

TGF- β 1 activating antibodies to treat graft-versus-host disease

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ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
aGVHD	Acute graft-versus-host disease
AICD	Activation-induced cell death
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
APC	Antigen presenting cell
BAFF	B cell activating factor
BCR	B cell receptor
Bim	Bcl-2 interacting mediator of cell death
BM	Bone marrow
BTK	Bruton tyrosine kinase
CDC	Complement-depend cytotoxicity
cGVHD	Chronic graft-versus-host disease
CNI	Calcineurin inhibitor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DTR	Diphtheria toxin receptor
ECM	Extracellular matrix
EMA	European Medicines Agency
FDA	Food and Drug Administration
GARP	Glycoprotein A repetition predominant
GC	Glucocorticoids
GI	Gastro-intestinal
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
Haplo	HLA haplo-identical
HLA	Human leukocyte antigen
HPE	Homeostatic peripheral expansion
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IFN-γ	Interferon- γ
IDO	Indoleamine 2,3 dioxygenase
IL	Interleukin

ITK	IL-2 inducible T cell kinase
MMF	Mycophenolate mofetil
MTX	Methotrexate
LAP	Latency-associated peptide
LRRC	Leucine-rich repeat-containing protein
LTBP	Latent transforming growth factor beta binding protein
mAb	Monoclonal antibody
MAC	Myeloablative conditioning
MHC	Major histocompatibility complex
MSD	Matched sibling donor
mTOR	Mammalian target of rapamycin
MUD	Matched unrelated donor
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor-kappa B
NIH	National Institutes of Health
NMA	Non-myeloablative conditioning
NO	Nitric oxid
ORR	Overall response rate
OS	Overall survival
pDC	Plasmacytoid dendritic cell
PBSC	Peripheral blood stem cell
PRR	Pattern recognition receptor
PTCy	Post-transplant cyclophosphamide
pTreg	Peripherally-induced regulatory T cell
RA	Retinoic acid
RIC	Reduced intensity conditioning
SRE	SMAD-responsive elements
TCR	T cell receptor
TEC	Thymic epithelial cell
TGF-β1	Transforming growth factor beta 1
Treg	Regulatory T cell
TSP-1	Thrombospondin-1
tTreg	Thymic-derived regulatory T cell
UCB	Umbilical cord blood
xGVHD	Xenogeneic graft-versus-host disease

ABSTRACT

Transforming growth factor beta 1 (TGF- β 1) is a potent immunosuppressive cytokine, playing critical roles in the regulation of immune responses. Virtually all cell types produce TGF- β 1 as an inactive complex, composed of mature TGF- β 1 non-covalently bound to the latency-associated peptide (LAP). Binding to the TGF- β receptor requires release of mature TGF- β 1 from the LAP, a process known as “TGF- β 1 activation”. Only a few cell types can activate TGF- β 1 via cell type-specific mechanisms. Our laboratory previously showed that TCR-stimulated regulatory T cells (Tregs) activate TGF- β 1 via transmembrane proteins GARP and integrin α v β 8. We developed monoclonal antibodies (mAbs) against the GARP:TGF- β 1 complex that block TGF- β 1 activation and immunosuppression by Tregs. These blocking mAbs enhanced anti-tumor T cell responses and displayed therapeutic efficacy when used as a monotherapy in murine models of myeloproliferative neoplasms, and in combination with anti-PD1 therapy in solid tumors.

Here, we derived mAbs that activate rather than inhibit TGF- β 1 by inducing its release from the LAP, with the objective to address diseases characterized by deleterious T cell activity insufficiently controlled by Tregs. Such diseases include autoimmune and alloimmune disorders. Biochemical and structural analyses by single-particle cryo-EM revealed that TGF- β 1 activating mAbs required bivalent binding close to the LAP dimerization interface and crosslinking of two membrane-bound GARP:TGF- β 1 complexes on the same cell or across different cells. Administration of TGF- β 1 activating mAbs to mice with acute graft-versus-host-disease (aGVHD) reduced disease severity and increased survival. The therapeutic effect in aGVHD was associated with increased TGF- β 1 activity and Treg numbers in the spleen 9 days after initiation of the treatment, and was lost in the absence of Tregs. TGF- β 1 activating mAbs also exerted therapeutic activity in a mouse model of chronic GVHD (cGVHD) characterized by severe cutaneous damage. Expression of TGF- β 1 target genes tended to increase in thymus and spleen after 6 weeks of treatment in cGVHD, indicating that activating mAbs also enhanced TGF- β 1 signaling in lymphoid organs at this later time-point. We observed that the therapeutic effect of TGF- β 1 activating mAbs in cGVHD was associated with enhanced thymic function, manifested as increased development of single positive T cells, including thymic Tregs (tTregs). The therapeutic effect in cGVHD was also associated after 6 weeks of treatment with decreased proportions of activated cells within CD8⁺ T lymphocytes in spleen and blood, and reduced expression of genes associated with type 1 adaptive immune responses in several organs (skin, kidney, lung and liver).

Collectively, our findings indicate that TGF- β 1 activating mAbs increase TGF- β 1 activity in lymphoid organs shortly after treatment initiation, appear to improve thymic function and reduce adaptive immune responses after longer term treatment. We thus suggest that TGF- β 1 activating mAbs may serve to treat acute or chronic GVHD in patients.

INTRODUCTION

1 TGF- β 1 biology

The transforming growth factor beta 1 (TGF- β 1) belongs to the “TGF- β super-family”, a group of proteins essential to maintain organism integrity (1). Three TGF- β isoforms (-1, -2 and -3) are encoded by distinct genes, signal through the same receptor complex, but exert different functions in the organism. TGF- β 1 plays key roles in tissue homeostasis and immunity after birth. Highlighting those critical functions, mice deficient in TGF- β 1 (*Tgfb1*^{-/-}) either die in utero due to developmental defects or are born alive but succumb within 3-4 weeks after birth to a severe, multi-organ inflammatory disease (2, 3). TGF- β 1 activity is tightly regulated. Although produced by virtually all cell types in an inactive form called latent TGF- β 1, only a few cell subsets can release the mature TGF- β 1 cytokine, which is biologically active, in response to specific stimuli.

1.1 Production, structure, and activation of latent TGF- β 1

1.1.1 Production of TGF- β 1

TGF- β 1 is synthesized as a pre-pro-peptide composed of a signal peptide, the latency-associated peptide (LAP) and the mature TGF- β 1 cytokine (**Figure 1**) (4). After cleavage of the signal peptide, the pro-peptide dimerizes via three intrachain disulfide bonds: two stabilize the LAP homodimer, and another links the two monomers of mature TGF- β 1. In the Golgi, a furin protease cleaves the pro-TGF- β 1, separating the LAP from the mature cytokine. Nevertheless, LAP remains non-covalently associated to mature TGF- β 1, forming an inactive complex named “latent TGF- β 1”. In this stable conformation, LAP sterically masks the receptor-binding sites of mature TGF- β 1, thereby preventing its biological activity (5, 6). The release of mature TGF- β 1 from the latent complex is called “TGF- β 1 activation”.

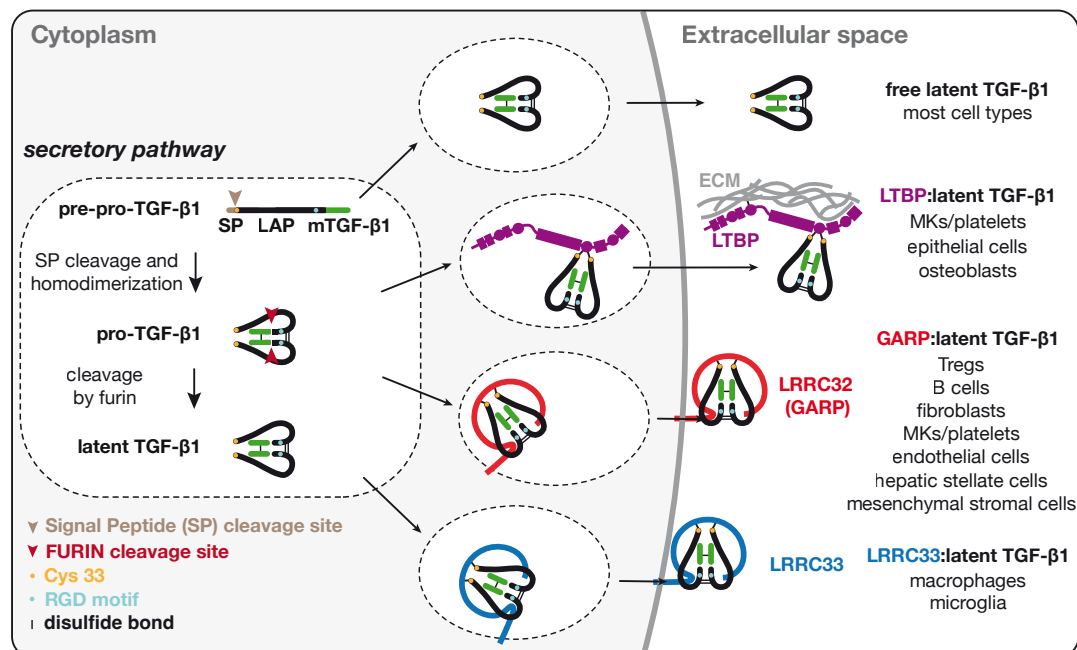


Figure 1: Schematic representation of TGF- β 1 processing and trafficking

Figure from F. Lambert's thesis (7). SP, signal peptide; LAP, latency-associated peptide; mTGF- β 1, mature TGF- β 1; MK, megakaryocyte.

1.1.2 Structure of TGF- β 1

The structure of latent TGF- β 1 provides important insights into the mechanisms of TGF- β 1 activation (5). The mature TGF- β 1 homodimer exhibits a butterfly-like shape, with each monomer resembling a rigid palm hand bearing 3 “fingers” that constitute the main regions involved in TGF- β 1 receptor binding (**Figure 2**) (8, 9). Each LAP monomer is composed of two major domains: an arm and a straitjacket domain. The arm domain consists of a rigid structure formed by two antiparallel four-stranded β -sheets and includes the RGD(LXX(L/L)) motif on the shoulder region, which mediates binding to specific integrins involved in TGF- β 1 activation. The straitjacket domain, formed by the α 1-helix and the latency lasso (i.e. a flexible loop connecting the α 1-helix to the arm domain), prevents interaction of mature TGF- β 1 with receptor binding sites. The arm domains are covalently bound at the upper neck of the homodimer, forming a strong dimerization interface essential for TGF- β 1 latency. Mutations in amino acids within this interface cause the Camurati-Engelmann disease, characterized by long bone and skull malformations resulting from an abnormal TGF- β 1 activity (10).

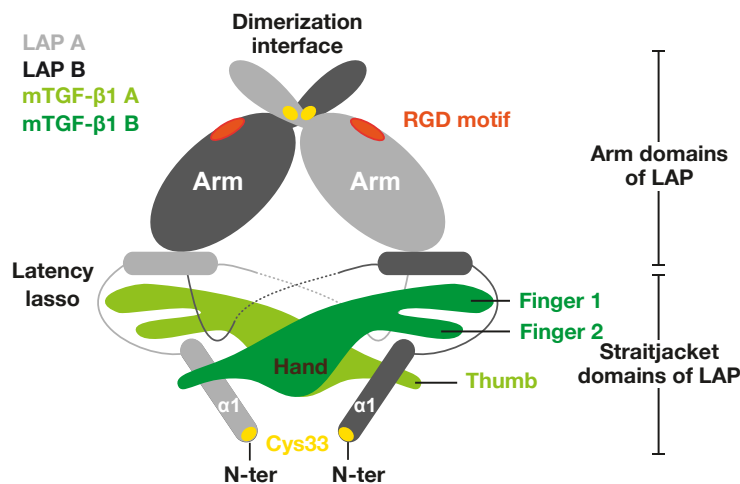


Figure 2. Structure of latent TGF- β 1

Schematic representation of the porcine latent TGF- β 1 structure. Figure adapted by M. Jamez.

