CD4^{IL\text{-}10}$ cell immunotherapy prevents leukemia development in conditioned humanized mice, in which $CD4^{IL\text{-}10}$ cells are present in the bone marrow as early as seven days post-infusion. The anti-leukemic effect exerted by $CD4^{IL\text{-}10}$ cells is not achieved in un-conditioned humanized mice in which $CD4^{IL\text{-}10}$ cells are present in limited numbers and for a short period of time in the bone marrow. These data indicate that to control leukemia progression, which occurs at later time points, $CD4^{IL\text{-}10}$ cells needs to be present in the bone marrow. The limited lifespan of $CD4^{IL\text{-}10}$ cells could be beneficial for their future clinical use as immunotherapy, since it could avoid the risk of general immunosuppression.

Recognition of host alloAgs by donor T lymphocytes causes GvHD after allo-HSCT, a major cause of transplant related morbidity and mortality. On the other hand, donor T cells recognize also alloAgs on leukemic cells thus mediating a beneficial GvL immune response [40]. Prevention and treatment of GvHD currently relies on depletion of T cells from the graft or general immunosuppression, which however abrogates the GvL effect, increasing the incidence of leukemia relapse [41]. Establishing a regimen that prevents GvHD without affecting GvL represents a key challenge in allo-HSCT

treatment of hematological malignancies. Several studies indicate that both $CD8^+$ and $CD4^+$ donor T cells, recognizing peptide-HLA complexes on the surface of host blasts, mediate GvL [40]. $CD4^+$ HLA-class II-specific T cells isolated from patients after allo-HSCT [42,43], have been shown to contribute to GvL effects [44]. Therefore, the anti-leukemic effects mediated by donor $CD4^+$ T cells require TCR-mediated activation. However, it is still unclear whether donor T cells recognize the same alloAg peptide on host APC and on leukemic blasts, or if $CD4^+$ T cells are activated by tumor-derived specific peptide presented by HLA-class II^+ host cells. Here we show that $CD4^{IL\text{-}10}$ cells prevent xeno-GvHD without hampering the anti-leukemic effect of allogeneic PBMC. Although this peculiar effect of $CD4^{IL\text{-}10}$ cells is intriguing an deserves further investigation, we can postulate that following TCR-mediated activation $CD4^{IL\text{-}10}$ cells increase the IL-10 production [19], which directly inhibits the proliferation and expansion of allo(xeno)-reactive human T cells, thus preventing GvHD, and express and secrete GzB, which is necessary for the cytolysis [6]. In addition, IL-10-derived from activated $CD4^{IL\text{-}10}$ cells does not prevent the cytotoxicity mediated by allogeneic PBMC against leukemic cells. It has been shown that IL-10 increases the proliferative response of activated human $CD8^+$ T cells [45], the frequency of CTL after polyclonal activation [46], and IL-10-activated $CD8^+$ T cells mediate anti-tumor activity *in vivo* [47]. Notably, $CD4^{IL\text{-}10}$ cells exert an anti-leukemic effect, which does not require TCR-mediated activation. Therefore, activated $CD4^{IL\text{-}10}$ cells directly kill monocytes, myeloid

DC and $CD13^+CD54^+CD112^+$ leukemic blasts in an alloAg-nonspecific manner.

In humanized models it has been shown that co-infusion of *in vitro* expanded or freshly isolated human $CD4^+CD25^+$ Tregs with allogeneic PBMC [48] or conventional donor T cells [17] rescued mice from leukemia, which survived without xeno-GvHD. In the first study, human $CD4^+CD25^+$ Tregs have been show to migrate into the bone marrow, where they are converted into IL-17-producing T cells and lose the ability to suppress anti-tumor activity of human T cells [48]. Conversely, Martelli et al. [17] proposed that the failure of human $CD4^+CD25^+$ Tregs to home to the bone marrow allows allogeneic T cells to control leukemia development. Our data suggest that $CD4^{IL\text{-}10}$ cells, differently from human $CD4^+CD25^+$ Tregs, migrate to the bone marrow where they eliminate myeloid leukemia cells without limiting the GvL mediated by allogeneic PBMC.

Overall, our data show that immunotherapy with $CD4^{IL\text{-}10}$ cells can: i) inhibit GvHD, ii) mediate anti-leukemic effects, and iii) spare the GvL effect mediated by allogeneic T cells.

These findings pave the way for the use of $CD4^{IL\text{-}10}$ cell immunotherapy after allo-HSCT for hematological malignancies aimed at inhibiting GvHD while sparing GvL.

Acknowledgements

We thank Prof. Luigi Naldini for the kind gift of the bidirectional LV and Dr. Cristina Tresoldi for providing clinical samples. This work was supported by a grant from the Italian Telethon Foundation "Comitato Telethon Fondazione Onlus", Core grant TIGET TeleE2, and the Italian Association for Cancer Research project IG 10439 (Associazione Italiana per la Ricerca sul Cancro, or AIRC)

Authorship contributions

The authors' individual contributions were the following G.L. and G.A. performed experiments, analyzed the data, and contributed to the writing the manuscript. F.R., and B.C. performed the *in vivo* experiments. A.B supervised the *in vivo* experiments, provided scientific advises and editing; M.G.R. supervised the experiments, analyzed the data, provided scientific advises, and wrote the manuscript; S.G designed the study, coordinated and supervised the project and wrote the manuscript.

References

1. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). *J. Immunol.* **155,** 1151–64 (1995).

2. Groux, H. *et al.* A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389,** 737–742 (1997).

3. Gagliani, N. *et al.* Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med* **19,** 739–746 (2013).

4. Bacchetta, R. *et al.* High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *J Exp Med* **179,** 493–502 (1994).

5. Roncarolo, M. G. *et al.* Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological Reviews* **212,** 28–50 (2006).

6. Magnani, C. F. *et al.* Killing of myeloid APCs via HLA class I, CD2 and CD226 defines a novel mechanism of suppression by human Tr1 cells. *Eur. J. Immunol.* **41,** 1652–1662 (2011).

7. Bacchetta, R. *et al.* Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy. *Haematologica* **95,** 2134–2143 (2010).

8. Gregori, S. *et al.* Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* **116,** 935–944 (2010).

9. Hoffmann, P., Ermann, J., Edinger, M., Fathman, C. G. & Strober, S. Donor-type CD4+CD25+ Regulatory T Cells Suppress Lethal Acute Graft-Versus-Host Disease after Allogeneic Bone Marrow Transplantation. *Journal of Experimental Medicine* **196,** 389–399 (2002).

10. Edinger, M. *et al.* CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat. Med.* **9,** 1144–1150 (2003).

11. Cohen, J. L., Trenado, A., Vasey, D., Klatzmann, D. & Salomon, B. L. CD4(+)CD25(+) immunoregulatory T Cells: new therapeutics for graft-versus-host disease. *J. Exp. Med.* **196,** 401–406 (2002).

12. Gregori, S., Goudy, K. S. & Roncarolo, M. G. The cellular and molecular mechanisms of immuno-suppression by human type 1 regulatory T cells. *Frontiers in Immunology* **3,** (2012).

13. Trzonkowski, P. *et al.* First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. *Clin. Immunol.* **133,** 22–26 (2009).

14. Edinger, M. & Hoffmann, P. Regulatory T cells in stem cell transplantation: Strategies and first clinical experiences. *Current Opinion in Immunology* **23,** 679–684 (2011).

15. Di Ianni, M. *et al.* Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* **117,** 3921–3928 (2011).

16. Brunstein, C. G. *et al.* Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood : safety profile and detection kinetics Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood : safety profile and . **117,** 1061–1070 (2011).

17. Martelli, M. F. *et al.* HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. *Blood* **124,** 638–644 (2014).

18. Bacchetta, R. *et al.* Immunological Outcome in Haploidentical-HSC Transplanted Patients Treated with IL-10-Anergized Donor T Cells. *Front Immunol* **5,** 16 (2014).

19. Andolfi, G. *et al.* Enforced IL-10 Expression Confers Type 1 Regulatory T Cell (Tr1) Phenotype and Function to Human CD4+ T Cells. *Molecular Therapy* **20,** 1778–1790 (2012).

20. De Palma, M. & Naldini, L. Transduction of a gene expression cassette using advanced generation lentiviral vectors. *Methods Enzymol.* **346,** 514–529 (2002).

21. Bondanza, A. *et al.* Suicide gene therapy of graft-versus-host disease induced by central memory human T lymphocytes. *Blood* **107,** 1828–1836 (2006).

22. Hambach, L. *et al.* Human cytotoxic T lymphocytes specific for a single minor histocompatibility antigen HA-1 are effective against human lymphoblastic leukaemia in NOD/scid mice. *Leuk. Off. J. Leuk. Soc. Am. Leuk. Res. Fund, U.K* **20,** 371–374 (2006).

23. Pegoraro, L. *et al. Establishment of a Ph1-positive human cell line (BV173). Journal of the National Cancer Institute* **70,** 447–453 (1983).

24. Casucci, M. *et al.* CD44v6-targeted T cells mediate potent antitumor effects against acute myeloid leukemia and multiple myeloma. *Blood* **122,** 3461–72 (2013).

25. Dar, A., Kollet, O. & Lapidot, T. Mutual, reciprocal SDF-1/CXCR4 interactions between hematopoietic and bone marrow stromal cells regulate human stem cell migration and development in NOD/SCID chimeric mice. *Experimental Hematology* **34,** 967–975 (2006).

26. Tavor, S. *et al.* CXCR4 Regulates Migration and Development of Human Acute Myelogenous Leukemia Stem Cells in Transplanted NOD/SCID Mice. *Cancer Res.* **64,** 2817–2824 (2004).

27. Kollet, O. *et al.* Rapid and efficient homing of human CD34+CD38-/lowCXCR4+ stem and progenitor cells to the bone marrow and spleen of NOD/SCID and NOD/SCID/B2mnull mice. *Blood* **97,** 3283–3291 (2001).

28. Ponomaryov, T. *et al.* Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. *J. Clin. Invest.* **106,** 1331–1339 (2000).

29. Grossman, W. J. *et al.* Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* **104,** 2840–8 (2004).

30. Grossman, W. J. *et al.* Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* **21,** 589–601 (2004).

31. Pende, D. *et al.* Analysis of the receptor-ligand interactions in the NK-mediated lysis of freshly isolated myeloid or lymphoblastic leukemias. Evidence for the involvement of the poliovirus receptor (CD155) and Nectin-2 (CD112). *Blood* 2004–2009 (2004). doi:10.1182/blood-2004-09-3548.Supported

32. Shibuya, K. *et al.* Physical and functional association of LFA-1 with DNAM-1 adhesion molecule. *Immunity* **11,** 615–623 (1999).

33. Lozano, E., Dominguez-Villar, M., Kuchroo, V. & Hafler, D. A. C.-P. C.-N. The TIGIT/CD226 axis regulates human T cell function. *J Immunol* **188,** 3869–3875 (2012).

34. Yu, X. *et al.* The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat. Immunol.* **10,** 48–57 (2009).

35. Georgiev, H. *et al.* To the Editor TIGIT versus CD226: Hegemony or coexistence? *Eur. J. Immunol.* **44,** 307–308 (2014).

36. Stanietsky, N. *et al.* Mouse TIGIT inhibits NK-cell cytotoxicity upon interaction with PVR. *Eur. J. Immunol.* **43,** 2138–2150 (2013).

37. Sanchez-Correa, B. *et al.* Decreased expression of DNAM-1 on NK cells from acute myeloid leukemia patients. *Immunology and Cell Biology* **90,** 109–115 (2012).

38. Vago, L. *et al.* Loss of mismatched HLA in leukemia after stem-cell transplantation. *N. Engl. J. Med.* **361,** 478–488 (2009).

39. Villalobos, I. B. *et al.* Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation. *Blood* **115,** 3158–3161 (2010).

40. Appelbaum, F. R. Haematopoietic cell transplantation as immunotherapy. *Nature* **411,** 385–389 (2001).

41. Warren, E. H. & Deeg, H. J. Dissecting graft-versus-leukemia from graft-versus-host-disease using novel strategies. *Tissue Antigens* **81,** 183–193 (2013).

42. Faber, L. M., van Luxemburg-Heijs, S. A., Veenhof, W. F., Willemze, R. & Falkenburg, J. H. Generation of CD4+ cytotoxic T-lymphocyte clones from a patient with severe graft-versus-host disease after allogeneic bone marrow transplantation: implications for graft-versus-leukemia reactivity. *Blood* **86,** 2821–2828 (1995).

43. Griffioen, M. *et al.* Identification of phosphatidylinositol 4-kinase type II beta as HLA class II-restricted target in graft versus leukemia reactivity. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 3837–3842 (2008).

44. Stevanović, S. *et al.* Human allo-reactive CD4+ T cells as strong mediators of anti-tumor immunity in NOD/scid mice engrafted with human acute lymphoblastic leukemia. *Leukemia* **26,** 312–322 (2012).

45. Groux, H., Bigler, M., de Vries, J. E. & Roncarolo, M. G. Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. *J. Immunol.* **160,** 3188–3193 (1998).

46. Chen, W. F. & Zlotnik, A. IL-10: a novel cytotoxic T cell differentiation factor. *J. Immunol.* **147,** 528–534 (1991).

47. Groux, H. *et al.* A transgenic model to analyze the immunoregulatory role of IL-10 secreted by antigen-presenting cells. *J. Immunol.* **162,** 1723–1729 (1999).

48. Guichelaar, T. *et al.* Human regulatory T cells do not suppress the antitumor immunity in the bone marrow: a role for bone marrow stromal cells in neutralizing regulatory T cells. *Clin Cancer Res* **19,** 1467–75 (2013).

Table 1. Patients' characteristics.

	Leu #	Sex/age	[1]FAB	[2]WHO	Cytogenetics	Molecular Markers	Source	Blasts (%)
#1	AML #61	F/71	M0	AML	N.A.	Flt3 ITD+/NPMI+	PB	92%
#2	AML #63	F/83	M0	AML with minimal maturation	del7, t(1;7), t(4;12)	Flt3 ITD+/NPMI+	PB	98%
#3	AML #39	F/47	M1	AML without maturation	46, XX	Flt3 ITD-/NPMI-	BM	79%
#4	AML #60	F/64	M1	AML	46, XX	Flt3 ITD+/NPMI-	BM	78%
#5	AML #1	F/50	M1	AML without maturation	N.A.	Flt3 ITD-/NPMI+	PB	98%
#6	AML #64	M/60	M2	AML with t(8;21)RUNX1-RUNKX1T1	del9, t(8;21)	Flt3 ITD+/NPMI+	PB	26%
#7	AML #37	F/59	M2	AML with maturation	46, XX	Flt3 ITD-/NPMI+	BM	54%
#8	AML #5	F/66	M2	AML with maturation	dup8	Flt3 ITD+/NPMI+	PB	65%
#9	ALL 57	M/38	ALL	ALL-T	N.A.	N.A.	PB	46%
#10	ALL #48	F/22	ALL	ALL	N.A.	N.A.	BM	85%
#11	ALL 62	F/76	ALL	ALL-B L2	N.A.	Flt3 ITD+/NPMI+	PB	91%

[1]AML and ALL subtypes according to the French-American-British (FAB) classification.
[2]AML and ALL chategories according to the World Health Organization (WHO) classification.
N.A. Not Applicable; Flt3 ITD, Flt3 internal-tandem duplication; NPM1, nucleophosmin; BM, bone marrow, PB, peripheral blo

Figure legends

Figure 1. $CD4^{IL\text{-}10}$ cells express CD18, CD2, and CD226 and are cytotoxic. A. The expression of CD18, CD2 and CD226 on freshly isolated $CD4^+$ T, $CD4^{IL\text{-}10}$, and $CD4^{GFP}$ cells was evaluated by FACS. Un-stained $CD4^{IL\text{-}10}$ cells (filled light gray histogram, isotype control), freshly isolated $CD4^+$ T cells (dashed black line histogram), $CD4^{GFP}$ cells (black line histogram), $CD4^{IL\text{-}10}$ cells (red solid line histogram). Numbers indicate the MFI on $CD4^+$ gated cells. One representative donor out of 5 tested is shown. **B.** $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells were co-cultured with CD3, CD14, U937, BV-173, Daudi, K562 and ALL-CM target cell lines at 5:1 (E:T) ratio. After 6 hours, degranulation of $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells was measured by the co-expression of CD107a and Granzyme (GzB). Numbers in quadrants indicate percentage of positive cells, one representative donor is depicted. **C.** Cytotoxicity of $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells against U937, BV-173, Daudi, K562, and ALL-CM cells was determined by ^{51}Cr-release assay. Mean±SEM of cytotoxicity performed in duplicated is reported; mean±SEM of n=6 and of n=4 independent donors for $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells against ALL-CM and U937 cells, and of n=4 and of n=2 for $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells against BV-173, Daudi, and K562 cells. *P<0.05, Mann Whitney t-test.

Figure 2. $CD4^{IL\text{-}10}$ cells specifically lyse myeloid cell lines in HLA-class and I-GzB-dependent manner. A. $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells were co-cultured with CD14, U937, BV-173, K562, THP-1,

and ALL-CM cells at 1:1 ratio. After 3 days, residual leukemic cell lines ($CD45^{low}CD3^{-}$) were analyzed and counted by FACS. Cytolysis mediated by $CD4^{IL\text{-}10}$ cells was measured as elimination index (see Material and Methods) for each target cells. Analysis was performed at least in three independent experiments. Dots represent the elimination index of $CD4^{IL\text{-}10}$ cells generated from different healthy donors. Lines represent mean values of the elimination index. Gray area indicates the threshold of cytotoxicity. **B.** $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells were co-cultured with ALL-CM or U937 target cell line at 1:1 (E:T) ratio in the presence of 10 μg/ml of anti-HLA class I or isotype control mAbs. After 3 days, residual leukemic cell lines ($CD45^{low}CD3^{-}$) were analyzed and counted by FACS. Cytolytic effect by $CD4^{IL\text{-}10}$ cells was measured as elimination index for each target cells. Dots represent $CD4^{IL\text{-}10}$ cells generated from different healthy donors. Lines represent mean values of the elimination index. $*P<0.05$, Mann Whitney t-test. **C.** $CD4^{IL\text{-}10}$ cells were pre-incubated with Z-AAD-CMK at the indicated concentrations and co-cultured with ALL-CM and U937 target cell at 50:1 (E:T) ratio, and cytotoxicity was determined by ^{51}Cr release. Mean±SEM of n=3 independent donors performed in triplicates is reported.

Figure 3. $CD4^{IL\text{-}10}$ cells specifically kill *in vitro* primary blasts expressing CD13, CD54, and CD112. A. The expression of the CD13, CD54, HLA-class I, CD58, CD155, and CD112 on U937, BV-173, K562, Daudi, THP-1, and ALL-CM cell lines was analyzed by FACS. Numbers indicate the percentages of positive cells (black

solid line) according to isotype control (filled light gray histogram). **B.** $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells were co-cultured with primary blast at 1:1 (E:T) ratio. After 3 days, residual leukemic blasts ($CD45^{low}CD3^{-}$) were analyzed and counted by FACS. Cytolytic effect of $CD4^{IL\text{-}10}$ cells was measured as elimination index (see Material and Methods) for each target cells. Dots represent each primary blast co-cultured with $CD4^{IL\text{-}10}$ cells. Gray area indicates the threshold of cytotoxicity. **C.** Correlation between cytotoxicity and expression of specific markers on primary blasts. Plots represent percentages of CD13, CD54, HLA-class I, CD58, CD155, and CD112 positive primary blasts versus elimination index of $CD4^{IL\text{-}10}$ cells for each primary blast tested in a co-culture assay (mean±SEM). The line represents the linear regression. The *P* value of the correlation and the coefficient of determination (R2) are reported (two-tailed test).

Figure 4. The *in vivo* localization of $CD4^{IL\text{-}10}$ cells influences their anti-leukemic activity. A. $CD4^{IL\text{-}10}$ cells delay the subcutaneous ALL-CM tumor growth. NSG mice were sub-cutaneously (s.c.) injected with ALL-CM ($2x10^6$cells/mouse). Three days later mice received allogeneic PBMC ($2x10^6$cells/mouse), or $CD4^{IL\text{-}10}$ cells, or $CD4^{GFP}$ cells ($1x10^6$cells/mouse). Tumor growth was measured at the indicated time points after ALL-CM injection. Statistical analysis was performed by comparing $CD4^{IL\text{-}10}$ cells treated mice (ALL-CM + $CD4^{IL\text{-}10}$) versus un-treated control mice (ALL-CM): $*P<0.05$, $**P\leq 0.01$; $*** P\leq 0.001$; Mann Whitney t-test. **B.** $CD4^{IL\text{-}10}$ cells do

not mediate GvL effect in the ALL-CM leukemia model of T-cell therapy. NSG mice were intravenously (i.v.) injected with ALL-CM ($5x10^6$ cells/mouse) alone or, on day three, with allogeneic PBMC ($5x10^6$cells/mouse), $CD4^{IL\text{-}10}$ cells, or $CD4^{GFP}$ cells ($2.5x10^6$cells/mouse). Leukemia free mice (less than 50% of circulating human blast $hCD45^+hCD3^-$ in peripheral blood) were followed over time after cell injection. Data obtained from four independent experiments are presented;*** $P \leq 0.001$, Log-rank (Mantel-Cox) test. **C.** *In vivo* localization of $CD4^{IL\text{-}10}$ cells. NSG mice were intravenously (i.v.) injected with ALL-CM ($5x10^6$ cells/mouse) alone or, on day three, with allogeneic PBMC ($5x10^6$cells/mouse), $CD4^{IL\text{-}10}$ cells, or $CD4^{GFP}$ cells ($2.5x10^6$cells/mouse). On day 7 and 14 the percentages of human T cells ($hCD45^+hCD3^+$) in peripheral blood, bone marrow, spleen, lung, and liver were evaluated by FACS. Mean±SD of n=6 mice for ALL-CM + PBMC and for ALL-CM + $CD4^{GFP}$ cells, and n=7 mice for ALL-CM + $CD4^{IL\text{-}10}$ cells in the peripheral blood on day 7, and mean±SD n=3 mice for ALL-CM + PBMC and ALL-CM + $CD4^{GFP}$ cells, and n=4 mice for ALL-CM + $CD4^{IL\text{-}10}$ cells, on day 7 in other organs (upper panel). Mean±SD of n=3 mice per group on day 14 (lower panel). **D.** $CD4^{IL\text{-}10}$ cells mediate anti-leukemic effect in a model of extramedullary tumor. NSG mice were intravenously (i.v.) injected with THP-1 leukemia cells ($2x10^6$cells/mouse). Three days later mice received allogeneic PBMC ($2x10^6$cells/mouse), or fourteen days later mice were injected with allogeneic PBMC ($2x10^6$cells/mouse), $CD4^{IL\text{-}10}$ cells, or $CD4^{GFP}$ cells ($1x10^6$cells/mouse). Tumor growth was analyzed by

measuring the weight of THP-1-infiltrated livers. Mean±SEM of n=12 mice for THP-1, n=10 mice THP-1 + PBMC (injected on day 3), n=3 mice THP-1 + PBMC (injected on day 14), n=8 mice for THP-1 + $CD4^{IL\text{-}10}$ cells, and n=7 mice for THP-1 + $CD4^{GFP}$ cells are presented; $^{**}P \leq 0.01$; Mann Whitney t-test.

Figure 5. Adoptive transfer of $CD4^{IL\text{-}10}$ cells prevents GvHD while spearing the Graft-versus-Leukemia of allogeneic T cells. A. *In vivo* localization of $CD4^{IL\text{-}10}$ cells in conditioned mice. NSG mice were sub-lethally irradiated and intravenously (i.v.) injected with ALL-CM ($5x10^6$cells/mouse). Three days later mice received allogeneic PBMC ($5x10^6$cells/mouse), or $CD4^{IL\text{-}10}$ cells, or $CD4^{GFP}$ cells ($2.5x10^6$cells/mouse). On day 7 and 14 the percentages of human T cells ($hCD45^+hCD3^+$) and ALL-CM ($hCD45^+hCD3^-$) in bone marrow and peripheral blood were evaluated by FACS. Mean±SEM of n=9 mice for ALL-CM + PBMC, and for ALL-CM + $CD4^{IL\text{-}10}$ cells, and n=7 for ALL-CM + $CD4^{GFP}$ cells on day 7, and n= 14 for ALL-CM + PBMC, n= 11 for ALL-CM + $CD4^{IL\text{-}10}$ cells, and n=7 for ALL-CM + $CD4^{GFP}$ cells on day 14 in the bone. Mean±SEM of n=17 mice for ALL-CM + PBMC and for ALL-CM + $CD4^{IL\text{-}10}$ cells, and n=10 for ALL-CM + $CD4^{GFP}$ cells on day 7, and n= 16 for ALL-CM + PBMC, n= 14 for ALL-CM + $CD4^{IL\text{-}10}$ cells, and n=8 for ALL-CM + $CD4^{GFP}$ cells on day 14 in the peripheral blood. Data were obtained from two to five independent experiments. **B**. Adoptive transfer of $CD4^{IL\text{-}10}$ cells prevents xeno-GvHD while sparing GvL mediated by human allogeneic PBMC. NSG mice were sub-lethally irradiated and intravenously (i.v.)

injected with ALL-CM (5x10^{6}cells/mouse). Three days later mice received allogeneic PBMC (5x10^{6}cells/mouse) alone or in combination with CD4$^{IL\text{-}10}$ cells (2.5x10^{6}cells/mouse). As control mice were injected with CD4$^{IL\text{-}10}$ cells or CD4^{GFP} cells (2.5x10^{6}cells/mouse) alone. Leukemia free mice (presence of less than 50% of circulating human blast hCD45$^+$hCD3$^-$ in peripheral blood) and xeno-GvHD free mice (presence of less than 50% of circulating human T lymphocytes hCD45$^+$hCD3$^+$ in peripheral blood with a loss of weight higher than 20%) were followed over time after cell injection. Statistical analysis were performed by comparing treated mice versus un-treated control mice (ALL-CM): *P<0.05, **** P≤0.0001; two-way ANOVA plus Bonferroni post test.