1.1.3 Reservoirs of latent TGF- β 1

Latent TGF- β 1 can be secreted as a soluble complex or covalently bound by tethering proteins on the cell surface or the extracellular matrix (ECM) (**Figure 1**). LTBP (Latent TGF- β Binding Protein) are large glycoproteins secreted by megakaryocytes, platelets, epithelial cells, and osteoblasts that anchor latent TGF- β 1 to the ECM (11-14). The critical role of LTBP in TGF- β 1 activation *in vivo* is revealed in mice deficient in LTBP-1, -3 or -4, which develop cardiac, skeletal or vascular malformations associated with reduced TGF- β 1 signaling (11). Leucine-rich repeat-containing proteins 32 and 33 (LRRC32 and LRRC33) are transmembrane proteins sharing 34% of sequence homology and a horseshoe-shape structure in their extracellular domain. LRRC33:(latent)TGF- β 1 complexes are present on the surface of macrophages and microglial cells (15), while LRRC32, also named glycoprotein A repetition predominant (GARP), displays latent TGF- β 1 on the surface of several cell types including TCR-stimulated regulatory T cells (Tregs) (16, 17), BCR-stimulated B cells (18), megakaryocytes and platelets (17, 19, 20), endothelial cells (19, 21, 22), fibroblasts (21, 23), mesenchymal stromal cells (24) and hepatic stellate cells (25, 26).

1.1.4 Activation of TGF- β 1

Activation of TGF- β 1 from the latent complex occurs in response to various stimuli through distinct mechanisms, including proteolytic cleavage by metalloproteinases, interface destabilization by thrombospondin-1 (TSP-1) (27, 28), or profound conformational changes induced by RGD-binding integrins. These specific integrins (α v β 1, α v β 6, α v β 8) are heterodimeric transmembrane proteins which bind with high affinity to the Arg-Gly-Asp (RGD) motif located in the “arm” of the LAP (29). The functional relevance of integrins is demonstrated in mice carrying an RGE mutation, which phenocopy *Tgfb1*^{-/-} mice and die from an early-onset inflammatory disease (30). While integrin α v β 1, expressed by activated fibroblasts, mediates TGF- β 1 activation specifically in fibrosis, α v β 6 and α v β 8 also participates in immune homeostasis. Integrin α v β 6 is expressed on epithelial cells upon tissue damage or inflammation, whereas α v β 8 is exhibited by Tregs (31), myeloid cells (32), epithelial cells (33) and fibroblasts (34). Structural analyses of α v β 6 binding to latent TGF- β 1 suggest that the integrin β subunit transmits mechanical tension from the actin cytoskeleton to the LAP anchored to the cell membrane or the ECM via covalent linkage to LRRC32, LRRC33 or LTBP, generating opposing forces that can unfold the LAP and release mature TGF- β 1. In contrast to α v β 6, the mechanism by which α v β 8 mediates TGF- β 1 activation remains under debate.

1.1.5 Signaling by TGF- β 1

Mature TGF- β 1 binds to a hetero-tetrameric receptor composed of 2 pairs of TGF β R1 and TGF β R2 proteins. Upon ligand binding, the intracellular serine/threonine kinase domain of TGF β R2 becomes phosphorylated and subsequently recruits TGF β R1. It triggers the engagement of the Smad signaling cascade by phosphorylating Smad2 and Smad3, that will bind Smad4. Trimeric complexes migrate into the nucleus to exert as a transcription factor, binding to Smad-responsive elements (SREs) in target gene promoters. Among the genes regulated by the Smad pathway, *SMAD7* is induced as part of a negative feedback loop that limits TGF- β 1 signaling. In addition to this canonical signaling pathway, TGF- β 1 is also able to deliver signals via MAPK, PI3K or Rho GTPase pathways (35).

1.2 Effects of TGF- β 1 signals on immune cells

The immune system constitutes an integrated defense network that detects, responds to, and eliminates foreign antigens to protect the organism against infectious pathogens and malignant cells, while maintaining self-integrity. It involves specialized circulating and resident immune cells coordinated through cytokines. The innate immune system represents the first line of defense, providing a rapid and robust, yet nonspecific, response to any pathogen. In contrast, adaptive immunity offers highly specific and long-lasting protection through antigen receptor-mediated recognition. TGF- β 1 acts as a key regulator of activation, proliferation, differentiation and function of virtually all immune cell types that is essential to preserve immune homeostasis (**Figure 3**). Although TGF- β 1 is also involved in wound healing and fibrotic processes, the following section will only address its roles within the immune system.

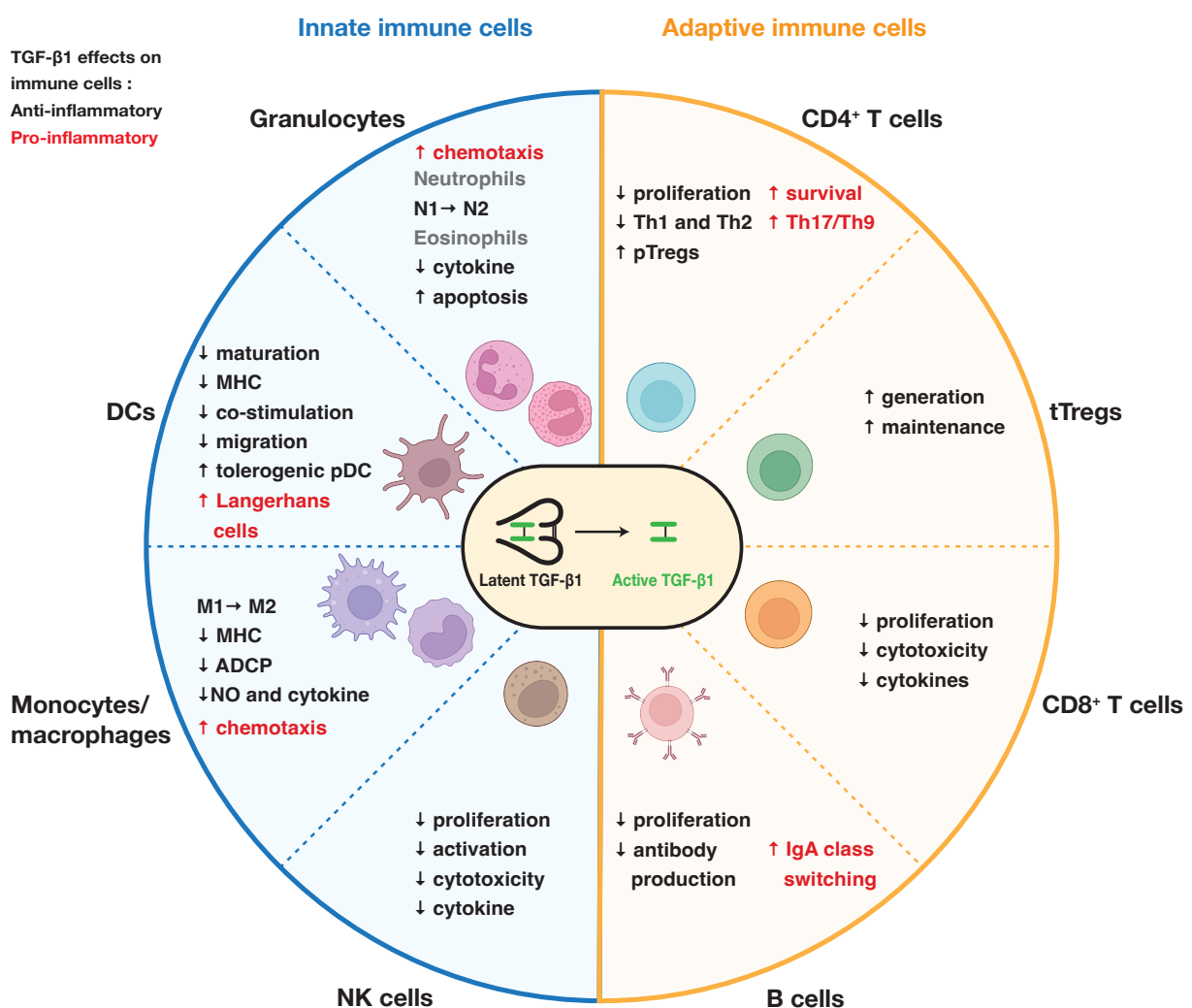


Figure 3. Effects of TGF- β 1 signals on immune cells

TGF- β 1 regulates activation, proliferation, differentiation and function of virtually all innate and adaptive immune cell types to preserve homeostasis. For each cell type, anti-inflammatory effects of TGF- β 1 are highlighted in black, pro-inflammatory effects in red.

1.2.1 Innate immune cells

1.2.1.1 Granulocytes

The effects of TGF- β 1 on granulocytes are poorly studied. TGF- β 1 appears to inhibit cytokine production in human eosinophils and to increase their apoptosis (36). In neutrophils, TGF- β 1 promotes a phenotypic switch from pro-inflammatory N1 to anti-inflammatory N2 subsets (37). *In vitro* and *in vivo* experiments showed that TGF- β 1 promotes the chemotaxis of both eosinophils and neutrophils (38, 39).

1.2.1.2 Dendritic cells

In vitro experiments showed that TGF- β 1 decreases the ability of dendritic cells (DCs) to activate T cells by downregulating expression of chemokine receptors, major histocompatibility complex-II (MHC-II) and co-stimulatory molecules such as CD80 and CD86 (37). Moreover, TGF- β 1 can induce a tolerogenic phenotype in plasmacytoid DCs (pDCs) by inducing expression of the indoleamine 2,3 dioxygenase (IDO) enzyme. However, these effects have not been consistently confirmed *in vivo*. Mice lacking TGF- β 1 signaling in DCs (*CD11c^{Cre}Tgfb β 2^{fl/fl}*) do not exhibit reduced expression of MHC-II or co-stimulatory molecules (40). Instead, mutant mice display alteration in Treg homeostasis and develop a fatal systemic inflammatory disease, suggesting a functional link between TGF- β 1 signaling in myeloid cells and Tregs. Moreover, the absence of Langerhans cells in *Tgfb β 1^{-/-}* mice suggest that TGF- β 1 is essential for the differentiation of this specialized DC subset residing in epithelial tissues (41).

1.2.1.3 Monocytes/macrophages

TGF- β 1 promotes monocyte migration, particularly toward inflammatory sites, but decreases MHC expression and favors their differentiation into non-inflammatory (M2) macrophages (42). TGF- β 1 also reduces the activity of Fc receptors of macrophages, and therefore antibody-dependent phagocytosis (ADPC). In addition, TGF- β 1 downregulates the production of cytotoxic molecules such as TNF- α and nitric oxide (NO), indicating a global reduction in macrophage cytotoxicity (43-45).

1.2.1.4 NK cells

TGF- β 1 acts as a negative regulator of NK cell activity. In *CD11c^{dn}Tgfb β 2* mice, loss of TGF- β signaling in innate immune cells results in the expansion of mature NK cells and increase resistance to viral infection (46). TGF- β 1 downregulates cell-surface activating receptors such as NKG2D and NKp30, thereby reducing NK cell activation (35). Furthermore, TGF- β 1 suppresses cytolytic functions and IFN- γ production (37, 47).

1.2.2 Adaptive immune cells

1.2.2.1 T cells

TGF- β 1 prevents excessive T cell proliferation and activation. The central role of TGF- β 1 in T cell regulation is highlighted by several transgenic mouse models. The absence of TGF- β 1 signaling specifically in T cells recapitulates the fatal inflammatory disease exhibit by *Tgfb1*^{-/-} mice, as shown by studies involving mice expressing a dominant negative TGF β R2 (*CD4^{dnTgfb2}*) (48, 49) or a selective deletion of TGF β R2 in T cells (*CD4^{Cre}xTgfb2^{fl/fl}*) (50, 51). TGF- β 1-deficient SCID mice (i.e. lacking functional T and B cells) survive to adulthood without inflammation, in contrast to *Tgfb1*^{-/-} mice (52). Furthermore, CD8⁺ T cell depletion in *Tgfb1*^{-/-} mice attenuates inflammation (53). Collectively, these findings establish TGF- β 1 as a crucial regulator of T cell homeostasis, including development and differentiation.