Figure S1. CD4$^{IL\text{-}10}$ cells are phenotypically and functionally super-imposable to Tr1 cells. A. Scheme of the LV-IL-10/ΔNGFR and LV-GFP/ΔNGFR vectors. The presence of the bidirectional promoter (mCMV/PGK) allows the co-regulated expression of ΔNGFR and human IL-10 or GFP genes. Ψ, encapsidation signal including the 5' portion of GAG gene (GA); RRE, Rev-responsive element; cPPT, central poly-purine tract; polyA, poly-adenylation site from the Simian Virus 40; CTE, constitutive transport element; WPRE, woodchuck hepatitis virus post-transcription regulatory element. **B**. CD4$^{IL\text{-}10}$ and CD4^{GFP} cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 mAbs for 48 hours and IL-10, IL-4, IFN-g, and IL-17 levels were measured by ELISA in culture supernatants. All samples were tested in

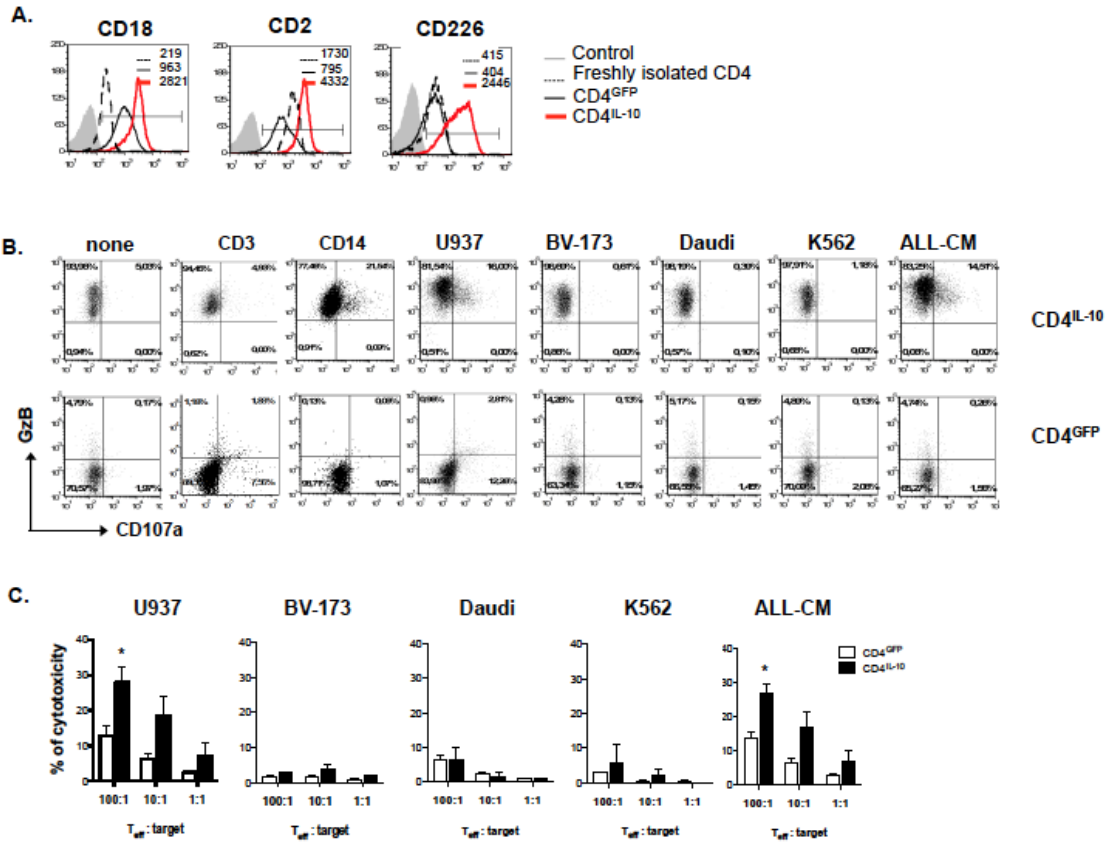
duplicate-triplicate. Mean±SEM of n=35 for IL-10 and IL-4, n=29 for IFN-g, and n=7 for IL-17 of $CD4^{IL\text{-}10}$ cells and of n=19 for IL-10 and IL-4, n=16 for IFN- g, and n=12 for IL-17 of $CD4^{GFP}$ cells are presented. ** $P \leq 0.01$; **** $P \leq 0.0001$, Mann Whitney t-test. **C**. $CD4^{IL\text{-}10}$ cells suppress T cell proliferation *in vitro*. Allogeneic PBMC cells were labeled with CSFE and stimulated with immobilized anti-CD3 and anti-CD28 mAbs alone (filled light grey histogram) or in the presence of $CD4^{IL\text{-}10}$ cells (red solid line) or $CD4^{GFP}$ cells (black solid line) at 1:1 ratio. After 4 days of culture, the percentage of proliferating PBMC was determined by CSFE dilution. One representative donor out of six tested is shown. The suppression mediated by $CD4^{IL\text{-}10}$ cells or $CD4^{GFP}$ cells was calculated as follows: ([proliferation responder-proliferation transduced)/proliferation responder] x 100).

Figure S2. $CD4^{IL\text{-}10}$ cells specifically degranulate when co-cultured with myeloid cells. $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells were co-cultured with CD3, CD14, U937, BV-173, Daudi, K562 and ALL-CM target cell lines at 5:1 (E:T) ratio. After 6 hours, degranulation of $CD4^{IL\text{-}10}$ and $CD4^{GFP}$ cells was measured by the co-expression of CD107a and Granzyme (GzB). Mean±SEM of n= 15 for $CD4^{IL\text{-}10}$ cells, and n=12 for $CD4^{GFP}$ cells cultured alone or with ALL-CM or U937 cells, n= 5 for $CD4^{IL\text{-}10}$ cells and n=2 for $CD4^{GFP}$ cells co-cultured with CD3 cells, n=10 for $CD4^{IL\text{-}10}$ cells and n=8 for $CD4^{GFP}$ cells co-cultured with CD14 cells, n=5 for $CD4^{IL\text{-}10}$ cells and n=6 for $CD4^{GFP}$ cells co-cultured with BV-173 cells, n=4 for $CD4^{IL\text{-}10}$ cells and n=6 for $CD4^{GFP}$ cells co-cultured with Daudi cells, and n=11 or

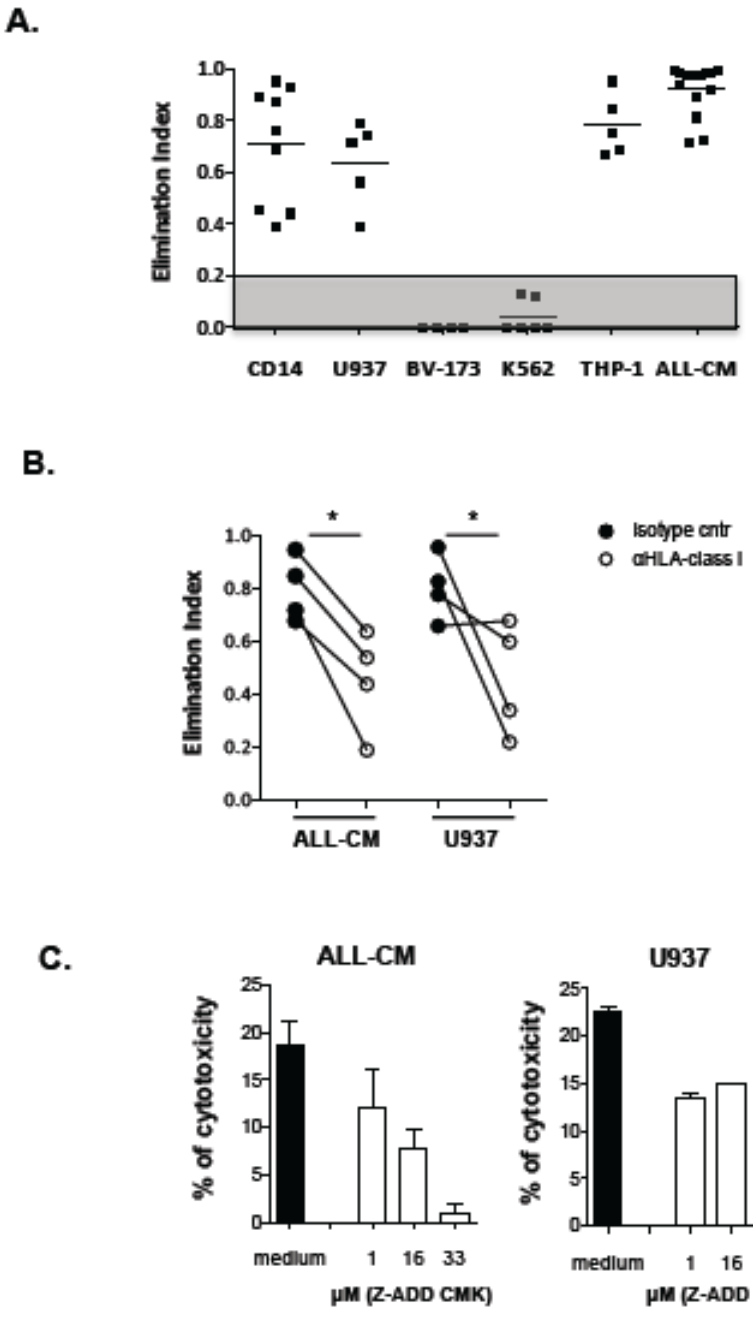
$CD4^{IL\text{-}10}$ cells and n= 9 for $CD4^{GFP}$ cells co-cultured with K562 cells is reported. *** $P \leq 0.001$; **** $P \leq 0.0001$, Mann Whitney t-test.

Figure S3. The molecular mechanism underlying the lytic activity of $CD4^{IL\text{-}10}$ cells is comparable to that of *bona fide* Tr1 cells. This mechanism requires stable adhesion between $CD4^{IL\text{-}10}$ cells and blasts mediated by LFA-1/CD54 interaction, activation of $CD4^{IL\text{-}10}$ cells *via* HLA class I (Signal 1), *via* CD2 (Signal 2), and *via* CD226 (Signal 3), leading to GzB release and killing of leukemic blasts.

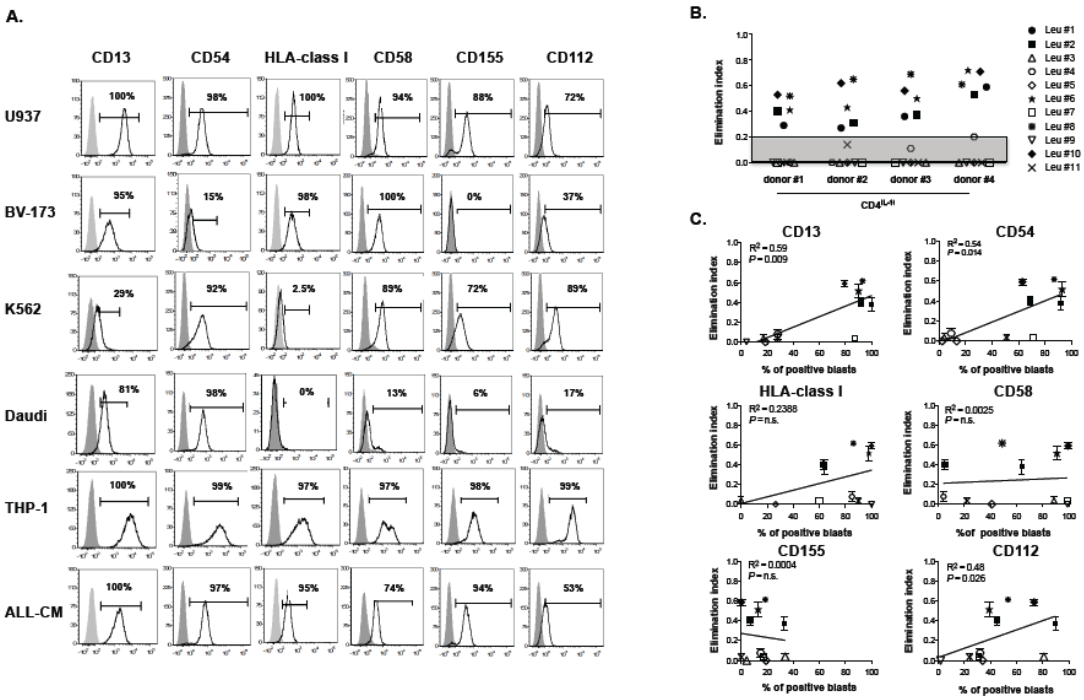
Figure S4. $CD4^{IL\text{-}10}$ cells do not express CXCR4. $CD4^{IL\text{-}10}$ cells, ALL-CM, and PBMC were analyzed for the expression of CXCR4 by FACS. Numbers indicate the percentage of positive cells (black solid line) according to isotype control (filled light gray histogram). One representative out of three tested is shown.