• Thymic development

The thymus supports the development of T lymphocytes derived from hematopoietic stem cells, enabling them to recognize foreign antigens presented by self-MHC molecules while remaining tolerant to self-antigens, thereby preserving self-tissue homeostasis. During thymic development, T cell progenitors express a T cell receptor (TCR) generated by random recombination of *TCRA* and *TCRB* gene segments, and both CD4 and CD8 co-receptors (54). These double-positive (CD4⁺CD8⁺; DP) progenitors undergo sequential positive and negative selection. Positive selection preserves DP thymocytes whose TCR binds a self MHC molecule with sufficient affinity. Approximately 95% of developing T cells die by neglect at this stage. Surviving DP thymocytes retain only one of the two co-receptors (the one that binds to the self MHC molecule), and are named CD4⁺ or CD8⁺ single positive (SP) T cells. Subsequently, negative selection eliminates SP thymocytes expressing TCRs that bind with high affinity to self peptide-MHC (pMHC) complexes, allowing deletion of nearly, but not all autoreactive T cells. Interestingly, some CD4⁺ SP T cells which recognize self pMHC complexes with an intermediate affinity differentiate into so-called thymic regulatory T cells (tTregs).

Tregs are a specialized subset of CD4⁺ T cells exerting immunosuppressive capacity through multiple mechanisms, including production and activation of TGF- β 1. They are defined by stable expression of the transcription factor FOXP3 and constitutive high surface expression of CD25 (IL-2 receptor alpha-chain). Loss of *FOXP3* results in fatal autoimmune disease in both mice and humans, a phenotype closely resembling TGF- β 1 deficiency (55, 56). Tregs comprise two main subsets : thymic-derived Tregs (tTregs) and peripherally-induced Tregs (pTregs). As indicated above, tTregs differentiate in the thymus from CD4⁺ SP thymocytes following an intermediate affinity interaction of the TCR with a self pMHC in the presence of cytokines (IL-2, IL-7 and IL-15) and co-stimulatory signals from APCs (**Figure 4**). Experimental evidence supports a two-step model of tTreg development : an initial TCR signal inducing CD25 expression, followed by IL-2–dependent upregulation of FOXP3 (57, 58). CD4⁺CD25⁺FOXP3⁻ thymocytes thus represent an intermediate precursor stage capable of differentiating into FOXP3⁺ tTregs upon IL-2 exposure. An alternative pathway has also been described in which FOXP3 expression precedes CD25 induction (59, 60). Co-stimulatory signaling via CD28 is important for tTreg development, as both CD28- and CD80/86-deficient mice display reduced tTreg numbers (61). CD28 signaling protects tTreg precursors from apoptosis by downregulating the pro-apoptotic protein Bim. However, Bim

deficiency alone does not restore tTregs in *Cd28*^{-/-} mice, suggesting that several survival pathways are involved (62).

In addition, *Foxp3* expression, and thus Treg development, are influenced by intronic enhancer elements within the *Foxp3* locus, which are subjected to epigenetic modifications and are referred to as conserved noncoding sequences (CNS). Even before thymocytes receive signals that commit them to the Treg lineage, DP and CD4⁺ SP thymocytes acquire permissive histone modifications at CNS0 and CNS3 that will facilitate *Foxp3* expression later during tTreg development (63, 64). Consistently, *Satb1*-deficient mice and *Mll4*-deficient mice exhibit a significant decrease in tTreg numbers. These studies suggest that *Satb1* and *Mll4* bind to CNS0, promoting chromatin opening and recruitment of transcriptional regulators that establish a permissive epigenetic landscape for tTreg development. These elements do not directly induce tTreg development, but instead confer thymocytes the potential to become tTregs once appropriate additional signals are received during thymic selection.

The functions of the other CNS (CNS1, 2 and 3) have also been investigated in vivo through the generation of mice carrying individual deletions of these noncoding regions, associated with insertion of GFP reporter in the *Foxp3* locus (65). Experiments demonstrated that CNS1 and CNS3 are required for the induction of *Foxp3* expression, whereas CNS2 is specifically required to stabilize *Foxp3* expression in the progeny of dividing Tregs. Indeed, although young CNS2-deficient (*Foxp3*^{ΔCNS2-gfp}) mice display normal Tregs, these numbers progressively decline with age. Moreover, following co-transfer of CNS2-deficient Tregs with wild-type naïve T cells into T cell-deficient mice, CNS2-deficient Tregs lose *Foxp3* expression over several weeks. Together, these findings indicate that CNS2 is dispensable for Treg generation but essential for Treg maintenance, ensuring stable *Foxp3* expression across cell divisions through the full demethylation of CpGs in CNS2, which are uniquely found in Tregs.

As mentioned above, CNS3 acquires permissive histone modifications before initiation of tTreg differentiation, modifications that are absent from CNS1 and CNS2. This suggests that CNS3 acts as a pioneer element at the *Foxp3* locus. Accordingly, CNS3-deficient mice (*Foxp3*^{ΔCNS3-gfp}) exhibit significantly reduced frequencies of both tTregs and pTregs (65). Upon TCR activation, c-Rel binding to CNS3 is thought to favor the establishment of open *Foxp3* chromatin conformation, thereby initiating *Foxp3* expression in CD4⁺ SP thymocytes and naïve CD4⁺ T cells. Finally, CNS1 is bound by NFAT and Smad3 and is also required for *Foxp3* expression, at least in pTregs. This role will be discussed in the following section.

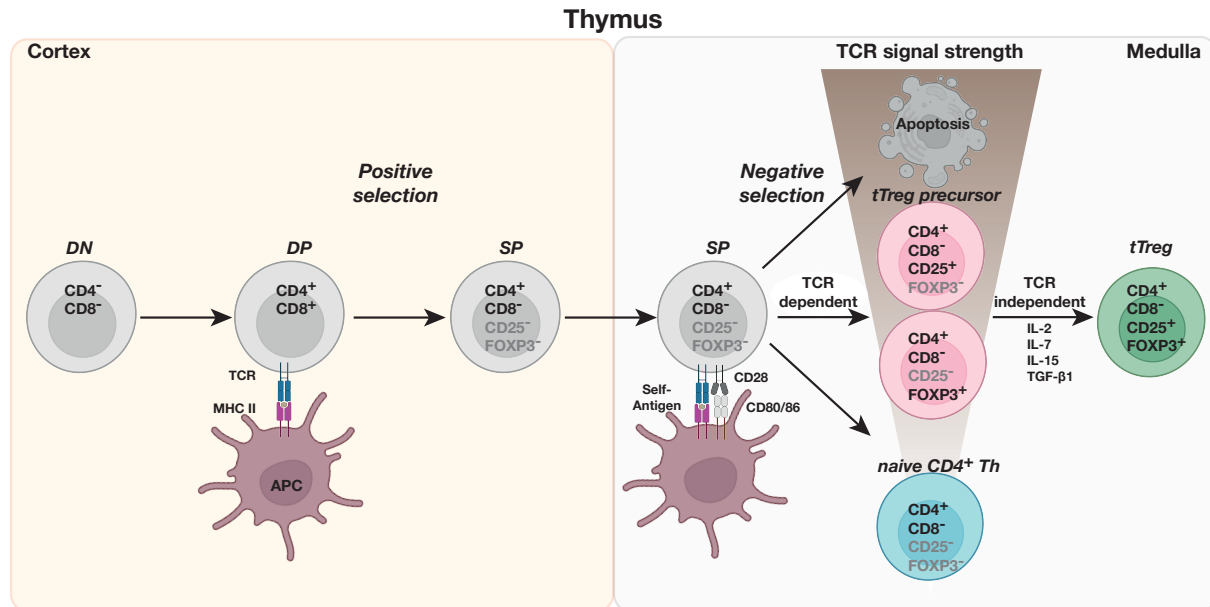


Figure 4. Thymic development of tTregs

After positive selection, some CD4⁺ SP thymocytes recognize self-pMHC complexes with intermediate affinity in presence of co-stimulatory signals, and differentiate into tTreg precursors (CD4⁺CD8⁻CD25⁺FOXP3⁻ or CD4⁺CD8⁻CD25⁻FOXP3⁺). Completion of tTreg development requires IL-2, IL-7, IL-15 or TGF-β1.

TGF-β1 signaling in T cell negative selection and Treg development in the thymus

It was initially proposed that TGF-β1 promotes the survival of tTregs in the periphery rather than promoting their development in the thymus. Early studies showed that loss of TGF-β signaling in T cells (*CD4^{Cre}xTgfb2^{fl/fl}*) resulted in reduced Treg frequencies in peripheral lymphoid organs in mice of 17 days, while thymic tTreg numbers were preserved (51, 66, 67). However, subsequent experiments showed that tTreg numbers are decreased at 3-5 days of age in mice lacking TGF-β1 signaling in T cells (*Lck^{Cre}xTgfb1^{fl/fl}* and *CD4^{Cre}xTgfb2^{fl/fl}* mice, in which TGF-β signaling is ablated from DN or DP stage, respectively) (67, 68). In these mice, after the first postnatal week, tTreg numbers rebounded, correlating with increased intrathymic IL-2 levels and enhanced proliferation of the remaining Tregs. Consistently, co-deletion of IL-2 and TGFβR1 prevented this tTreg rebound (*Lck^{Cre}xTgfb1^{fl/fl}xIL2^{-/-}*) (68). In parallel, TGF-β1 expression temporally correlates with neonatal waves of thymocyte negative selection and tTreg differentiation, suggesting a functional role of TGF-β1 during these thymic processes (69, 70). Apoptotic thymocytes during negative selection were suggested to represent a main source or trigger of TGF-β1 secretion in the thymus. However, identification of cells allowing TGF-β1 activation remains to be determined. While several studies support a role for TGF-β1 in Treg development and negative selection, the exact underlying molecular mechanisms remain incompletely understood.

Two studies proposed that TGF-β1 signaling directly drives tTreg differentiation by inducing Foxp3 expression. Mechanical studies showed that Smad3 binds to CNS1 within the *Foxp3* locus, and induce Foxp3 expression in collaboration with NFAT (68, 71). However, CNS1-deficient (*Foxp3^{ΔCNS1-gfp}*) mice do not exhibit reduced tTreg numbers, Foxp3 expression or suppressive function, indicating that CNS1 is dispensable for direct Foxp3 induction in tTreg precursors. Nevertheless, TGF-β1 induced-Foxp3 expression may still occur via non-canonical pathways, independently of direct Smad binding to the *Foxp3* locus. In support of this idea, several negative

regulators of *Foxp3* have been identified whose expression may be influenced by TGF- β 1 signaling. One such factor is the homeobox protein Hhex, whose expression is lower in Tregs than in conventional T cells. Hhex expression is downregulated by TGF- β /Smad3 signaling, and Hhex directly binds to the *Foxp3* promoter, suggesting that TGF- β 1 may indirectly promote *Foxp3* expression by reducing transcriptional negative regulators (72). Additional chromatin regulators could also be modulated by TGF- β 1 signaling, given the pleiotropic expression of TGF- β receptors ; however, their identity and functional relevance remain to be formally demonstrated.

Beyond its role in *Foxp3* expression, TGF- β 1 signaling has been proposed to influence negative selection, although this role remains controversial. Ouyang and colleagues suggested that TGF- β 1 protects self-reactive thymocytes, including tTregs, from apoptosis during negative selection (62). In double-transgenic mice expressing a TCR specific for a chicken ovalbumin-derived peptide (OT-II) and the cognate antigen in medullary thymic epithelial cells (TECs), they showed increased deletion of OT-II T cells in the absence of TGF- β signaling in T cells (*CD4^{Cre}Tgfb β 2^{fl/fl}*), suggesting that TGF- β 1 signaling impaired negative selection. They also showed that deletion of the pro-apoptotic Bim restored normal tTreg numbers in the thymus of 4-day-old *CD4^{Cre}Tgfb β 2^{fl/fl}* mice, which was likely a consequence of reduced T cell negative selection. Based on these findings, the authors proposed that TGF- β 1 does not directly drive tTreg development, but instead preserves self-reactive thymocytes from apoptosis by Bim, including tTregs. They suggest that the strength of T cell negative selection can be modulated to ensure correct generation of tTregs.

In contrast, McCarron and colleagues showed that the thymus of *CD4^{Cre}Tgfb β 2^{fl/fl}* mice exhibited increased numbers of autoreactive CD4⁺ T cells (defined as CD4⁺CD8^{low}Helios⁺Foxp3⁻), which had escaped negative selection and therefore drove the lethal systemic inflammation observed in these mice. It indicates that TGF- β 1 signaling in thymocytes is required for efficient negative selection (67). The authors suggested that impaired negative selection reduces the availability of TGF- β 1 released from apoptotic thymocytes, thereby contributing to impairment of tTreg development, possibly through diminished *Foxp3* expression (69).