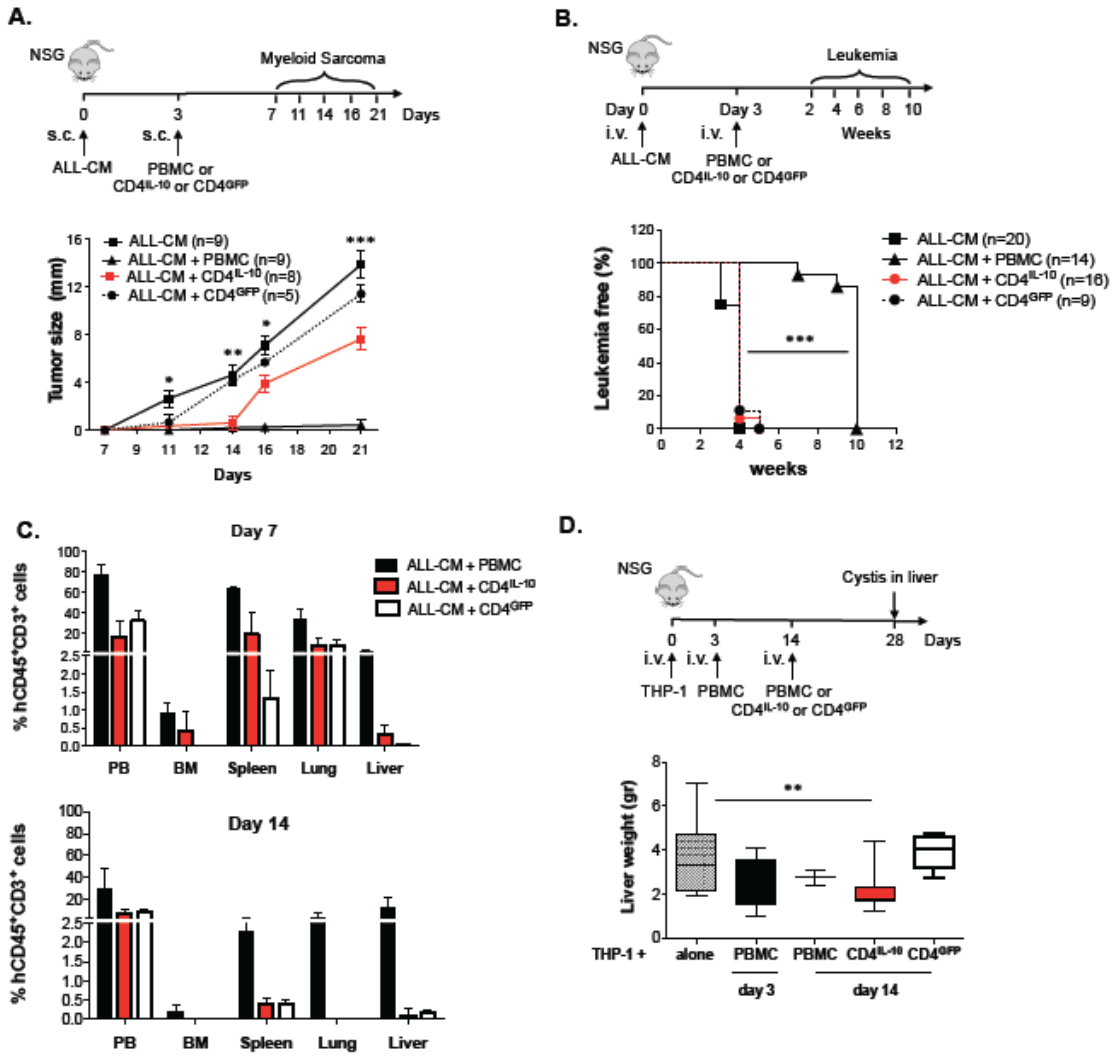
Locafaro G. and Andolfi G. et al. Figure 1



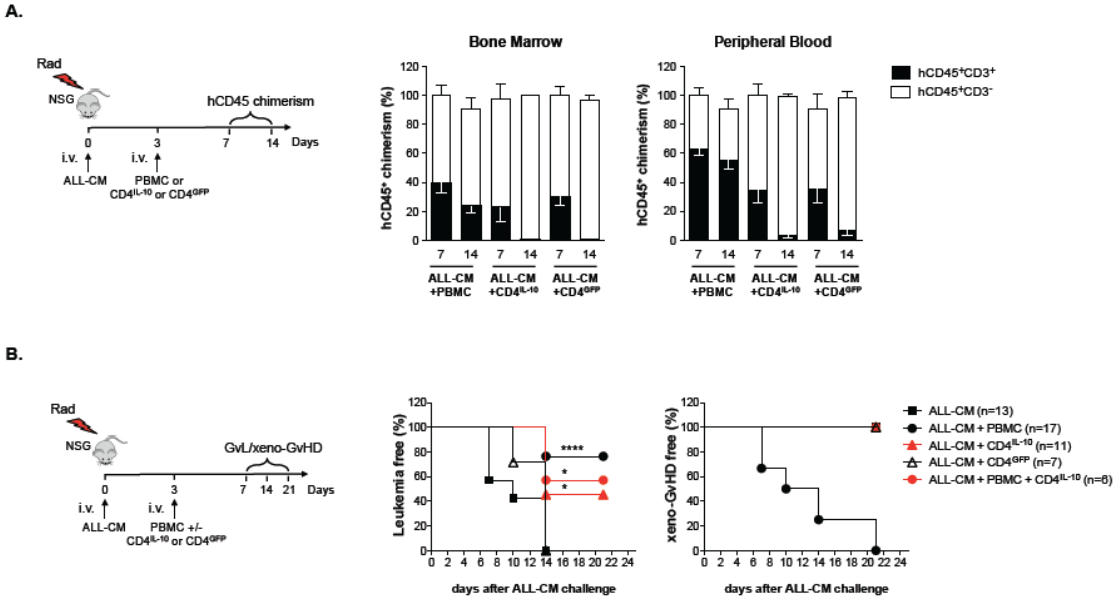
Locafaro G. and Andolfi G. et al. Figure 2



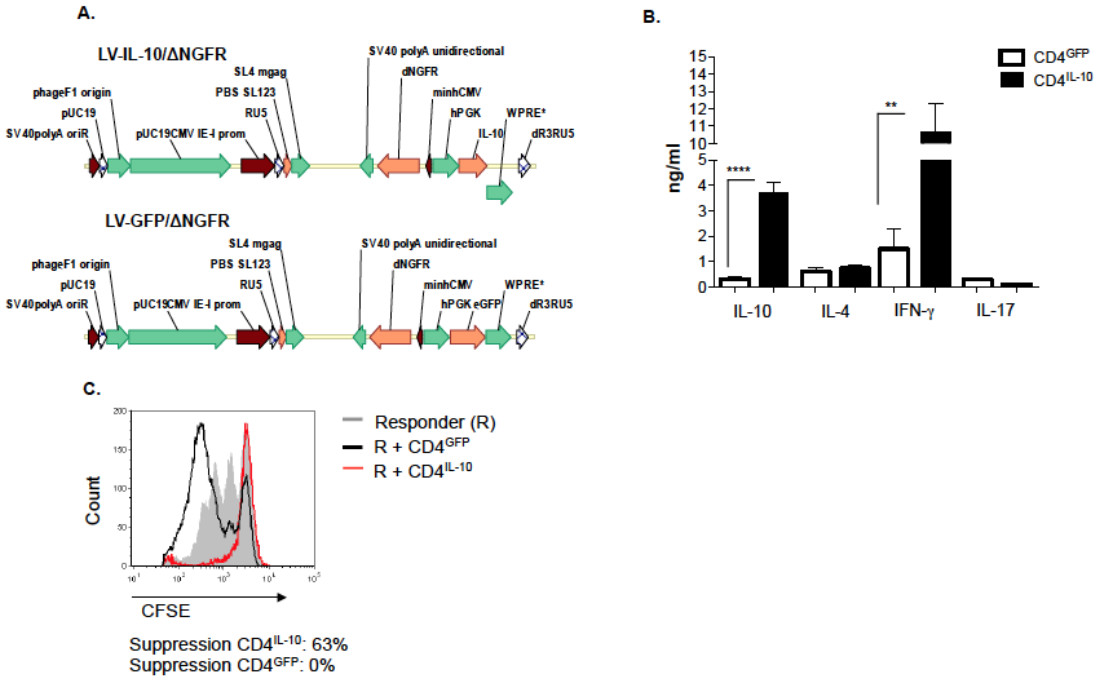
Locafaro G. and Andolfi G. et al. Figure 3



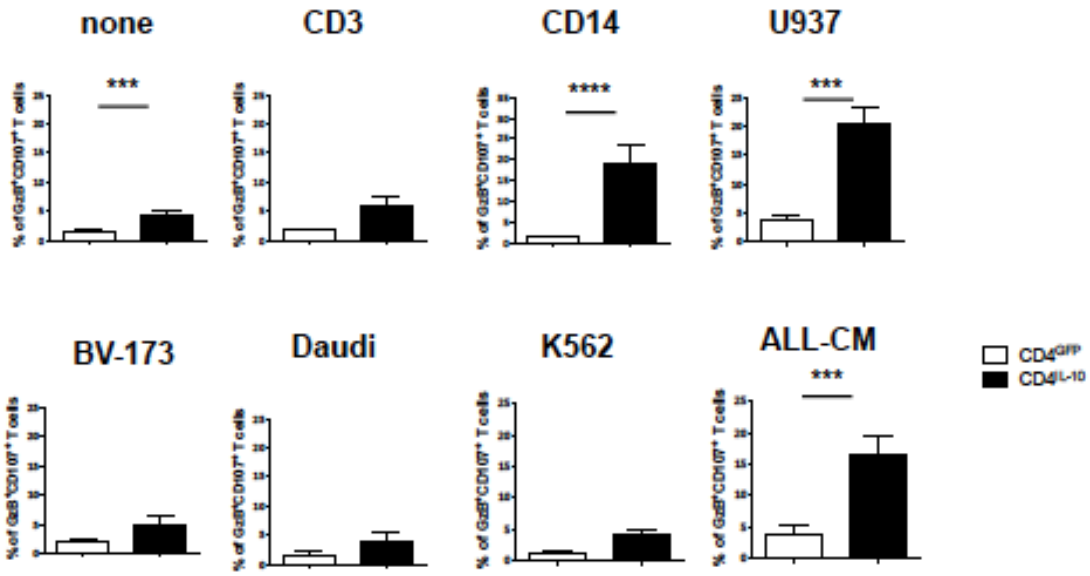
Locafaro G. and Andolfi G. et al. Figure 4



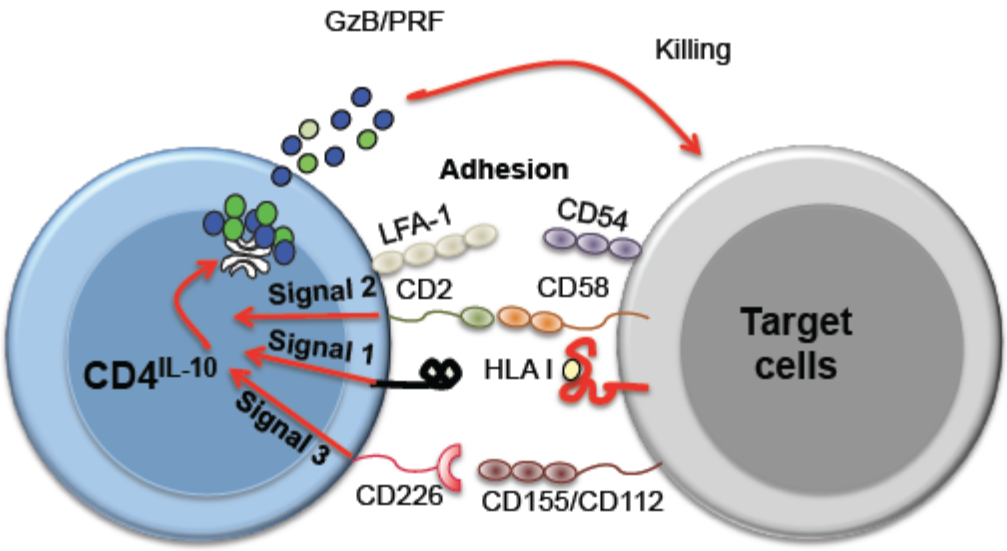
Locafaro G. and Andolfi G. et al. Figure 5



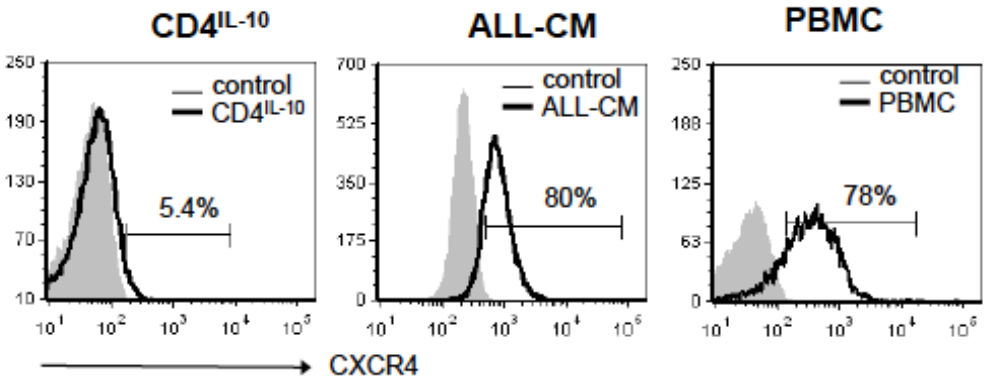
Locafaro G. and Andolfi G. et al. Figure S1



Locafaro G. and Andolfi G. et al. Figure S2



Locafaro G. and Andolfi G. et al. Figure S3



Locafaro G. and Andolfi G. et al. Figure S4

Chapter 3

Induction of allo-specific Tr1 cells by lentiviral vector-mediated IL-10 gene transfer

G. Locafaro[1-2], G.Andolfi[1], S. Gregori[1], and M.G. Roncarolo[1,3]

[1]San Raffaele Telethon Institute for Gene Therapy (TIGET), Department of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute (HSR-DREaM-GENE), Milan, Italy; [2]Ph.D Program in Translational and Molecular Medicine (DIMET), University of Milan-Bicocca, Italy; [3]Department of Pediatrics, Stanford University School of Medicine, Stanford, CA.

Abstract

T regulatory type 1 (Tr1) cells are a subset of inducible $CD4^+$ T regulatory cells characterized by high levels of IL-10 and the ability to suppress T-cell responses in an antigen-specific manner. In a proof-of-principle clinical trial we demonstrated that immunotherapy with allo-specific IL-10-anergized T cells, containing Tr1 cells and generated with recombinant human IL-10 or tolerogenic DC-10, is feasible and promote tolerance. Despite these promising results, we are aware that the lack of homogeneity in the culture preparation limits the number of Tr1 cells administered. Therefore, to truly optimize the effectiveness of Tr1 cell treatment, we esteblished a protocol to generate a pure population of polyclonal Tr1 cells, termed $CD4^{IL\text{-}10}$, by lentiviral vector-mediated IL-10 (LV-IL-10) gene transfer into conventional $CD4^+$ T cells. In the present study we applied the LV-IL-10 platform to generate human allo-specific Tr1 cells. We showed that upon allo-specific stimulation $CD4^{IL\text{-}10}$ cells, obtained by transducing $CD4^+$ T cells with LV-IL-10 during a secondary allo-stimulation, secrete high levels of IL-10, are anergic, and suppress allo-specific T-cell responses *in vitro*. These results represent the first step for the generation of a pure population of allo-specific Tr1 cells to be used as cell immunotherapy for promoting tolerance after cell and organ transplantation.