Collectively, these studies demonstrate that TGF- β 1 signaling is required for efficient tTreg development, either by promoting *Foxp3* expression or by increasing resistance of tTreg precursors to apoptosis during negative selection, or both. The potential co-existence of these processes cannot be excluded and may be important for the establishment of effective central tolerance. Further studies using thymus-specific modulation of TGF- β 1 signaling are required to delineate its precise role in thymic functions.

- **Differentiation**

Once thymocytes have passed through positive and negative selection, they exit the thymus as mature naïve T cells capable of responding to foreign antigens in the periphery. Upon activation, naïve CD4⁺ T cells differentiate into distinct T cell subsets, contrasting by cytokine profiles and effector functions.

Differentiation in Th1, Th2, Th9 and Th17

TGF-β1 negatively regulates naïve T cell differentiation into the pro-inflammatory Th1 and Th2 subsets. Th1 cells express the transcription factor T-bet and secrete IFN-γ. *CD4^{Cre}xTgfb^{2fl/fl}* mice develop a systemic inflammatory disorder characterized by massive T cell proliferation and exaggerated Th1 responses (51). In the Th2 lineage, TGF-β1 downregulates *GATA3* expression and inhibits Th2 cytokine production (IL-4, IL-5 and IL-13) through the SMAD signaling pathway (4, 37). In contrast, TGF-β1 can, under certain conditions, favor differentiation into Th9 and Th17 lineages, mostly recognized as pro-inflammatory T cell subsets. Th9 cells differentiate in the presence of TGF-β1 and IL-4, and secrete IL-9 and IL-10. They have been implicated in promoting tissue inflammation, in colitis and intestinal infections by helminths (73, 74). Differentiation of Th17 cells, mostly present in mucosal tissues for defense against bacteria and fungi, are driven by the cooperation of TGF-β1 and IL-6. Their production of IL-17A, IL17-F, IL-21 and IL-22 promotes tissue inflammation. Reduced Th17 cell numbers in mice deficient for Smad proteins in CD4⁺ T cells highlight the role of TGF-β1 in Th17 differentiation *in vivo* (75).

Differentiation in pTregs

TGF-β1 signaling is essential for the differentiation of naïve CD4⁺ T cells into pTregs, which are particularly important in gastrointestinal (GI) tract homeostasis. Upregulating the expression of *FOXP3* in naïve CD4⁺ T cells, and therefore the differentiation into pTregs, requires IL-2 upon exposure to antigenic stimulation. In addition, retinoic acid (RA) produced by DCs in the intestinal environment also favor pTreg differentiation (76). Mice deficient in pTregs develop spontaneous inflammation in the intestine, lung and liver (77, 78).

TGF-β1 signaling is clearly required for pTreg differentiation. Consistently, CNS1 bound by Smad3/NFAT complexes is indispensable for pTreg differentiation, but has no apparent role in tTreg development or maintenance of *Foxp3* expression (65). CNS1-deficient naïve T cells failed to induce *Foxp3* expression *in vitro*, and mice deficient in CNS1 showed a complete absence of pTregs, which led to the development of allergic inflammation in GI tract and lungs, and modification of commensal microbiota (79). In contrast, CNS1-deficient mice exhibited preserved tTregs, which are sufficient to prevent autoimmune disease development. The authors thus suggest that tTregs are likely responsible for tolerance to self-antigens, while pTregs are required to control immune responses to non-self antigens, such as allergens, commensal microbiota and dietary antigens at mucosal interfaces. In addition, as described for tTregs, CNS3 is also implicated in *Foxp3* expression in pTregs and CNS2 participates in stable *Foxp3* expression across Treg division. In addition, mice deficient for both Smad2 and Smad3 develop fatal inflammatory diseases with reduced *Foxp3* expression in CD4⁺ T cells in the periphery, reinforcing the role of Smad signaling through CNS1 in *Foxp3* expression for pTreg differentiation (75).

Although TGF- β 1 is involved in pTreg development, several studies do not show a major role for TGF- β 1 in Treg maintenance or function under homeostatic conditions. Adult mice deficient for TGF- β 1 signaling in FOXP3⁺ cells (*Foxp3^{Cre}Tgfb1^{fl/fl}*) do not show any inflammation or decreased Treg numbers (69).

- **Proliferation and effector functions**

TGF- β 1 inhibits proliferation and effector functions of activated T cells. Transfer of T cells lacking TGF β R2 into lymphopenic recipients induces a lymphoproliferative disease characterized by enhanced proliferation and differentiation (80). TGF- β 1 also inhibits the effector functions of CD8⁺ T cells by decreasing expression and production of perforin, granzyme A, granzyme B, Fas ligand and IFN- γ (81).

1.2.2.2 B cells

TGF- β 1 exerts an anti-proliferative effect on B cells by downregulating cyclin A expression and by inactivating Cdk2, two regulators of the cell cycle (37). In addition, TGF- β 1 is essential for class switch recombination leading to IgA production, an important mechanism in mucosal immunity. Old (>8 months) mice with a conditional deletion of the TGF- β receptor in B cells (*CD19^{Cre}Tgfb2^{fl/fl}* mice) exhibit a complete deficiency in plasmatic IgA. While they do not develop any sign of autoimmunity, these mice display hyperactivated and hyperresponsive B cells with elevated production of antibodies, including anti-double stranded DNA antibodies (82). These results indicate that TGF- β 1 regulates various aspects of B cell biology, including proliferation and antibody production.

2 Targeting TGF- β 1 with monoclonal antibodies (mAbs)

2.1 Blocking TGF- β 1 mAbs

Therapeutic inhibition of TGF- β 1 could be beneficial in cancer and chronic infectious diseases. However, systemic targeting is challenging considering the pleiotropic activities of TGF- β 1, and results in significant adverse effects. Therefore, strategies designed to selectively inhibit cell-specific TGF- β 1 activation appear more promising. In particular, the GARP:TGF- β 1 complex represents an attractive target, as it mediates TGF- β 1 activation only in specific cell types, including TCR-stimulated Tregs, BCR-activated B cells, and platelets (16, 18, 20).

Our group has long been interested in the immunosuppressive mechanisms of Tregs. By comparing the transcriptome of human Tregs, identified through demethylation of a regulatory intronic region of *FOXP3*, with those of other human T cells, we showed that Tregs but not other T cells produce active TGF- β 1 after TCR stimulation (83). We further demonstrated that direct contact between Tregs and their target cells is required for TGF- β 1 signaling in the target cells, indicating that TGF- β 1 activation occurs very close to the Treg surface. Additionally, we found that human Tregs bind and present latent TGF- β 1 on their surface thanks to binding to the transmembrane protein GARP (16). To investigate the role of GARP in TGF- β 1 activation and in Treg-mediated immunosuppression, our group generated anti-human GARP:(latent)TGF- β 1 mAbs, using hybridoma and llama phage-display libraries (22, 23). Among 31 mAbs produced, only two effectively blocked GARP-dependent TGF- β 1 activation by Tregs *in vitro* (23). These blocking mAbs recognize human, but not murine, GARP:TGF- β 1 complexes.

To elucidate the structural basis of TGF- β 1 activation by GARP, we sought to determine the structure of the GARP:TGF- β 1 complexes. We successfully solved the crystal structure of the human GARP:TGF- β 1 complex bound to the Fab fragment derived from one of the blocking mAbs. These analyses revealed that blocking mAbs recognize a mixed conformational epitope, simultaneously contacting residues on GARP, LAP and mature TGF- β 1 (6). Our findings suggest that the blocking mAbs act as a molecular “glue”, preventing the elongation of the LAP latency lasso, and thereby the release of mature TGF- β 1 (**Figure 5**).

To assess whether these mAbs block TGF- β 1 activation *in vivo*, we used a mouse model of xenogeneic graft-versus-host disease (xGVHD), caused by injection of PBMCs from healthy human donors into immunodeficient NSG mice (i.e. lacking T and B cells). In this model, co-transfer of human Tregs decreased xGVHD severity. Treatment with blocking anti-human GARP:TGF- β 1 mAbs abrogated the protective effect of co-transferred human Tregs, similarly to neutralizing anti-mature TGF- β 1,2,3 mAbs. These results demonstrated the ability of anti-human GARP:TGF- β 1 mAbs to block TGF- β 1 activation by human Tregs *in vivo* (23).

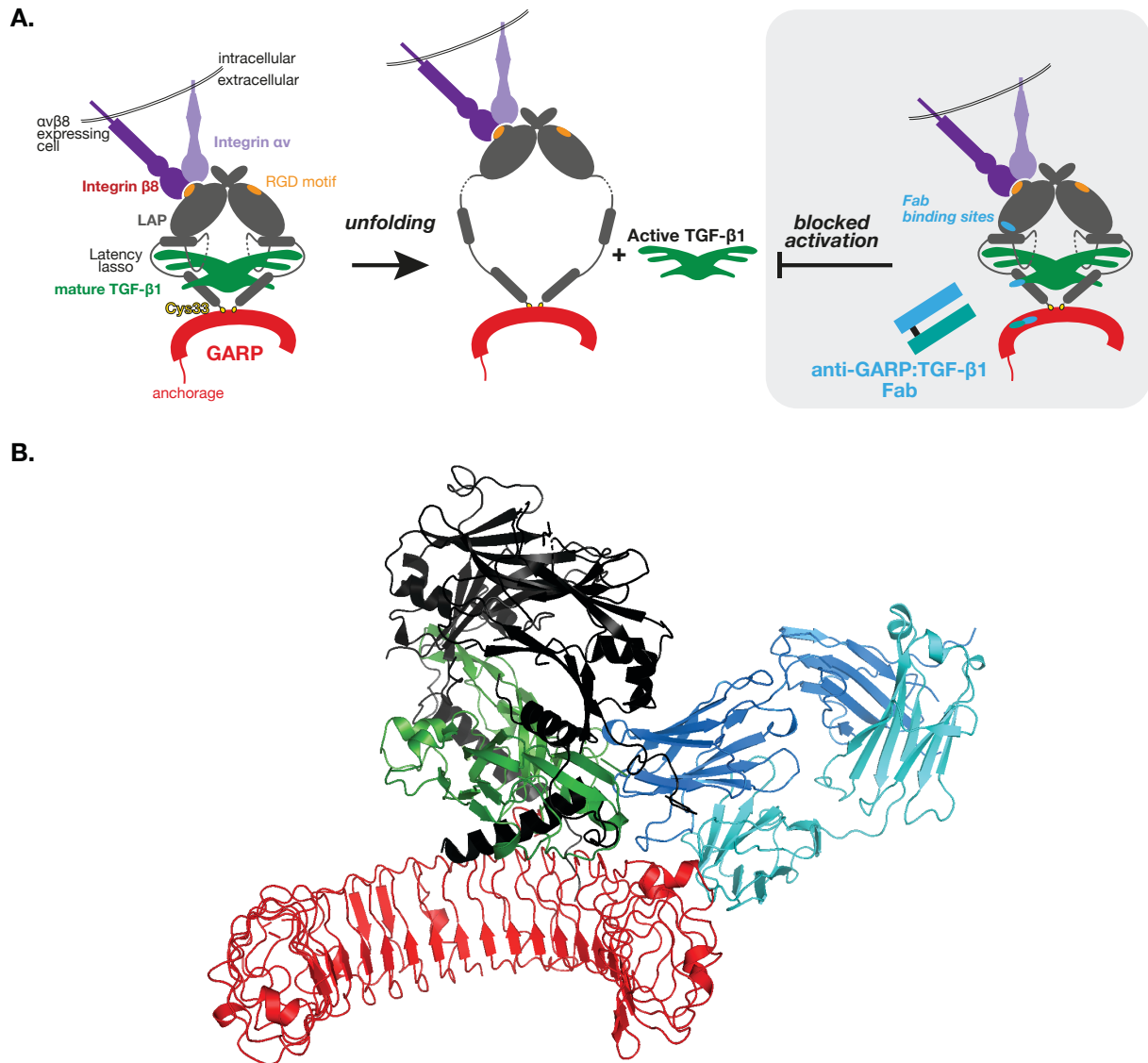


Figure 5. Blocking TGF- $\beta 1$ activation with anti-GARP:TGF- $\beta 1$ mAbs

(A) Proposed mechanism of latent TGF- $\beta 1$ activation by GARP and integrin $\alpha v \beta 8$ (figure adapted from S. Liénart's thesis (84)). Blocking GARP:TGF- $\beta 1$ mAbs prevent deformation of the latency lasso by recognizing a mixed conformational epitope comprising amino acids from GARP, LAP and mature TGF- $\beta 1$. (B) Cartoon representation of GARP:TGF- $\beta 1$ structure bound by Fab from blocking anti-GARP:TGF- $\beta 1$ mAb (here MHG-8), as determined by x-ray crystallography. Representation performed using PyMol.