Introduction

T regulatory type 1 (Tr1) cells are a discrete population of $CD4^+$ T regulatory (Tregs) cells critically involved in promoting and maintaining of immune tolerance [1]. Tr1 cells are induced in the periphery in the presence of interleukin (IL)-10, express CD49b and LAG-3, and are characterized by the ability to secrete high levels of IL-10 and low levels of IL-4 and IL-17 (Groux, Bigler, de Vries, & Roncarolo, 1996; Nicola Gagliani et al., 2013; Roncarolo et al., 2014). Tr1 cells suppress effector T cell responses via IL-10- and TGF-β-dependent mechanisms and by killing of myeloid antigen-presenting cell via granzyme (Gz)-B [1,4]. Adoptive transfer of antigen-specific Tr1 cells has been shown to be efficacious in suppressing immune responses in pre-clinical models of colitis, type 1 diabetes, multiple sclerosis, allergy, and transplantation (Groux et al., 1997; Zeller et al., 1999; Barrat et al., 2002; Cottrez, Hurst, Coffman, & Groux, 2000; Foussat et al., 2003; N Gagliani et al., 2013). In humans, Tr1 cells have been shown to be involved in promoting tolerance after allogeneic hematopoietic stem cell transplantation (HSCT) [11,12], organ transplantation [13,14], and towards non-harmful antigens (Ags), such as gliadin [15], allergens [16,17], and auto-Ags of type 1 diabetes (T1D) [18]. The presence of increased or decreased frequency of Tr1 cells have been associated with tolerance [11,12,15], or with autoimmune diseases such as Pemphigus Vulgaris [19], T1D [18], rheumatoid arthritis [20], and allergy [16,17], respectively. Moreover, the genetic loss of IL-10 or IL-10R in humans [21,22] leads to a devastating inflammatory disease with predominant pathology in the gut where it is now well

known that Tr1 cells are required to maintain tolerance to commensal antigens and bacteria.

Overall these evidences suggest the rationale for cell therapies with Tr1 cells to restore/promote tolerance in T-cell mediated diseases. In the last decade much effort has been dedicated to establish methods to isolate/expand or to induce Tr1 cells for clinical use [1,23,24]. Tr1 cells were initially induced *in vitro* by stimulating peripheral blood mononuclear cells (PBMC) or $CD4^+$ T cells with irradiated allogeneic monocytes in the presence of recombinant human IL-10 (mixed lymphocytes reactions-MLR/IL-10) (Groux et al., 1997b; Rosa Bacchetta et al., 2010). Tr1 cells can be also differentiated by repetitive stimulation of naive $CD4^+$ T cells with allogeneic immature monocyte-derived DC [27] or by a single stimulation with IL-10-treated tolerogenic DC, named DC-10 [26,28]. A reproducible manufacturing protocol for the GMP production of allo-specific IL-10-anergized T cells generated *in vitro* through activation of T cells by allo-antigen presenting cells (APCs) in the presence of IL-10 has been developed (Rosa Bacchetta et al., 2010). An improved protocol for the generation of Tr1 cells, which foresees the use of tolerogenic DC-10 has been subsequently developed [29]. Notably, the above-mentioned methods allow the induction of bulk cultures containing Tr1 cells and non-anergic T cells that are non-alloAg specific and maintain their ability to respond to other Ags, such as pathogens or third party allo-Ags (Bejarano et al., 1992; Rosa Bacchetta et al., 2010). As an alternative to generate a homogeneous IL-10–producing Tr1 cells population, the direct IL-10 gene transfer using a

bidirectional lentiviral vector (LV-IL-10) co-encoding for human IL-10 and GFP, as marker gene, has been developed [31]. Resulting engineered T cells, termed $CD4^{IL\text{-}10}$ cells, resemble the phenotype and functions of *bona fide* Tr1 cells, including the suppressive activities both *in vitro* and *in vivo* in NOD.scid mice in which xeno graft-versus-host disease (xeno-GvHD) was induced [31]. Moreover, we recently demonstrated that adoptive transfer of polyclonal $CD4^{IL\text{-}10}$ cells in NSG mice prevents xeno-GvHD without affecting the graft-*versus*-leukemia (GvL) mediated by allogeneic PBMC and mediates a GvL effect *in vivo* (Locafaro and Andolfi, submitted).

A growing body of evidence from animal models suggests that the efficacy of Tr1 cell therapy is strictly dependent on the Ag specificity. Indeed, adoptive transfer of Ag-specific Tr1 cells induce stable, graft-specific, and IL-10–dependent tolerance in a stringent model of allogeneic islet transplant, while non–Ag-specific polyclonal Tr1 cells fail to provide this same activity [32]. Therefore the induction of a pure population of Ag-specific Tr1 cells is highly warranted.

In the present study, we apply the LV-IL-10 platform to obtain human allo-specific Tr1 cells. We showed that allo-specific $CD4^+$ T cells can be easily transduced with LV-IL-10 and that the resulting IL-10-transduced cells, named allo-$CD4^{IL\text{-}10}$ cells, acquired a Tr1-like cytokine profile and an anergic phenotype upon allo-specific stimulation. Moreover, preliminary data suggested that allo-specific $CD4^{IL\text{-}10}$ cells suppress T cell proliferation in an allo-specific manner. Results from this study represent the first step for the development of a homogeneous population of human allo-

specific Tr1 cells to be used as Tr1-based immunotherapy for promoting tolerance after allogeneic HSCT and organ transplantation.

Material and Methods

Plasmid construction. The coding sequence of human IL-10 was excised from pH15C (ATCC n° 68192). The resulting 549bp fragment was cloned into the multiple cloning site of pBluKSM (Invitrogen) to obtain pBluKSM-hIL-10. A fragment of 555bp was obtained by excision of hIL-10 from pBluKSM-hIL-10 and ligation to 1074.1071.hPGK.GFP.WPRE.mhCMV.dNGFR.SV40PA (here named LV-ΔNGFR), to obtain LV-IL-10/ΔNGFR. The presence of the bidirectional promoter (human PGK promoter plus minimal core element of the CMV promoter in opposite direction) allows co- expression of the two transgenes. The sequence of LV-IL-10/ ΔNGFR was verified by pyrosequencing (Primm).

Vector production and titration. VSV-G-pseudotyped third generation LVs were produced by Ca_3PO_4 transient four-plasmid co-transfection into 293T cells and concentrated by ultracentrifugation as described [33] with a small modification: 1 μM sodium butyrate was added to the cultures for vector collection. Titer was estimated on 293T cells by limiting dilution, and vector particles were measured by HIV-1 Gag p24 antigen immune capture (NEN Life Science Products; Waltham, MA). Vector infectivity was calculated as the ratio between $5x10^8$ to $6x10^9$ titer and particle. For concentrated vectors, titers ranged from transducing units/ml, and infectivity from transducing $5x10^4$ to 10^5 units/ng of p24.

Patients and donors. All protocols were approved by the Institutional Review Board and samples collected under written informed consent according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were prepared by centrifugation over Ficoll-Hypaque gradients. $CD4^+$ T cells were purified with the $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) with a resulting purity of >95%. Naive $CD4^+$ T cells were isolated from purified $CD4^+$ T cells by negative selection with $CD45RO^+$ Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) with a resulting purity of >95%.

Dendritic cell differentiation. $CD14^+$ monocytes were isolated from peripheral blood mononuclear cells by positive selection using $CD14^+$ MicroBeads (Miltenyi Biotech, Germany) according to the manufacturer's instructions. Cells were cultured in RPMI 1640 (Lonza, Italy) supplemented with 10% fetal bovine serum (FBS) (Lonza, Italy), 100 U/mL penicillin/streptomycin (Lonza, Italy), 2 mM L-glutamine (Lonza, Italy), at 37°C in the presence of 10 ng/mL recombinant human (rh) IL-4 (R&D Systems, Minneapolis MN, USA) and 100 ng/mL rhGM-CSF (Genzyme, Seattle, WA, USA) for 5 days. On day 5 cells were matured with 1 mg/mL of lipopolysaccharide (LPS, Sigma, CA, USA) for 2 more days to generate mature dendritic cells (mDC). At day 7, DC were collected, phenotypically analyzed, and used to stimulate T cells. The purity and maturation state of DC were checked by flow cytometry to determine expression of CD1a, CD14, CD86, CD83 and HLA-DR.

Enrichment in allo-specific $CD4^+$ T cells. Naive $CD4^+$ T cells were stimulated with allogeneic mDC (allo-mDC) for fourteen days. Naive $CD4^+$ T cells were plated at a 10:1 ratio: 10^6/well naive $CD4^+$ T cells with 10^5/well of mDC in a final volume of 1 mL in 24-well plates. At day 7 and at day 10, half of the medium was replaced by fresh medium supplemented with 25 U/ml of rhIL-2 (PROLEUKIN, Novartis, Italy). On day 14, cells were collected, washed and re-stimulated for transduction.

Transduction of human $CD4^+$ T cells. Enriched allo-specific $CD4^+$ T cells were activated and transduced with LV-GFP/ΔNGFR ($CD4^{GFP}$), LV-IL-10/ΔNGFR ($CD4^{IL\text{-}10}$) with MOI of 20 after 24 hours of secondary stimulation with the same allo-mDC used for priming. Transduced $CD4^+$ $\Delta NGFR^+$ T cells were purified 14 days after transduction using $CD271^+$ Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and expanded in X-VIVO 15 medium supplemented with 5% human serum (Lonza, Italy), 100 U/ml penicillin–streptomycin (Lonza, Italy), and 50 U/ml rhIL-2 (PROLEUKIN, Novartis, Italy). $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells were stimulated every two weeks in the presence of an allogeneic feeder mixture containing 10^6 PBMC (irradiated at 6,000 rad) per ml, 10^5 JY cells (an Epstein–Barr virus– transformed lymphoblastoid cell line expressing high levels of human leukocyte antigen and co-stimulatory molecules, irradiated at 10,000 rad) per ml, and 1 μg/ml of soluble anti-CD3 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany). Cultures were maintained in 50 U/ml rhIL-2. All FACS phenotypic analysis and *in*

vitro experiments were performed with cells obtained after at least 12 days feeder addition.

Cytokine determination. $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells were plated in 96-well plates at a 10:1 ratio: 10^5/well $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells with 10^4/well of allo or third party mDC in a final volume of 200 µL in round-bottomed 96-well plates to measure cytokines production. The levels of IL-4, IL-10, and IFN-γ secreted were determined by ELISA according to the manufacturer's instructions (BD Biosciences).

Proliferation assays. $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells were plated in 96-well plates at a 10:1 ratio: 10^5/well $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells with 10^4/well of allogeneic or third party mDC in a final volume of 200 µL in round-bottomed 96-well plates. After the indicated time, cells were pulsed for 16 h with 1 µCi/well ^{3}H-thymidine to evaluate their allo-specific proliferative response.

Suppression assays. To test the suppressive capacity of $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells, autologous $CD4^+$ T cells were labeled with eFluor670 (Invitrogen) before stimulation with allogeneic or third party mDC at 10:1 ratio. Suppressor cells were added at 1:1 ratio. After 4-6 days of culture, proliferation of eFluor670-labeled responder cells was determined by flow cytometry.

Flow cytometry analysis. For the detection of cell surface antigens $CD4^+$ T cells or $CD4^{GFP}$ and $CD4^{IL\text{-}10}$ cells were stained with the specified mAbs after a 2.4G2 blocking step. Cells were

incubated with mAbs for 30 min at 4°C in PBS 2% FBS, washed twice and fixed with 0.25% formaldehyde. Samples were acquired using a FACS Canto II flow cytometer (Becton Dickinson, USA), and data were analyzed with FCS express (De Novo Software, CA, USA). We set quadrant markers to unstained controls.

Statistical Analysis. Statistical analyses on the functional data were performed using Wilcoxon matched-pairs signed rank test, a test for non-parametric data. All results are presented as mean values ± standard error of mean (SEM). Differences were regarded as statistically significant at $*p<0.05$, $**p<0.01$, and $***p<0.001$. Statistic calculations were performed with the Prism program 5.0 (GraphPad Software).

Results

Bidirectional LVs encoding for human IL-10 and ΔNGFR can efficiently transduce allo-specific CD4$^+$ T cells. We previously demonstrated that transduction of polyclonal CD4$^+$ T cells with a bidirectional LV co-encoding for human IL-10 and GFP or ΔNGFR, as marker gene, generates a population of Tr1-like cells ([31] and Locafaro and Andolfi, submitted). Here, we applied the LV-IL-10 platform to generate allo-specific Tr1 cells. Naïve CD4$^+$ T cells were stimulated with allogeneic mature dendritic cells (allo-mDC), differentiated from CD14$^+$ cells in the presence of GM-CSF and IL-4 for 5 days and activated for additional two days with LPS, for 14 days (**Figure 1A**). After culture, CD4$^+$ T cells were re-stimulated with the same allo-mDC used for priming and at day +1 (15 after the initial culture) were infected overnight (multiplicity of infection of 20) with a bidirectional LV-IL-10/ΔNGFR (**Figure S1A**). As control, a bidirectional LV co-encoding for GFP (in IL-10 position) and ΔNGFR, was used (**Figure S1B**). A mean of 55% of CD4$^+$ T cells were transduced, as confirmed by ΔNGFR expression (55±4%, and 49±3%, mean±SEM, n=14 for LV-IL-10 and LV-GFP, respectively; **Figure 1B and 1C**). As previously demonstrated, LV-IL-10 transduced CD4$^+$ T cells (CD4$^{IL\text{-}10}$) acquired the ability to release significantly higher levels of IL-10 compared to LV-GFP transduced CD4$^+$ T cells (CD4^{GFP}) in culture supernatant (4.49±0.64 ng/ml, and 0 ng/ml, mean±SEM, n=11, for LV-IL-10 and LV-GFP, respectively, $p<0.001$, **Figure 1D**). These data indicate that LV-IL-10 efficiently transduces primed CD4$^+$ T cells

during a secondary allo-specific stimulation.

CD4^{IL-10} cells are anergic allo-specific cells that secrete IL-10.

Allo-CD4^{IL-10} cells were beads-sorted according to ΔNGFR expression and expanded *in vitro* using feeder mixture, as described in Materials and Methods. In all experiments allo-CD4^{GFP} cells were included as controls. Upon stimulation with allo-mDC the levels of IFN-γ secreted by both allo-CD4^{IL-10} and allo-CD4^{GFP} cells were significantly higher compared to those produced upon a third party mDC stimulation (1.7±0.3 versus 0.7±0.1 ng/ml, mean±SEM, n=18 for CD4^{IL-10} cells, p≤0.05, and 1.8±0.5 versus 0.6±0.1 ng/ml, mean±SEM, n=18 for CD4^{GFP} cells, p≤0.05, **Figure 2A**), indicating that transduced T cells contained a high proportion of allo-specific CD4^{+} T cells. The enrichment of allo-specific CD4^{+} T cells was confirmed by the proliferative capacity of transduced cells: allo-CD4^{IL-10} and allo-CD4^{GFP} cells showed significantly higher proliferative response to the allo-mDC compared that to third party mDC (22.2±7.1 versus 3.2±1.3× 10^3 cpm, mean±SEM, n=19 and n=16 for CD4^{IL-10} cells stimulated with allo-mDC or third party mDC respectively, p≤0.001 and 56±11.3 versus 6.9±3.1×10^3 cpm, mean±SEM, n=19 and n=16 for CD4^{GFP} cells stimulated with allo-mDC or third party mDC respectively, p≤0.001, **Figure 2B**). Importantly, proliferation in response to allo-mDC of allo-CD4^{IL-10} cells was significantly lower compared to that of allo-CD4^{GFP} cells (22.2±7.1, versus 56±11.3×10^3 cpm, mean±SEM, n= 19, respectively, p≤0.001, **Figure 2B**), with a mean of reduction in CD4^{IL-10} cells proliferation of 54% compared to

$CD4^{GFP}$ cells. Overall, these data indicate that enforced expression of IL-10 confers to allo-specific $CD4^+$ T cells an anergic phenotype.

Although allo-$CD4^{IL\text{-}10}$ cells secreted spontaneously significantly higher level of IL-10 compared to allo-$CD4^{GFP}$ cells (0.32±0.06 versus 0.058±0.03 ng/ml, mean±SEM, n=8 for $CD4^{IL\text{-}10}$ cells and n=7 for $CD4^{GFP}$ cells, $p<0.05$, **Figure 2C**), upon stimulation with alllo-mDC the levels of IL-10 produced by allo-$CD4^{IL\text{-}10}$ cells increased, and resulted significantly higher that those produced by allo-$CD4^{GFP}$ cells (2.3±0.5 versus 0.5±0.2 ng/ml, mean±SEM, n=21 for $CD4^{IL\text{-}10}$ cells and n=18 for $CD4^{GFP}$ cells, $p\leq0.001$, **Figure 2C**). Conversely, stimulation of allo-$CD4^{IL\text{-}10}$ cells with a third party mDC did not significantly increase the levels of IL-10, which were not statistically different to those spontaneously released by $CD4^{IL10}$ cells, and resulted significantly lower compared to those released upon allo-specific stimulation ($p\leq0.0001$).

In addition, allo-$CD4^{IL\text{-}10}$ cells produced significantly lower levels of IL-4 compared to allo-$CD4^{GFP}$ cells both upon allo-mDC (0.4±0.2 versus 1.3±0.4 ng/ml of IL-4, mean±SEM, n=21, respectively, $p\leq0.01$, **Figure 2D**) and third party mDC stimulation (0.06±0.02 versus 0.3±0.1 ng/ml of IL-4, mean±SEM, n=21, respectively, $p\leq0.001$, **Figure 2D**).

Taken together these results showed that enforced IL-10 expression in allo-specific $CD4^+$ T cells promotes the conversion of effector T cells into allo-$CD4^{IL\text{-}10}$ cells that display an anergic phenotype and a Tr1-like cytokine profile.

Allo-$CD4^{IL\text{-}10}$ cells suppress allo-specific T-cell responses *in*

***vitro*.** We next analyzed the suppressive activity of allo-CD4$^{IL\text{-}10}$ cells *in vitro*. Autologous eFluor670-stained CD4^{+} responder T cells were stimulated with allo-mDC alone or in combination with allo-CD4$^{IL\text{-}10}$ or allo-CD4^{GFP} cells at 1:1 ratio and their proliferation (eFluor670 dilution) was determined by flow cytometry. Allo-CD4$^{IL\text{-}10}$ cells suppressed the proliferation of autologous T cells activated with allo-mDC at significantly higher levels compared to CD4^{GFP} cells (83.4±2.2% versus 55.3±1.5% of suppression for CD4$^{IL\text{-}10}$ cells and CD4^{GFP} cells mean±SEM, n=3, respectively, **Figure 3A-B**). Notably, the fact that at the end of the co-culture of autologous CD4^{+} T cells with allo-CD4^{GFP} cells the majority of cells were ΔNGFR^{+} (**Figure 3C**), indicates that proliferating allo-CD4^{GFP} cells consumed IL-2 in culture. It should be also noted that the kinetic of allo-specific response of autologous CD4^{+} T cells and allo-specific transduced CD4^{+} T cells are different, being a primary response and a secondary response, respectively. We therefore assessed the ability of allo-CD4$^{IL\text{-}10}$ cells to suppress *in vitro* primed autologous CD4^{+} T cells. As before, autologous primed eFluor670-stained CD4^{+} cells were stimulated with allo-mDC alone or in combination with allo-CD4$^{IL\text{-}10}$ or allo-CD4^{GFP} cells at 1:1 ratio and their proliferation (eFluor670 dilution) was determined by flow cytometry. Preliminary results showed that allo-CD4$^{IL\text{-}10}$, but not allo-CD4^{GFP,} cells suppressed the allo-specific proliferation of primed T cells (**Figure 4A**).

Overall, these data showed that allo-CD4$^{IL\text{-}10}$ cells were able to suppress primary and secondary responses in an allo-specific manner *in vitro*.

Discussion

In the present study we establish a protocol to generate allo-specific Tr1 (allo-CD4$^{IL\text{-}10}$) cells using a bidirectional LV encoding for human IL-10 and ΔNGFR, as clinical grade marker gene. Resulting allo-CD4$^{IL\text{-}10}$ cells, upon stimulation with allo-mDC, secrete higher levels of IL-10 and comparable amounts of IFN-γ than control allo-CD4^{GFP} cells, display an allo-specific anergic phenotype, and suppress primary and secondary allo-specific T-cell responses *in vitro*.

During the last decade, several protocols have been developed to generate *in vitro* human allo-specific Tr1 cells. Repetitive stimulations *via* TCR of naive CD4^{+} T cells in the presence of recombinant human IL-10 or IL-10–derived from tolerogenic DC-10 (Rosa Bacchetta et al., 2010; Gregori et al., 2010) favor the generation of a bulk population that is composed of allo-specific Tr1 cells and a significant fraction of contaminating non-Tr1 cells, which represent a major drawback for Tr1 clinical application. The recent identification of CD49b and LAG-3 as specific biomarkers for Tr1 cells, gives the possibility to purify the allo-specific Tr1 cells from these cultures [3]. However, the limitation to obtain a sufficient numbers of allo-specific Tr1 cells is still a hurdle. In this study we developed an efficient method to generate a large number of allo-specific Tr1-like cells using the recent developed LV-IL-10 platform ([31] and Locafaro and Andolfi, submitted) Allo-CD4$^{IL\text{-}10}$ cells acquire a Tr1 cytokine profile as upon allo-specific stimulation secrete IL-10 and IFN-γ, but not IL-

4. As expected and previously described ([31] and Locafaro and Andolfi, submitted), IL-10-transduced CD4$^+$ T cells spontaneously and constitutively secrete low levels of IL-10, which is also dependent on T-cell activation. Indeed, upon allo-specific, but not third party stimulation, allo-CD4$^{IL\text{-}10}$ cells released significantly higher levels of IL-10. Thus, TCR-mediated activation induced the glucose metabolism in lymphocytes [35] that enhance the IL-10 expression in allo-CD4$^{IL\text{-}10}$ cells. Moreover, as a consequence of IL-10 over-expression in these cells, allo-CD4$^{IL\text{-}10}$ cells also stably down-regulate IL-4 production, as was already proved for polyclonal CD4$^{IL\text{-}10}$ cells [31].

The high levels of IL-10 secreted by allo-CD4$^{IL\text{-}10}$ cells upon allo-specific stimulation resulted in an anergic/hypo-proliferative phenotype. This result support the notion that IL-10 over-expression into CD4$^+$ T cells, either polyclonal ([31] and Locafaro and Andolfi, submitted) or allo-specific, induces their conversion into Tr1-like cells. It is indeed generally approved that Tr1 cells upon activation with their cognate Ags secrete IL-10, which in an autocrine fashion limit their Ag-specific proliferation [5,11,30].

Similar to human Tr1 and polyclonal CD4$^{IL\text{-}10}$ cells, allo-CD4$^{IL\text{-}10}$ cells express and release high levels of Granzyme B (GzB) (data not shown). Since human Tr1 and polyclonal CD4$^{IL\text{-}10}$ specifically kill cells of myeloid origin ([4,31] and Locafaro and Andolfi, submitted), future investigations are warranted to verify whether also allo-CD4$^{IL\text{-}10}$ cells acquire the ability to kill myeloid cells by releasing GzB after target recognition.

Allo-CD4$^{IL\text{-}10}$ cells suppress the proliferation of autologous CD4$^+$ T

cells both during a primary or a secondary response to allo-mDC. Although highly preliminary, these data clearly suggest that over-expression of IL-10 into allo-specific $CD4^+$ T cells confers them the ability to specifically suppress allo-specific T-cell responses. Future studies are required to confirm these data by testing additional donors, and by lowering the ratio of LV-transduced cells to responder cells, in order to better define the allo-specific suppressive capacity of allo-$CD4^{IL\text{-}10}$ cells compared to mock transduced control cells. Moreover, several protocols have been implemented for ex vivo expansion of allo-specific Tregs through stimulation with mDC [36,37], PBMCs [38,39] or with CD40-ligand activated B cells [40,41]. Notably, *in vitro* [37,42,43] and *in vivo* in humanized mouse models [42,44] allo-specific Tregs have been shown to be superior to polyclonal Tregs in modulating allo-specific T-cell responses. Therefore, it would be interesting to compare the suppressive capacity of allo-$CD4^{IL\text{-}10}$ cells with polyclonal $CD4^{IL\text{-}10}$ cells in order to define the efficacy, the safety and to identify the more suitable cell product to be used in future clinical trials.

Overall, results from this study show the feasibility of generating *in vitro* human allo-specific Tr1 cells by LV-mediated IL-10 gene transfer for cellular immunotherapy approaches aimed at modulating Ag-specific immune responses. Human allo-$CD4^{IL\text{-}10}$ cells may help patients receiving an HLA mismatched or haploidentical HSCT, where allo-reactivity plays an important role in GvHD [45,46]. A clinical trial of adoptive therapy with ex vivo generated host-specific Tr1 cells to provide fast immune-

reconstitution and to prevent GvHD in leukemia patients who received a transplant of haploidentical HSCT demonstrated the feasibility and safety of this approach [47]. The advantage of using allo-CD4$^{IL\text{-}10}$ cells in this setting would be the homogeneity of the cell product to be infused, the easily *in vitro* generation and expansion that could be beneficial in case of multiple infusions. In addition, human allo-CD4$^{IL\text{-}10}$ cells could be used also in organ transplantation, such as liver or pancreatic islet transplant where the purity of the cell product is critically important.

References

1. Roncarolo, M. G., Gregori, S. & Bacchetta, R. Interleukin-10 in Health and Disease. **380,** 39–68 (2014).

2. Groux, H., Bigler, M., de Vries, J. E. & Roncarolo, M. G. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J. Exp. Med.* **184,** 19–29 (1996).

3. Gagliani, N. *et al.* Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat. Med.* **19,** 739–46 (2013).

4. Magnani, C. F. *et al.* Killing of myeloid APCs via HLA class I, CD2 and CD226 defines a novel mechanism of suppression by human Tr1 cells. *Eur. J. Immunol.* **41,** 1652–1662 (2011).

5. Groux, H. *et al.* A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389,** 737–742 (1997).

6. Zeller, J. C. *et al.* Induction of CD4+ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-beta. *J. Immunol.* **163,** 3684–3691 (1999).

7. Cottrez, F., Hurst, S. D., Coffman, R. L. & Groux, H. T regulatory cells 1 inhibit a Th2-specific response in vivo. *J. Immunol.* **165,** 4848–4853 (2000).

8. Barrat, F. J. *et al.* In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J. Exp. Med.* **195,** 603–616 (2002).

9. Foussat, A. *et al.* A Comparative Study between T Regulatory Type 1 and CD4 + CD25 + T Cells in the Control of Inflammation. *J. Immunol.* **171,** 5018–26 (2003).