To explore therapeutic applications in mouse models, we derived anti-mouse GARP:TGF- β 1 mAbs and demonstrated their capacity to block TGF- β 1 activation on murine Tregs *in vitro*. In preclinical experiments, combination of blocking anti-mouse GARP:TGF- β 1 mAbs with anti-PD1 therapy effectively induced rejection of solid tumors that were otherwise resistant to anti-PD-1 alone (22, 85). In addition, monotherapy with blocking GARP:TGF- β 1 mAbs reduced tumor burden in mouse models of myeloproliferative neoplasms (20). Using mice bearing a conditional deletion of *Garp* in specific cell types, we showed that the therapeutic efficacy of anti-GARP:TGF- β 1 mAbs was lost when GARP is absent on Tregs, but not on platelets, in mice bearing solid tumors. This indicated that blocking TGF- β 1 activation from GARP expressing Tregs is required for anti-tumor efficacy by the mAbs. These findings highlight the important role of GARP-expressing Tregs in promoting tumor immune evasion through TGF- β 1 activation. Phase 1 and 2 clinical trials are currently ongoing to evaluate blocking anti-human GARP:TGF- β 1 mAbs in patients with advanced or metastatic solid tumors, as a novel form of cancer immunotherapy (ClinicalTrials.gov: NCT03821935, NCT05822752, and NCT06109272).

2.2 Activating TGF- β 1 mAbs

Building on the mechanistic insights obtained by studying GARP-mediated TGF- β 1 activation by Tregs and blocking GARP:TGF- β 1 mAbs, our group aimed to develop mAbs designed to enhance, rather than inhibit, TGF- β 1 activation. The objective is to reinforce Treg-mediated immunoregulation in diseases characterized by excessive T cell activity insufficiently controlled by Tregs.

To date, all therapeutic mAbs targeting TGF- β 1 have been engineered to block its immunosuppressive and fibrotic activities. Attempts to administer recombinant active TGF- β 1 have been unsuccessful, because the mature cytokine has an extremely short plasma half-life (of the order of minutes in preclinical models), leading to rapid clearance and a minimal pharmacological effect (86). To our knowledge, no therapeutic antibodies have been developed to increase TGF- β 1 activation. However, controlled increase of TGF- β 1 activity may be therapeutically interesting in autoimmune and alloimmune diseases, characterized by disruption of self-tolerance, inappropriate T et B cell activation, leading to chronic inflammation. Targeting cell-specific tethering proteins that present latent TGF- β 1, such as GARP on Tregs, offers a particularly appealing approach. Antibodies that selectively promote TGF- β 1 activation from defined cell subsets could potentiate immunosuppressive mechanisms while minimizing systemic adverse effects such as fibrosis.

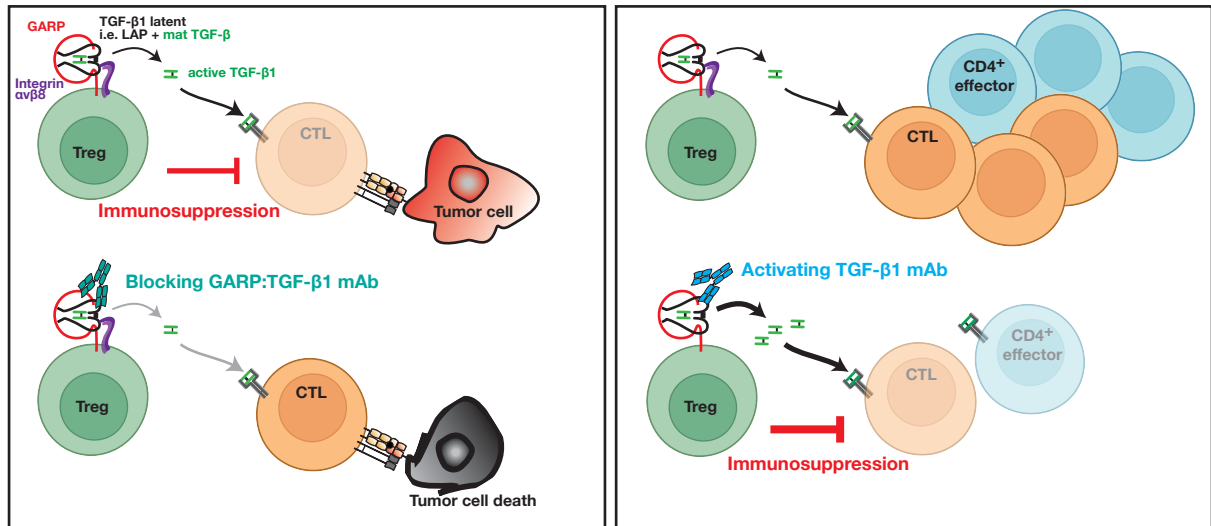


Figure 6. Targeting TGF- β 1 activation with mAbs

Left panel : Blocking anti-GARP:TGF- β 1 mAbs inhibit TGF- β 1 activation by GARP-expressing Tregs and restore anti-tumor T cell immunity by cytotoxic T lymphocytes (CTL). Figure adapted from G. de Streel's thesis.

Right panel : TGF- β 1 activating mAbs enhance TGF- β 1 activation and could suppress deleterious excessive T cell activity insufficiently control led by Tregs.

3 Graft-versus-host disease after allogeneic hematopoietic stem cell transplantation

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) constitutes a major therapeutic strategy to cure malignant hematologic diseases. Immune cells and hematopoietic progenitors from a healthy donor are transplanted into a recipient to establish donor-derived immunity and hematopoiesis. The success of allo-HSCT against hematological neoplasms comes from the ability of patients to receive intensive (radio)-chemotherapy followed by hematopoiesis reconstitution, coupled with a graft-versus-leukemia (GVL) effect mediated by donor immune cells. Allo-HSCT remains indispensable for patients with high-risk hematologic malignancies who lack effective alternative therapies. Moreover, infusion of donor hematopoietic progenitors may cure nonmalignant diseases such as inborn errors of immunity or bone marrow failure syndromes. Nevertheless, common use of allo-HSCT is limited by several factors, including graft-versus-host disease (GVHD).

GVHD is the most frequent life-threatening complication of allo-HSCT, manifesting when donor mature T lymphocytes recognize recipient cells as foreign. The allogeneic immune reaction activates donor T cells which differentiate and acquire effector functions to eliminate recipient cells. Clinically, GVHD is classified into two principal forms: acute and chronic. Acute GVHD (aGVHD) typically occurs within the first 100 days after transplantation and mostly affects epithelia and lymphoid organs. aGVHD is characterized by an important immune cell infiltration and inflammation. In contrast, chronic GVHD (cGVHD) is characterized by autoimmune manifestations and fibrotic lesions. cGVHD generally occurs several months after allo-HSCT and can affect virtually all organs.

GVHD is a substantial cause of death in patients undergoing allo-HSCT. Despite prophylactic treatment aiming to limit donor T cell alloreactivity, 30-50% of patients develop aGVHD, with 15% of them experiencing a severe form. The cumulative incidence of cGVHD is similarly high, occurring in up to 40% of recipients (87). Despite improvement in pathophysiology knowledge, the first line treatment of both acute and chronic GVHD has remained unchanged for decades, and relies on glucocorticoids (GC). However, broad immunosuppressive effects of GC lead to significant adverse events, notably impaired immune reconstitution and infection risk, which negatively affect the quality of life and contribute to increased mortality. Moreover, 30-60% of patients become resistant or refractory to GC, with a 2-year overall survival (OS) of 25% despite second and further line therapies (88).

3.1 Allo-HSCT : clinical considerations

According to the most recent report from the European Society for Blood and Marrow Transplantation, approximately 20 000 allo-HSCTs were performed in Europe in 2023 (89), whereas more than 25 000 patients received autologous stem cell transplantations (**Figure 7**). In an allo-HSCT procedure, recipients first undergo a conditioning regimen, followed by the intravenous infusion of donor cells. The main indications are myeloid malignancies, particularly acute myeloid leukemia (AML). Once the indication for allo-HSCT has been established, several key parameters must be defined, as they will influence the success of allo-HSCT. These include the conditioning regimen, type of donor, and source of stem cells.

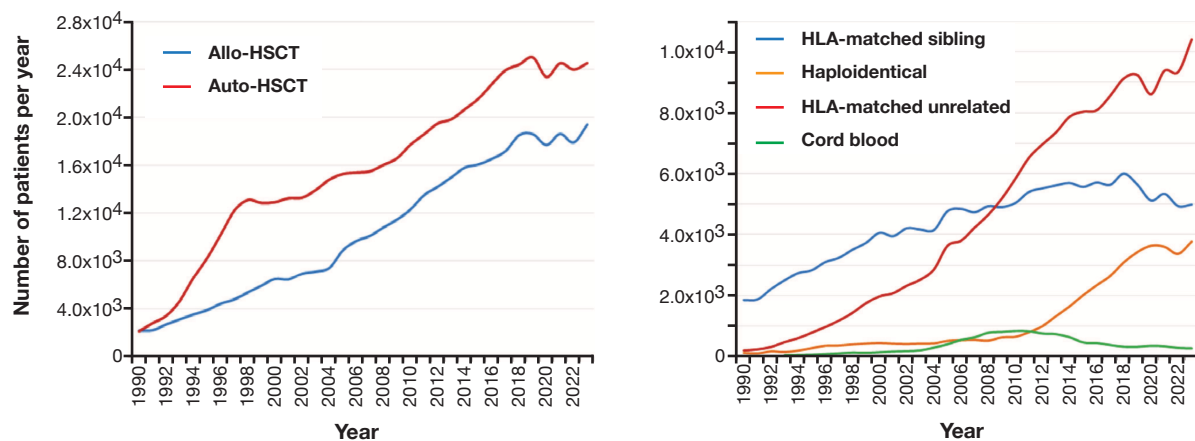


Figure 7. Epidemiology of allo-HSCT in Europe

Left graph : total number of allogeneic-HSCT (allo-HSCT) and autologous-HSCT (auto-HSCT). Right graph : number of allo-HSCT classified by type of donor.

3.1.1 Conditioning

Pre-transplant conditioning has several functions in allo-HSCT : eradicate residual malignant cells; provide sufficient immunosuppression by depleting recipient lymphocytes to prevent graft rejection; and clear BM niches to facilitate donor stem cell engraftment. Conditioning regimens are categorized into myeloablative (MAC), reduced intensity (RIC) and non-myeloablative (NMA) (90). MAC consists of intensive chemotherapy (e.g. busulfan, melphalan), sometimes combined with high-dose total body irradiation (TBI). Given the profound and irreversible myeloablation induced by MAC, patients must receive stem cell infusion to restore hematopoiesis. MAC is associated with profound toxicities, including mucositis, veno-occlusive disease and severe GVHD, and is therefore reserved for young and fit patients. To extend allo-HSCT applicability to older and more fragile patients, lower-toxicity regimens have been developed. In these approaches, patients receive lymphocytotoxic agents to suppress recipient immune cells and prevent graft rejection (e.g. fludarabine, low-dose TBI). NMA relies exclusively on lymphocytotoxic agents, whereas RIC combines these drugs with low-dose of myeloablative chemotherapy to provide some anti-tumoral activity and partially clear BM niches. With low toxicity regimens, anti-tumoral efficacy relies specifically on the GVL effect mediated by donor immune cells. However, the incidence of relapse could be slightly higher in the absence of fully cytotoxic conditioning (91). Importantly, the severity of GVHD is decreased in RIC and NMA regimens, contributing to lower transplant-related mortality compared to MAC.