10. Gagliani, N. *et al.* Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med* **19,** 739–746 (2013).

11. Bacchetta, R. *et al.* High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *J Exp Med* **179,** 493–502 (1994).

12. Serafini, G. *et al.* Type 1 regulatory T cells are associated with persistent split erythroid/lymphoid chimerism after allogeneic hematopoietic stem cell transplantation for thalassemia. *Haematologica* (2009). doi:10.3324/haematol.2008.003129

13. Weston, L. E., Geczy, A. F. & Briscoe, H. Production of IL-10 by alloreactive sibling donor cells and its influence on the

development of acute GVHD. *Bone Marrow Transplant.* **37,** 207–212 (2006).

14. VanBuskirk, A. M. *et al.* Human allograft acceptance is associated with immune regulation. *J. Clin. Invest.* **106,** 145–155 (2000).

15. Gianfrani, C. *et al.* Gliadin-specific type 1 regulatory T cells from the intestinal mucosa of treated celiac patients inhibit pathogenic T cells. *J. Immunol.* **177,** 4178–4186 (2006).

16. Akdis, M. *et al.* Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J. Exp. Med.* **199,** 1567–1575 (2004).

17. Akdis, M. T-cell tolerance to inhaled allergens: mechanisms and therapeutic approaches. *Expert Opin. Biol. Ther.* **8,** 769–777 (2008).

18. Arif, S. *et al.* Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. *J. Clin. Invest.* **113,** 451–463 (2004).

19. Veldman, C., Höhne, A., Dieckmann, D., Schuler, G. & Hertl, M. Type I regulatory T cells specific for desmoglein 3 are more frequently detected in healthy individuals than in patients with pemphigus vulgaris. *J. Immunol.* **172,** 6468–6475 (2004).

20. Yudoh, K., Matsuno, H., Nakazawa, F., Yonezawa, T. & Kimura, T. Reduced expression of the regulatory CD4+ T cell subset is related to Th1/Th2 balance and disease severity in rheumatoid arthritis. *Arthritis Rheum.* **43,** 617–627 (2000).

21. Glocker, E.-O. *et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. The New England journal of medicine* **361,** 2033–2045 (2009).

22. Glocker, E. O. *et al.* Infant colitis-its in the genes. *Lancet* **376,** 1272 (2010).

23. Battaglia, M., Gregori, S., Bacchetta, R. & Roncarolo, M. G. Tr1 cells: From discovery to their clinical application. *Seminars in Immunology* **18,** 120–127 (2006).

24. Roncarolo, M.-G. & Battaglia, M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat. Rev. Immunol.* **7,** 585–598 (2007).

25. Groux, H. *et al.* A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389,** 737–742 (1997).

26. Bacchetta, R. *et al.* Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy. *Haematologica* **95,** 2134–2143 (2010).

27. Levings, M. K. *et al.* Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. *Blood* **105,** 1162–1169 (2005).

28. Gregori, S. *et al.* Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* **116,** 935–944 (2010).

29. Petrelli, A. *et al.* Generation of Donor-Specific T Regulatory Type 1 Cells From Patients on Dialysis for Cell Therapy After Kidney Transplantation. **00,** 1–8 (2015).

30. Bejarano, M. T. *et al.* Interleukin 10 inhibits allogeneic proliferative and cytotoxic T cell responses generated in primary mixed lymphocyte cultures. *Int. Immunol.* **4,** 1389–1397 (1992).

31. Andolfi, G. *et al.* Enforced IL-10 Expression Confers Type 1 Regulatory T Cell (Tr1) Phenotype and Function to Human CD4+ T Cells. *Molecular Therapy* **20,** 1778–1790 (2012).

32. Gagliani, N. *et al.* Antigen-specific dependence of Tr1-cell therapy in preclinical models of islet transplant. *Diabetes* **59,** 433–439 (2010).

33. De Palma, M. & Naldini, L. Transduction of a gene expression cassette using advanced generation lentiviral vectors. *Methods Enzymol.* **346,** 514–529 (2002).

34. Levings, M. K. *et al.* IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *J. Immunol.* **166,** 5530–5539 (2001).

35. Frauwirth, K. a & Thompson, C. B. Regulation of T lymphocyte metabolism. *J Immunol* **172,** 4661–4665 (2004).

36. Yamazaki, S. *et al.* Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J. Exp. Med.* **198,** 235–247 (2003).

37. Litjens, N. H. R. *et al.* Allogeneic Mature Human Dendritic Cells Generate Superior Alloreactive Regulatory T Cells in the Presence of IL-15. *J. Immunol.* (2015). doi:10.4049/jimmunol.1402827

38. Peters, J. H., Hilbrands, L. B., Koenen, H. J. P. M. & Joosten, I. Ex vivo generation of human alloantigen-specific regulatory T cells from CD4posCD25high T cells for immunotherapy. *PLoS One* **3,** 1–13 (2008).

39. Veerapathran, A. *et al.* Human regulatory T cells against minor histocompatibility antigens: ex vivo expansion for prevention of graft-versus-host disease. *Blood* **122,** 2251–2261 (2013).

40. Putnam, A. L. *et al.* Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am. J. Transplant.* **13,** 3010–3020 (2013).

41. Chen, L. C., Delgado, J. C., Jensen, P. E. & Chen, X. Direct expansion of human allospecific FoxP3+CD4+ regulatory T cells with allogeneic B cells for therapeutic application. *J. Immunol.* **183,** 4094–4102 (2009).

42. Sagoo, P. *et al.* Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells. *Sci. Transl. Med.* **3,** 83ra42 (2011).

43. Peters, J. H., Hilbrands, L. B., Koenen, H. J. P. M. & Joosten, I. Ex vivo generation of human alloantigen-specific regulatory T cells from CD4posCD25high T cells for immunotherapy. *PLoS One* **3,** (2008).

44. Putnam, a. L. *et al.* Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am. J. Transplant.* **13,** 3010–3020 (2013).

45. Copelan, E. A. Hematopoietic Stem-Cell Transplantation. 1813–1826 (2015).

46. Reisner, Y., Aversa, F. & Martelli, M. F. Haploidentical hematopoietic stem cell transplantation: state of art. *Bone Marrow Transplant.* **50,** S1–S5 (2015).

47. Bacchetta, R. *et al.* Immunological outcome in haploidentical-HSC transplanted patients treated with IL-10-anergized donor T Cells. *Front. Immunol.* **5,** (2014).

Figure legends

Figure 1. **Enriched allo-specific CD4$^+$ T cells are efficiently transduced with LV-IL-10. A.** Protocol to generate and characterize allo-CD4$^{IL\text{-}10}$ cells. Enriched allo-specific CD4$^+$ T cells were transduced with LV-IL-10 or LV-GFP during a secondary stimulation with the same allo-mDC used for priming (see also Material and Methods). **B-C.** Human CD4$^+$ T cells were stimulated with allo-mDC for 14 days. After culture, CD4$^+$ T cells were re-stimulated with allo-mDC 24 hours before transduction with LV-IL-10 or LV-GFP at MOI of 20. After 6 days, the transduced cells were analyzed by FACS. The frequency of ΔNGR$^+$ cells are indicated. One representative donor of at least 14 analyzed is presented (**B**). Collective results from individual donors are presented. Each dot represent a single donor tested, lines indicate the mean±SEM of the percentage of transduction (**C**). **D**. Leves of IL-10 in culture supernatants were assessed by ELISA 6 days following infection (day 21 after the initiation of culture). *** p≤0.001, Wilcoxon matched-pairs signed rank test.

Figure 2. CD4$^{IL\text{-}10}$ cells are allo-specific, anergic cells with a Tr1-like cytokine profile. A. Allo-CD4$^{IL\text{-}10}$ or allo-CD4^{GFP} cells were stimulated with the same allo-mDC used for priming or a third party mDC (ratio 10:1), and IFN-γ production was determined by ELISA 48 hours after activation. Bars represent the mean value with SEM of n=17 donors tested in 5 independent experiments. **B.** The proliferative responses were evaluated 3 or 5

days after culture of allo-CD4$^{IL\text{-}10}$ and allo-CD4^{GFP} cells with allogeneic (allo-mDC) or third party mDC, respectively, by ^{3}HThy incorporation for additional 16 hours. Bars represent mean value with SEM of n=19 for allogeneic stimulation and n=16 for third party stimulation tested in more than 5 independent experiments. All samples were tested in duplicate-triplicate. **C**. Allo-CD4$^{IL\text{-}10}$ or allo-CD4^{GFP} cells were left inactivated or stimulated with the same allo-mDC used for priming or a third party mDC (ratio 10:1), and IL-10 production was determined by ELISA 48 hours after activation. Bars represent the mean value with SEM of n= 21 donors tested in 5 independent experiments. All samples were tested in duplicate-triplicate. **D**. Allo-CD4$^{IL\text{-}10}$ or allo-CD4^{GFP} cells were stimulated with the same allo-mDC used for priming or a third party mDC (ratio 10:1), and IL-4 production was determined by ELISA 48 hours after activation. Bars represent the mean value with SEM of n=21 donors tested in 5 independent experiments. All samples were tested in duplicate-triplicate. ** $p<0.01$, *** $p<0.001$, Wilcoxon matched-pairs signed rank test.

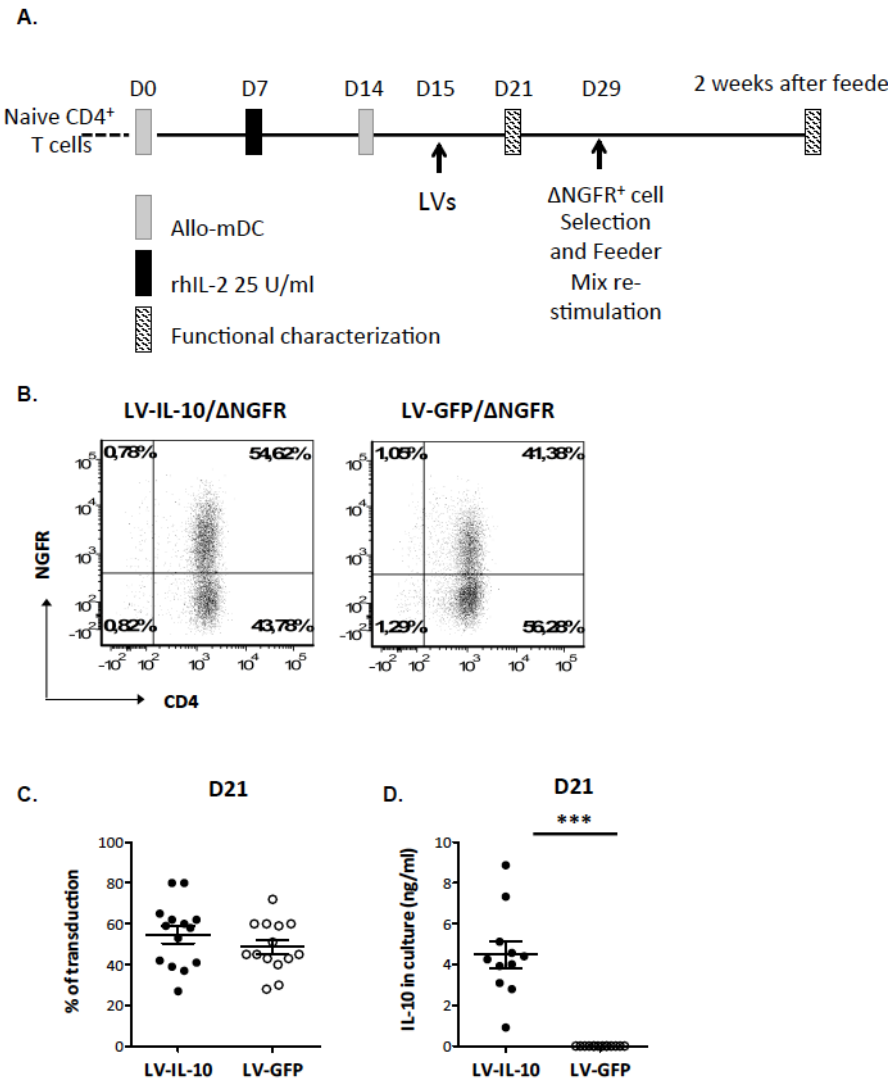
Figure 3. **Allo-CD4^{IL10} cells suppress allo-specific T-cell responses *in vitro*.** Allo-CD4$^{IL\text{-}10}$ or allo-CD4^{GFP} cells were tested for their ability to suppress proliferation of autologous CD4^{+} T cells activated with allo-mDC. Autologous CD4^{+} T cells (Responders) were labeled with eFluor dye and stimulated with allo-mDC (ratio 10:1) alone or in the presence of allo-CD4$^{IL\text{-}10}$ or allo-CD4^{GFP} cells at 1:1 ratio. After 4 days of culture the percentage of proliferating cells was determined by eFluor dilution. One

representative donor out of three tested is shown (**A**); the percentage of proliferating responder cells is reported. Collective results from individual donors are presented. Percentages of suppression mediated by allo-CD4$^{IL\text{-}10}$ or allo-CD4^{GFP} cells are shown. Each dot represent a single donor tested, lines indicate the mean±SEM of the percentage of suppression (**B**). The percentage of Responder cells (CD4^{+}ΔNGFR^{-}) among the CD4^{+} T cells during the suppression assay of one representative donor out of three tested is shown (**C**). ** p<0.01, Wilcoxon matched-pairs signed rank test.