3.1.2 Donor choice

The major determinant of successful allo-HSCT is the degree of MHC matching (also known as the human leucocyte antigen system, HLA) between donor and recipient. The first allo-HSCT procedures, performed in the 1950s and 1960s by the team of Donnall Thomas (who later received the Nobel Prize in Physiology and Medicine in 1990), were carried out before the discovery of the HLA system. Among the 200 patients grafted at the time, none achieved long-term survival, because of graft rejection, severe GVHD, or relapse (92, 93). The subsequent discovery of histocompatibility antigens and their central role in graft tolerance represented a major breakthrough in transplantation, enabling the first successful long-term allo-HSCTs in the 1970s using HLA-matched sibling donors (94, 95).

MHC molecules and MHC matching govern both graft rejection and development of GVHD. Preclinical experiments in mice showed that recipients of MHC-mismatched grafts invariably developed fatal GVHD, whereas syngeneic recipients did not (96). In humans, the HLA system comprises two classes of molecules: class I molecules (HLA-A, -B, and -C) are expressed on nearly all nucleated cells and present endogenous peptides to CD8⁺ T cells, whereas class II molecules (HLA-DP, -DQ, and -DR) are displayed mostly by APCs and present exogenous peptides to CD4⁺ T cells (96). HLA genes are among the most polymorphic in the human genome, a feature that underlies the strong allogeneic T cell responses observed after transplantation. Even a single mismatched HLA allele is sufficient to significantly increase GVHD and transplant-related mortality.

However, even when donor and recipient are fully matched for HLA molecules, GVHD can still occur. Observations in dogs and humans revealed that donor T cell recognition of minor histocompatibility antigens (miHAs) is sufficient to induce GVHD (97). miHAs correspond to polymorphic peptides presented by MHC molecules. They can elicit a GVH response despite full HLA matching (98). These polymorphisms typically originate from amino-acid substitutions encoded by single-nucleotide variants, insertions or deletions, or differences between Y- and X-linked genes (99, 100). Only a limited number of the most strongly immunogenic miHA mismatches appear to drive GVHD. Notably, the frequency of donor T cells specific for a given miHA is approximately 1 in 10⁶, whereas in HLA-mismatched HSCT, direct recognition of allogeneic HLA by donor T cells occurs at a frequency as high as 1 in 10²-10³ explaining why not only GVHD but also GVL are significantly reduced in HLA-matched sibling HSCT (101).

Given that allo-HSCT is used as consolidation therapy for high-risk hematologic malignancies, the ability of donor immune cells to mediate GVL is important for reducing relapse incidence. Nevertheless, relapse of the hematological malignancy remains the leading cause of mortality after allo-HSCT. Achieving a potent GVL effect without inducing excessive GVHD remains the “holy grail” of allo-HSCT. A number of leukemia associated antigens have been associated with tumor-specific T cell responses. These antigens are typically overexpressed on leukemia blasts while being absent in normal tissues, and include Wilms tumor 1 (WT-1), melanoma antigen family members (MAGE) and PR1 (a peptide derived from proteinase 3) (102). Cytotoxic T cells against NPM1, a frequent mutation in AML, have also been reported. In parallel to specific tumor antigens, the tissue-specific expression patterns of miHAs also contribute to GVL efficacy (103, 104). MiHAs restricted to the hematopoietic compartment, including leukemic cells, can promote GVL with minimal GVHD. Examples include BCL2A1 (an anti-apoptotic BCL2-family member frequently overexpressed in hematologic malignancies), and HB-1 (a polymorphic antigen highly expressed in B cell tumors such as ALL and lymphoma but only weakly expressed in most normal tissues).

Conversely, miHAs with broad tissue expression tend to induce both GVHD and GVL. This category includes antigens highly expressed on epithelial cells, as well as Y chromosome-derived antigens (HY), which differ significantly from their X-linked homologues and induce strong alloreactive responses in female-to-male transplantation. HY antigens are among the most clinically potent miHAs described.

Defining the suitable donor for a dedicated recipient primarily relies on the analysis of HLA class I and II. Currently, no identification of miHAs are used in clinics. Historically, the ideal donor was an HLA-matched sibling donor (MSD), defined by compatibility of 10/10 in HLA alleles between donor and recipient across HLA-A, -B, -C, -DRB1 and -DQB1 loci. The HLA-DPB1 locus was initially excluded due to its supposed poor immunogenicity, but increasing evidence supports the relevance of DPB1 matching in allo-HSCT (105). To note that in USA, matching is typically assessed at only 4 loci (HLA-A, -B, -C and -DRB1), and an 8/8 is therefore considered as a matched pair. The probability of identifying an MSD for any given patient is less than 25%, which often requires a search for alternative donor. Matched unrelated donors (MUD) are searched in Belgian and international donor databases. Survival outcomes were demonstrated nearly equivalent between MSD and MUD with current prophylaxis (106). However, the likelihood of finding a MUD is depending on patient ethnicity with success rates exceeding 75% for Caucasians but dropping below 20% for ethnic minorities. In the absence of MSD or MUD, alternative donor options include one-allele mismatched unrelated donors (MMUD), unrelated umbilical cord blood (UCB) or HLA-haploidentical family donors. All these options previously displayed inferior outcomes because of high rates of infections or GVHD. However, the recent implementation of early post-transplant cyclophosphamide in haploidentical HSCT has resulted in survival outcomes similar to those observed with MUD and MSD. The principal advantage of haploidentical donor is the rapid and almost universal donor availability, increasing the clinical use of this type of donor.

In addition to HLA matching, other donor and recipient characteristics play important roles in the success of allo-HSCT and the risk of developing GVHD. One of the most observed factors is donor age. Outcomes generally decline as donor age increases, particularly with unrelated donors, likely due to several mechanisms of HSC senescence (107, 108). This has contributed to the recent trend of preferential selection of younger haploidentical donors over older matched siblings (89). Other factors must be considered, although results are not fully consistent across studies : older recipients, female-to-male transplantation, cytomegalovirus (CMV) seropositivity, and ABO compatibility (109, 110).

3.1.3 Stem cell source

Another key factor affecting GVHD and overall outcomes is the stem cell source. Peripheral blood stem cells (PBSCs) are the predominant source of HSC in Europe, representing more than 80% of all transplants, while BM and umbilical cord blood (UCB) account for approximately 15% and 1%, respectively (89). The widespread use of PBSCs is largely driven by the easy collection of donor cells via cytapheresis after mobilization by G-CSF, and the robust GVL effect mediated by the high content of donor immune cells. However, these same immune cells increase the incidence of GVHD. In addition, G-CSF exposure in donors may further enhance cGVHD, presumably by promoting IL-17 production from T cells (111). In contrast, BM grafts are associated with a lower risk of GVHD, but provide higher relapse rates. Procurement of BM cells is also more demanding for donors. Consequently, BM transplantation is now mostly reserved for non-malignant indications.

The limited use of UCB in adults is mainly due to the significantly delayed engraftment and subsequent fatal invasive bacterial and fungal infections (112, 113).

3.2 Immune reconstitution

The success of allo-HSCT depends on the restoration of a competent donor-derived immune system in a recipient whose immunity has been compromised by the underlying disease, prior treatments and conditioning. The dynamics of immune reconstitution vary considerably among patients and are influenced by multiple intrinsic and extrinsic factors. The conditioning regimen modulates the depth of immune depletion and thereby the susceptibility to infections, which can further influence immune recovery. The stem cell source is also critical : UCB, characterized by a lower total nucleated cell count, is associated with delayed neutrophil engraftment (approximately 30 days) compared with PBSCs (14 days) and BM cells (around 21 days) (114). Moreover, both *ex vivo* and *in vivo* T cell depletion strategies delay immune reconstitution despite their efficacy in reducing GVHD. By damaging lymphoid organs, GVHD itself may postpone adaptive immune reconstitution. Additional determinants, including HLA compatibility, donor-recipient serostatus (notably CMV), nutritional status, and gut microbiota composition, also contribute to the heterogeneity of post-transplant immune reconstitution (115). The interplay of all these factors makes a precise prediction of immune recovery challenging. Nevertheless, innate immune cells generally reconstitute earlier than the adaptive immune system, which may require from several months to up to 2 years for a complete recovery (**Figure 8**) (116).

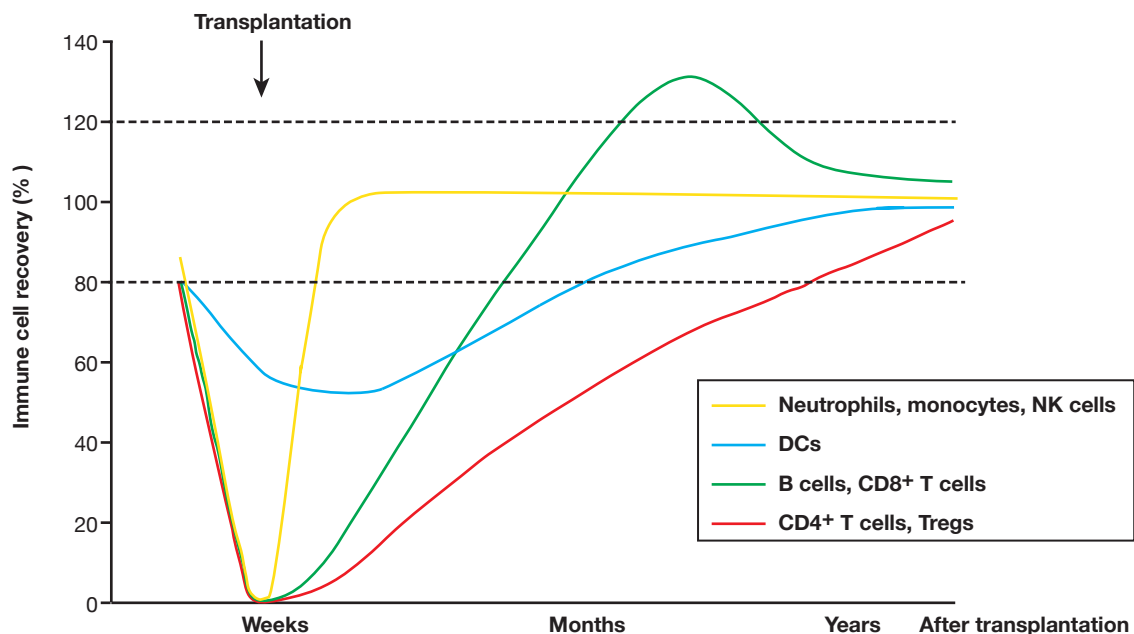


Figure 8. Immune reconstitution after allo-HSCT

Dynamics of immune cell reconstitution expressed by percentages of normal cell counts before and after myeloablative conditioning (MAC) followed by hematopoietic stem cell transplantation (HSCT). Figure adapted from Bosch *et al.* 2012.

3.2.1 Innate immune cells

3.2.1.1 Granulocytes

After MAC, patients experience an aplastic stage, defined by profound anemia, thrombopenia and neutropenia that typically lasts 2 to 3 weeks. Neutrophils are the first immune cell population to reconstitute from donor hematopoietic progenitors (117), and they rapidly acquire a functional phenotype (118). *Ex vivo* experiments on neutrophils isolated at day +28 after allo-HSCT demonstrated restoration of antimicrobial activity against *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli* (117, 118). This suggests an early and robust recovery of neutrophil effector function. However, this interpretation is nuanced by additional findings showing that oxidative burst activity during ingestion of *E. coli* was still impaired at day +30, but normalized by day +60 (117). The normalization of neutrophil functions is delayed in GVHD treated with GC. Data regarding the kinetics and functional recovery of eosinophils and basophils after allo-HSCT remain limited.

3.2.1.2 Dendritic cells

After allo-HSCT, donor DC recovery is slow, with significantly reduced numbers persisting for up to 18 months. This deficit is especially notable for plasmacytoid DCs, which produce IFN- γ during viral infections. Because host DCs are less sensitive to conditioning, they often persist alongside donor DCs and are capable of activating donor T cells (119). Impaired donor DC recovery has been associated with severe GVHD in clinical studies (120, 121).

3.2.1.3 Monocytes/macrophages

Monocyte reconstitution has been associated with improved OS after allo-HSCT (122). Their reconstitution occurs in parallel with neutrophils, typically reaching normal blood counts within the first month post-transplant. Macrophage reconstitution has been poorly documented, although it is generally assumed to follow monocyte regeneration, given that macrophages differentiate in part from circulating monocytes. *Ex vivo* experiments have reported both preserved and impaired functions among monocyte/macrophage populations. Culture of recipient PBMCs showed an increase in TNF α and IL-6 after lipopolysaccharide (LPS) stimulation, suggesting that cytokine production was maintained in monocytes as early as 10-14 days post-transplantation (123). In contrast, other experiments indicated impaired chemotaxis, phagocytosis and killing of pathogens by macrophages obtained from bronchoalveolar lavage approximately 4 months after allo-HSCT (124).

3.2.1.4 **NK cells**

NK cell counts recover more slowly than granulocytes, generally normalizing within 1-2 months after transplantation (125). They are the first lymphoid subset to regenerate, well before B and T cells. Most early post-transplant NK cells exhibit an immature phenotype (CD56^{bright}CD16^{dim}) with limited cytotoxic capacity, reflected by decreased IFN- γ production in *ex vivo* assays. Full functional development toward a mature phenotype (CD56^{dim}CD16^{bright}) may require more than 6 months (126). These observations suggest that early NK cells develop mainly from donor hematopoietic progenitors, rather than from expansion of mature NK cells infused with the graft. Nonetheless, mature donor NK cells may contribute to early immune reconstitution, as shown by the faster normalization of NK cell counts in patients receiving CD3/CD19-depleted grafts compared with CD34-selected grafts, the latter containing approximately 3-log fewer mature NK cells (127).

Clinically, NK cell reconstitution has been associated with improved OS (128). NK cells are important in the early post-transplant period by limiting relapse, and adoptive transfer of NK cells has shown beneficial effects on the GVL response, notably in myeloid malignancies (129-131). Their involvement in GVHD is generally considered protective, as NK cells can eliminate residual host APCs and activated alloreactive donor T cells (132). However, in specific contexts, NK cell hyperactivation may drive the production of pro-inflammatory cytokines that sustain pathogenic T cell responses (133).

3.2.2 **Adaptive immune cells**

3.2.2.1 **T cells**

Reconstitution of an efficient T cell compartment is crucial for the success of allo-HSCT as immune cellular therapy. As illustrated in **Figure 9**, T cell recovery occurs through two complementary mechanisms : a thymic-independent pathway, also referred to as homeostatic peripheral expansion (HPE), and a thymic-dependent pathway (116, 134).

Homeostatic peripheral expansion or thymic-independent T cell reconstitution

HPE involves the proliferation of mature donor T cells present in the graft, but also of residual recipient T cells. T cell expansion is triggered by profound lymphopenia induced by the conditioning regimen and is supported by elevated concentrations of IL-7 (135). HPE enables a rapid expansion of T cells and contributes to early immune surveillance. However, the number of clonotypes within the repertoire generated through HPE is restricted, being limited by the specificities of the initial T cell population and by the antigens that drive the clonal peripheral expansion. Consequently, immunity remains impaired. For unknown reasons, HPE preferentially expands effector memory CD8⁺ T cells, resulting in a faster recovery of CD8⁺ than CD4⁺ T cells (136). It explains the transient inversion of the CD4/CD8 ratio reported in the first months after allo-HSCT (137, 138). HPE is particularly important in older recipients given the severe thymic atrophy that occurs with aging (139).

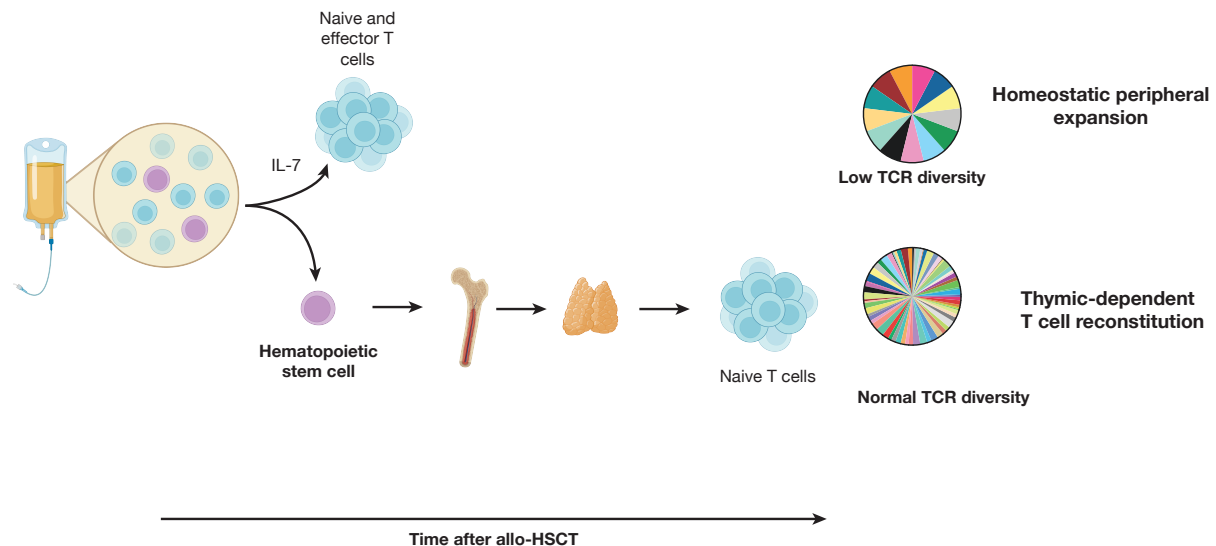


Figure 9. Thymic-independent and -dependent T cell reconstitution after allo-HSCT

After allo-HSCT, T cells reconstitute by a thymic-independent pathway, also named homeostatic peripheral expansion, which involves proliferation of mature donor T cells present in the graft and are characterized by a low diversity in the TCR repertoire. The thymic-dependent reconstitution starts approximately 6-12 months after allo-HSCT and generates naïve T cells derived from donor HSCs in the recipient thymus, leading to the establishment of a broad TCR repertoire tolerant against recipient antigens.

Thymic-dependent T cell reconstitution

The thymic-dependent pathway generates naïve T cells derived from donor HSCs and developed within the thymus of the recipient, leading to the establishment of a broad TCR repertoire tolerant against recipient antigens.

Conditioning regimens damage the thymic architecture, delaying thymopoiesis for at least 6 months (134). This means that HPE is the dominant mechanism of T cell reconstitution during the first months after transplantation. The delay of thymic recovery has been attested by an increase in recent thymic emigrant cells (CD4⁺CD45RA⁺CD62L⁺) and in TCR excision circles (i.e. circular episomal DNA fragment generated during TCR gene rearrangement; TREC) only 6 to 12 months after allo-HSCT (134, 140). Thymic regeneration and subsequent development of tolerant T cells are markedly influenced by multiple factors, including age-related thymic involution, cytotoxic effects of chemotherapy and irradiation, and GVHD. Consequently, the restoration of a normal T cell compartment often remains incomplete within the first year after allo-HSCT, especially in older recipients and those with GVHD. Moreover, CD4⁺ T cell recovery is highly dependent on thymic generation of naïve cells, making CD4⁺ reconstitution slower and more sensitive to thymic injury than CD8⁺ T cells (138). Reconstitution of Tregs parallels that of conventional CD4⁺ T cells, and their numbers remain lower than healthy controls for up to 2 years after allo-HSCT. Treg reconstitution is markedly better preserved with RIC, PBSC graft and in the absence of T cell depleting GVHD prophylaxis (141). Delayed Treg reconstitution has been correlated with an increased risk of acute GVHD, although it does not appear to be associated with the later development of cGVHD.

GVHD is a major determinant of impaired T cell reconstitution, by directly damaging thymic epithelial structures and disrupting microarchitecture. Thymopoiesis is impaired, as evidenced by decreased TRECs and naïve CD4⁺ T cell numbers in GVHD patients (142, 143). In addition, GVHD limits HPE by widespread bystander apoptosis of activated donor T cells, further narrowing the regenerated T cell repertoire diversity (139). Moreover, immunosuppressive agents required to prevent or control GVHD exacerbate these negative effects. Collectively, it results in prolonged immune deficiency in patients affected by GVHD. Importantly, beyond the increased susceptibility to infections, a deficient T cell reconstitution decreases the GVL effect, given that anti-tumor efficacy predominantly depends on the activity of donor-derived T cells.

3.2.2.2 B cells

Following allo-HSCT, B cell reconstitution recapitulates normal ontogeny (144). B cells become detectable within 2 months, but normalization of circulating B cell counts requires approximately one year. Initial B cell recovery predominantly consists of transitional B cells, which differentiate into naïve B cells over time. Consequently, the peripheral B cell compartment contains mainly naïve B cells after allo-HSCT, and memory B cells, which are essential for rapid recall responses, recover only 1 to 2,5 years later (145). As a result, full restoration of humoral immunity and robust antibody responses to vaccination commonly requires 2-3 years after allo-HSCT (146).

In patients with GVHD, B cell recovery and antibody production are significantly impaired, likely caused by alloreactive immune responses and prolonged immunosuppressive therapy, which contribute to the reduced vaccine responsiveness and increased susceptibility to infections.

3.3 Acute GVHD

The processes that drive immune recovery can paradoxically promote allogeneic reactions. The resulting GVHD is categorized into acute and chronic forms according to its pathophysiology, clinical features, and time of appearance.

3.3.1 Clinical presentation and risk factors

Acute GVHD predominantly affects the skin, liver and GI tract. Clinical manifestations include maculopapular erythematous skin rash; jaundice with hyperbilirubinemia leading to cholestasis; anorexia, nausea, vomiting and diarrhea associated with weight loss (147). Recent evidence suggests that additional organs may also be involved in aGVHD, including lymphoid organs (bone marrow, thymus, lymph nodes) and non-lymphoid organs (central nervous system, lungs, kidneys, ovaries, and testes) (148). Diagnosis relies on clinical presentation and biological parameters, requires exclusion of alternative etiologies, and should ideally be supported by characteristic histopathological findings (149). Severity is graded using a standardized scoring system assessing skin, liver, upper and lower GI tract involvement, resulting in an overall aGVHD score ranging from I (mild) to IV (life-threatening) (149).

3.3.2 Pathophysiology

The clinical presentation of aGVHD results from a well-established cascade of immunological events, initially described in mouse models and then validated in humans. The disease proceeds through three sequential and interconnected phases (**Figure 10**) : (1) conditioning-associated inflammation and activation of APCs, (2) activation of alloreactive donor T cells, and (3) T cell mediated organ damage (150).

(1) Conditioning-associated inflammation and activation of APCs

The intensity of the conditioning regimen is an important determinant of aGVHD severity, modulated by the underlying disease, prior therapies and infections. TBI and MAC produce extensive damage to fast-replicative epithelia, whereas RIC or NMA regimens exert less tissue damage, and are associated with attenuated GVHD severity (151). The intestinal epithelium is particularly damaged. Loss of the intestinal barrier permits translocation of bacterial products, including LPS and other pathogen-associated molecular patterns (PAMPs) derived from commensal bacteria (152, 153). In parallel, injured epithelial cells release damage-associated molecular patterns (DAMPs) including ATP, IL-1 α , IL-33, mitochondrial components, uric acid and heat shock proteins (154).

Tissue injury and microbial translocation recruit and activate neutrophils and monocytes/macrophages in the lamina propria (152, 154-158). Increased neutrophil infiltration in gastroduodenal biopsies correlates with intestinal GVHD and increased transplant-related mortality (155, 156). Given that activated neutrophils generate reactive oxygen species (ROS), this infiltration exacerbates epithelial injury (155). Patients with chronic granulomatous disease (a rare primary immunodeficiency disorder with defective ROS production), exhibit markedly reduced GVHD incidence after allo-HSCT (159), highlighting the role of granulocytes in the initiation of aGVHD.