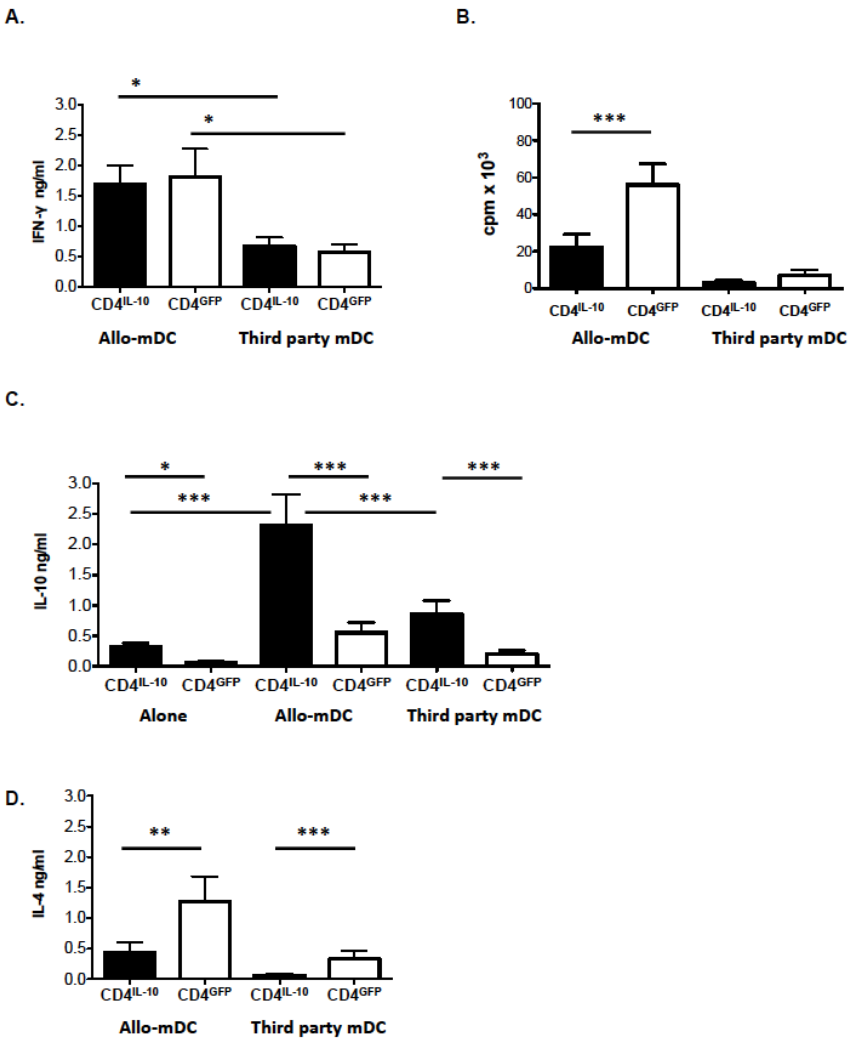
Figure 4. **Allo-CD4$^{IL\text{-}10}$ cells suppress secondary allo-specific T-cell responses *in vitro.*** Allo-CD4$^{IL\text{-}10}$ or allo-CD4^{GFP} cells were tested for their ability to suppress proliferation of autologous CD4^{+} T cells primed with allo-mDC. Autologous primed CD4^{+} T cells (Responders) were labeled with eFluor dye and stimulated with allo-mDC (ratio 10:1) alone or in the presence of allo-CD4$^{IL\text{-}10}$ or allo-CD4^{GFP} cells at 1:1 ratio. After 3 days of culture the percentage of proliferating cells was determined by eFluor dye dilution. One donor tested is shown; the percentage of proliferating responder cells is reported in the text box.

Figure S1. **Scheme of the LV-IL-10/ΔNGFR and LV-GFP/ΔNGFR vectors.** The presence of the bidirectional promoter (mCMV/PGK) allows the co-regulated expression of ΔNGFR and human IL-10 (**A**) or GFP (**B**) genes. Ψ, encapsidation signal including the 5' portion of GAG gene (GA); RRE, Rev-responsive element; cPPT,

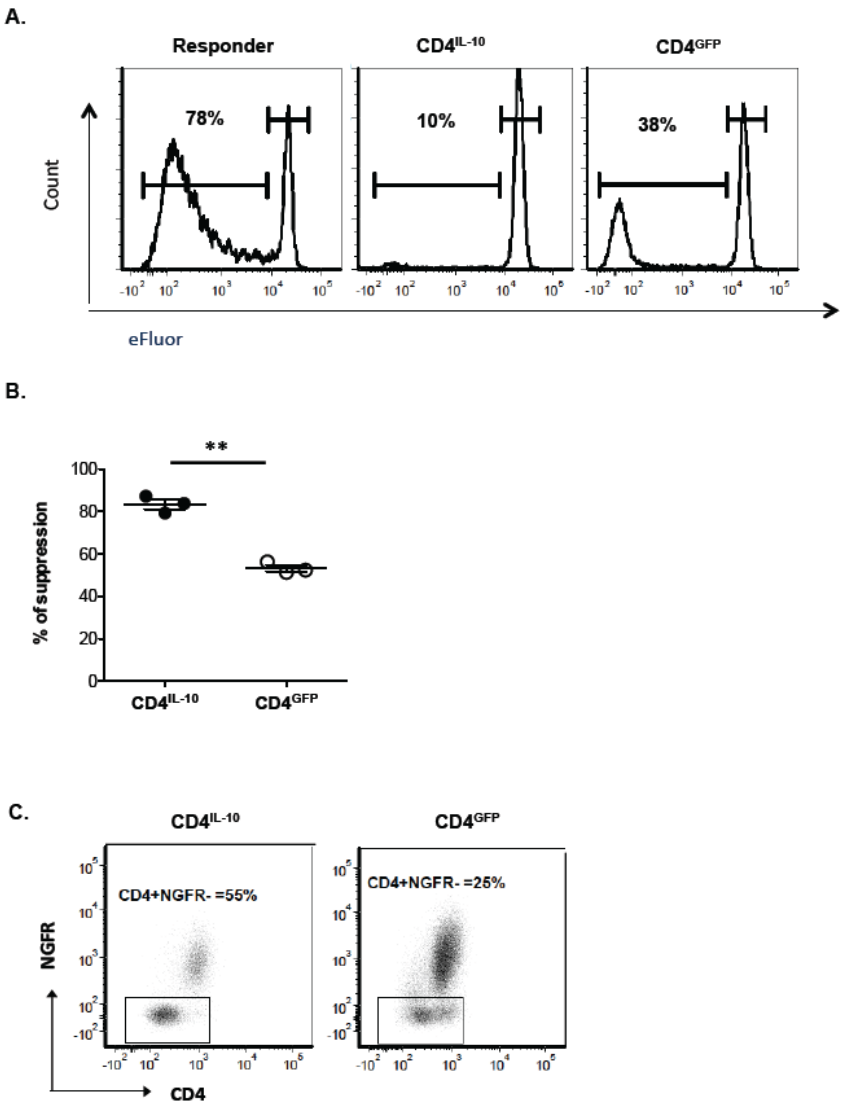
central poly-purine tract; polyA, poly-adenylation site from the Simian Virus 40; CTE, constitutive transport element; WPRE, woodchuck hepatitis virus post-transcription regulatory element.



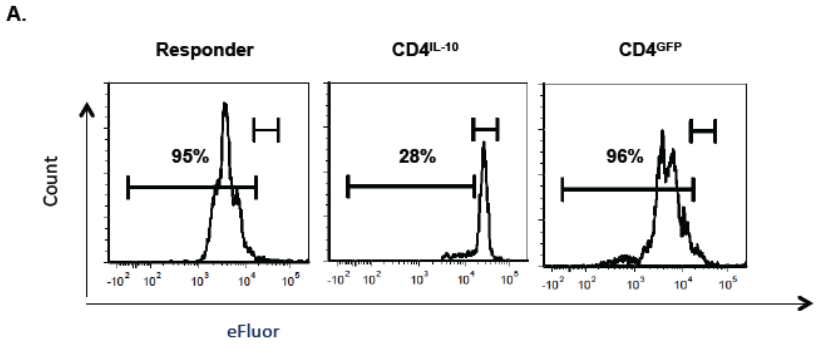
Locafaro G. et al., Figure 1



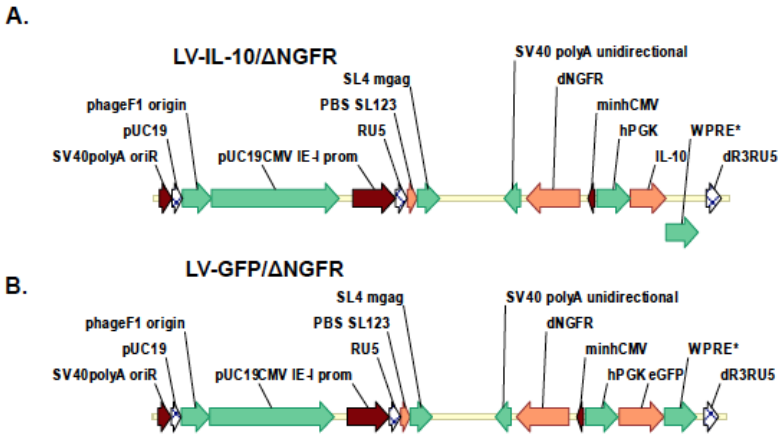
Locafaro G. et al., Figure 2



Locafaro G. et al., Figure 3



Locafaro G. et al., Figure 4



Locafaro G. et al., Figure S1

Chapter 4

Conclusions and Future perspectives

Growing evidences suggest that cellular adoptive immunotherapy with T regulatory cells (Tregs) is becoming an attractive and challenging approach for the treatment of patients with T cell-mediated diseases, such as autoimmunity, chronic inflammatory diseases, and other immune mediated pathologies including organ and hematopoietic stem cell transplantation. The advantages of the adoptive transfer of Tregs over conventional treatments are: i) the promise of avoiding many of the toxicities induced by immune-suppressive regimens, since it has the potential to modulate T cell responses in an antigen-specific manner, and ii) the potential to promote a 'physiological' long-lasting tolerance *in vivo*.

For this reason, during the last decade much effort has been dedicated to establish suitable methods for Tregs and Tr1 cell generation *in vitro* for Treg-cell based therapy. Our group focused on Tr1 cells, thus we validated a GMP protocol to generate Tr1 cells using recombinant IL-10 or tolerogenic DC-10 [1–3] and in proof-of-principle clinical trials in allogeneic hematopoietic stem cell transplantation (allo-HSCT) we demonstrated the safety of a cell therapy with these polarized Tr1 cells [4]. Despite these results, the IL-10-anergized T cells are a mixed population of cells containing both Tr1 and non-Tr1 cells, which can be detrimental is other clinical settings in which a pure population of antigen-specific Tr1 cells are envisaged.

The LV-mediated IL-10 gene transfer has been proved to be an innovative tool to convert human $CD4^+$ T cells in a homogeneous population of Tr1-like cells, named $CD4^{IL\text{-}10}$ cells, with regulatory

phenotype and functions, including the *in vitro* killing of myeloid cells and suppression of xeno Graft-*versus*-Host Disease (xeno-GvHD) [5]. In this thesis a deeper characterization of the mechanism underlying the cytotoxic capacity of polyclonal $CD4^{IL\text{-}10}$ cells has been conducted and has led us to: i) identify biomarkers of efficacy for their anti-leukemic effect *in vitro;* ii) demonstrate their Graft-*versus*-Leukemia (GvL) activity *in vivo*; ii) discover that $CD4^{IL\text{-}10}$ cells inhibit GvHD without affecting the GvL effect mediated by allogeneic cells. These results represent an important step forward the clinical application of polyclonal $CD4^{IL\text{-}10}$ cells as cellular therapy for patients affected by myeloid malignancies undergoing allo-HSCT. However, additional studies are warranted to better elucidate the mechanisms responsible for the $CD4^{IL\text{-}10}$-mediated modulation of GvHD but not of GvL mediated by allogeneic cells.

The demonstration of the feasibility to generate *in vitro* human allo-specific Tr1 cells by applying the LV-IL-10 platform will increase the application of Tr1 cells as cell therapy aimed at modulating allo-specific immune responses not only after allo-HSCT but also after organ transplantation. In the latter setting a pure population of allo-specific Tr1 cells are critically important for avoiding the potential detrimental effects mediated by the contaminating allo-specific but non-Tr1 cells present in the bulk populations generated with the current protocols. Moreover, these results imply new clinical applications for Tr1 cells, suggesting the possibility to convert auto-Ag-specific $CD4^+$ T cells isolated from patients suffering from autoimmune diseases into

Ag-specific $CD4^{IL\text{-}10}$ cells by applying the LV-IL-10 platform with the aim of inducing/restoring tolerance.

References

1. Bacchetta, R. *et al.* Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy. *Haematologica* **95,** 2134–2143 (2010).

2. Gregori, S. *et al.* Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* **116,** 935–944 (2010).

3. Petrelli, A. *et al.* Generation of Donor-Specific T Regulatory Type 1 Cells From Patients on Dialysis for Cell Therapy After Kidney Transplantation. **00,** 1–8 (2015).

4. Bacchetta, R. *et al.* Immunological outcome in haploidentical-HSC transplanted patients treated with IL-10-anergized donor T Cells. *Front. Immunol.* **5,** (2014).

5. Andolfi, G. *et al.* Enforced IL-10 Expression Confers Type 1 Regulatory T Cell (Tr1) Phenotype and Function to Human CD4+ T Cells. *Molecular Therapy* **20,** 1778–1790 (2012).

Chapter 5

Appendix

Manuscripts not included in the thesis

Enforced IL-10 expression confers type 1 regulatory T cell (Tr1) phenotype and function to human CD4(+) T cells.

Andolfi G, Fousteri G, Rossetti M, Magnani CF, Jofra T, **Locafaro G**, Bondanza A, Gregori S, Roncarolo MG.

Molecular Therapy 20(9): 1778-90, 2012

HLA-G expression on blasts and tolerogenic cells in patients affected by acute myeloid leukemia.

Locafaro G, Amodio G, Tomasoni D, Tresoldi C, Ciceri F, Gregori S.

J Immunol Res. Volume 2014, Article ID 636292, 10 pages, 2014