Pro-inflammatory cytokines, including TNF- α , IL-1, and IL-6, are secreted by activated myeloid cells. In combination with DAMPs and PAMPs which signal through pattern recognition receptors (PRRs), they promote the activation and maturation of APCs to facilitate antigen presentation by upregulating MHC-II and costimulatory molecules (157). These signals also induce antigen-presenting function in non-professional APCs within damaged tissues, such as MHC-II expression by intestinal epithelial cells (160, 161).

Activated APCs interact with donor T cells in secondary lymphoid organs, particularly in Peyer's patches within the GI tract. Supporting this concept, mice lacking Peyer's patches, or those with T cells unable to migrate to these sites due to CCR5 deficiency or integrin $\alpha 4\beta 7$ blockade, fail to develop aGVHD (162).

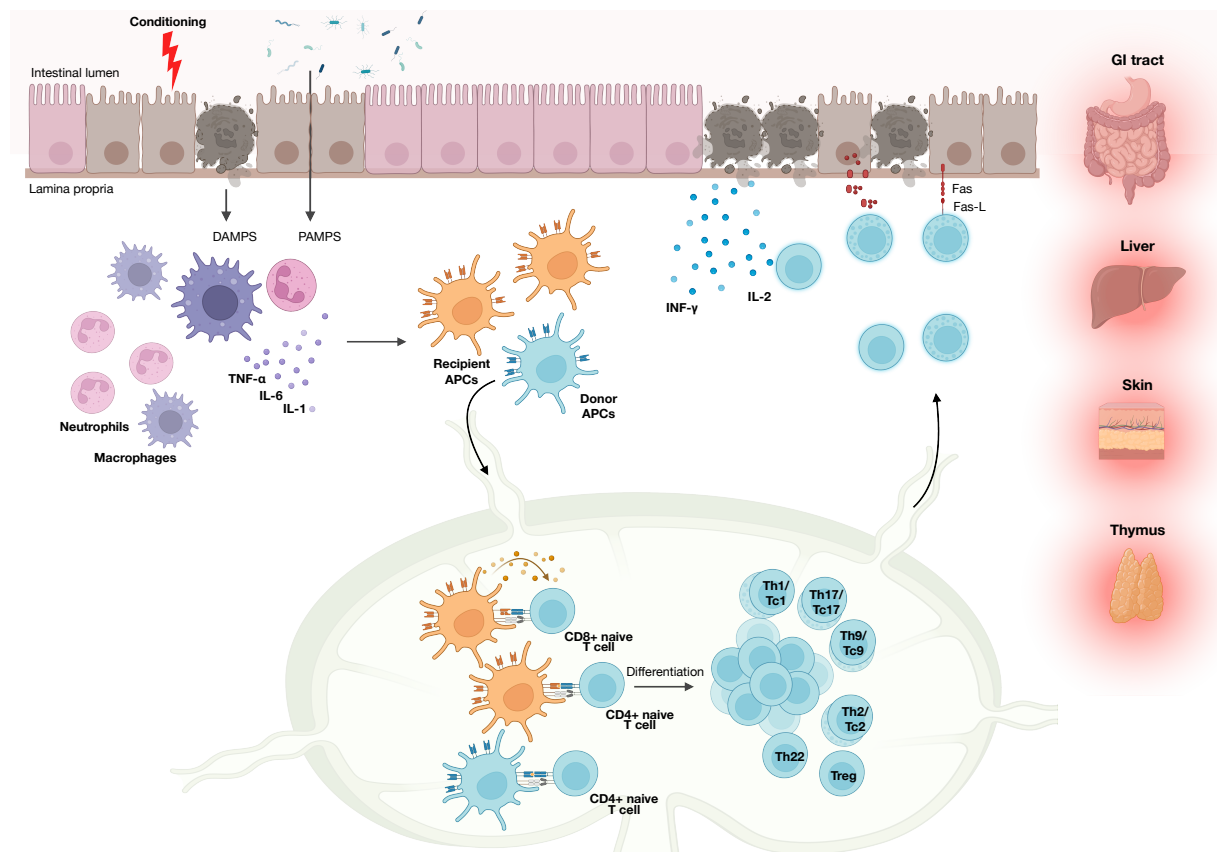


Figure 10. Pathophysiology of aGVHD

Conditioning-induced tissue damage releases pro-inflammatory cytokines and DAMPs, which activate host and donor antigen presenting cells (APCs), in combination with bacterial products. Recognition of mismatched MHC or minor histocompatibility antigens (miHAs) drives donor T cell activation, differentiation into effector cells and trafficking to target organs where cytokines and cytotoxic mechanisms increase tissue damage.

(2) Activation of alloreactive donor T cells

Donor T cells can be activated by allogeneic MHC molecules, or miHAs presented by matched MHC molecules. Murine models showed that recipient DCs are necessary and sufficient to activate T cells and initiate aGVHD, and that they present major and minor HAs directly to donor T cells more efficiently than donor DCs in the early post-transplant period (160, 163, 164). Nevertheless, once initiated, GVHD is intensified by donor DCs which present recipient antigens on MHC-II or on MHC-I via a cross-presentation mechanism, activating donor CD4⁺ and CD8⁺ T cells, respectively (119).

Donor T cells are central in aGVHD, as evidenced by the reduction of GVHD incidence in recipients of T cell-depleted grafts. To date, T cells remain the core target of most current therapies used to prevent or treat GVHD. *In vitro* and murine experiments showed that naïve T cells are more potent inducers of severe GVHD than effector T cells (165, 166). In murine models, transplantation of naïve T cells is sufficient to induce aGVHD, and in human allo-HSCT, high frequencies of naïve CD4⁺ T cells in the graft increase aGVHD severity (167). Consistent with this, naïve T cell-depleted grafts reduce both aGVHD severity and cGVHD incidence. One hypothesis proposes that the broad TCR diversity and rapid expansion capacity of naïve T cells make them major drivers of GVHD.

Effective activation of naïve donor T cells by APCs requires the integration of three signals. First, TCR engagement occurs through recognition of mismatched MHC and/or miHAs presented on matched MHC. Second, co-stimulatory signals are provided by interactions between members of the B7/CD28 family. Notably, the CTLA-4 agonist abatacept, which interferes with CD28-mediated co-stimulation, has recently been approved by the Food and Drug Administration (FDA) as GVHD prophylaxis (168). Third, cytokines produced by APCs in a paracrine manner and by T cells in an autocrine manner further drive complete T cell activation and polarization into different effector subsets. Several cytokines contribute to GVHD pathogenesis, including TNF- α , IFN- γ , IL-2, IL-17... (67).

Upon encountering their cognate antigen, donor naïve T cells proliferate and differentiate into various effector subsets : Th1/Tc1, Th2/Tc2, Th17/Tc17, Th9/Tc9, Th22, T follicular helper cells (Tfh), Tregs, and tissue-resident memory T cells (Trm) depending on antigen concentration, co-stimulation and cytokine signals (169). Pathophysiology of aGVHD is largely driven by Th1/Tc1 responses, particularly in intestinal disease (170). Their implication in GVHD has been demonstrated in murine models in which T-bet deficient donor T cells induced less severe GVHD (171). Furthermore, IFN γ and TFN α have been found in human aGVHD lesions, suggesting the importance of Th1/Tc1 responses in human aGVHD (172). Nevertheless, IFN γ may have context-dependent effects in GVHD. Transplantation of IFN γ -deficient donor cells delayed GVHD and reduced its severity in non-irradiated recipient mice, probably due to impaired CTL function (173). In contrast, GVHD was worsened in lethally irradiated recipients transplanted with IFN γ -deficient donor T cells (174). Another cytokine, IL-2 also displays dual roles in GVHD. On one hand, IL-2 promotes the activation and proliferation of T cells and, on the other hand, low-dose IL-2 therapy preferentially enhances Tregs and therefore reduces GVHD in mice and humans. Besides Th1/Tc1 responses, Th17/Tc17 cells are another main subset implicated in aGVHD, particularly in skin and lung lesions (175-177). The Th17/Tc17 differentiation is driven by TGF- β 1, IL-6 and IL-23. Mouse experiments showed that Th17 cells are sufficient to induce GVHD by themselves (175). Recent evidence showed that tissue-resident memory T cells (Trm) are highly resistant to depletion after

MAC or TBI and therefore persist in recipient tissues after allo-HSCT (178). Cytokine-producing recipient Trm cells were found in skin and colon of patients with aGVHD in the presence of donor APCs, suggesting that Trm could contribute to local inflammation (179, 180). A humanized model of GVHD-like dermatitis supports their role in cutaneous GVHD.

Despite the well-established pathogenic roles of Th1/Tc1 and Th17/Tc17 subsets, other T cell lineages may exert protective functions in aGVHD, even if results can sometimes be controversial. Several studies have reported that adoptive transfer of Th2/Tc2 cells into recipient mice decreased aGVHD severity, particularly in GI lesions (181). Likewise, recipients of Th2-cytokine-deficient donor cells (IL-4/5/9/13 knock-out) developed more severe GVHD than those of wild-type donor cells, supporting the protective contribution of Th2 responses (182). In contrast, other experimental models have implicated Th2 responses in cutaneous, hepatic and pulmonary GVHD (177, 183), highlighting the context-dependent effects of this subset. Th9/Tc9 cells, which secrete IL-9 and IL-10, remain poorly studied in GVHD. However, adoptive transfer of Th9/Tc9 has been reported to prevent GVHD (184). The impact of Th22 cells and their leading cytokine IL-22 on GVHD remains unresolved. IL-22 deficiency aggravated intestinal GVHD in a murine model, suggesting a protective effect of IL-22 (185). Conversely, in a distinct haploidentical mouse model, IL-22 deficiency decreased GVHD severity (186), and exogenous IL-22 administration worsened aGVHD by promoting Th1/Tc1 expansion and reducing Tregs (187).

In contrast to these variable outcomes, the role of Tregs in controlling aGVHD has proven far more consistent. Two landmark studies in the early 2000s demonstrated that depletion of donor Tregs (CD4+CD25+) from the graft exacerbated murine GVHD, whereas co-transfer of freshly isolated donor Tregs (at a ratio of 1:1 or 1:2 with conventional T cells) significantly delayed aGVHD onset, and even fully prevented disease in some mice (188, 189). Infusion of *ex vivo* expanded donor Tregs, cultured in the presence of allogeneic APCs and IL-2, reproduced these protective effects in mice, although their efficacy was time-limited (188). Importantly, co-transplantation of Tregs preserved GVL activity in murine models (190). These promising results laid the groundwork for clinical translation that will be discussed later in the present introduction.

Tregs exert immunosuppression through diverse mechanisms, including the production and activation of TGF- β 1. In xGVHD mouse model, we previously showed that administration of anti-TGF- β mAbs or anti-GARP:TGF- β 1 abolished the protective effect of Tregs on GVHD severity (23). Consistently, in a MHC-mismatched mouse model, blockade of TGF- β increased aGVHD severity and enhanced donor T cell proliferation, suggesting that TGF- β 1 could act by limiting T cell activation (191). TGF- β 1 could also promote differentiation of DCs into a tolerogenic phenotype capable of inducing pTregs (192). Clinical evidence supporting a protective role for TGF- β 1 remains limited, although an inverse correlation between serum TGF- β 1 level and aGVHD severity has been reported (193). Whether this reflects a causal contribution or is secondary to GVHD remains unclear. Additionally, several studies suggest that polymorphisms of *TGFB1* and *TGFB2* may influence aGVHD susceptibility and severity (194-196).

(3) Effector phase characterized by T cell mediated organ damage

Following activation, effector T cells gradually migrate from secondary lymphoid organs to GVHD target tissues, guided by gradients of chemokines (162, 197). Consistent with this, CXCR3 inhibition reduced GVHD in mice (198).

Tissue injury is mediated by donor T cells through a combination of cytokines and cytotoxic mechanisms. Cytotoxic T lymphocytes (CTLs) induce recipient cell apoptosis via both Fas/Fas ligand (FasL) and the perforin/granzyme pathway. In parallel, pro-inflammatory cytokines secreted by activated T cells, such as TNF- α and IL-1, directly induce apoptosis of recipient epithelium, amplifying local inflammation independently of alloantigen expression (164). Cellular damage and translocation of PAMPs participate to increase the recruitment of innate immune cells, which in turn will increase the production of pro-inflammatory cytokines. Altogether, these processes intensify tissue destruction and promote further release of pro-inflammatory cytokines, driving the amplification and propagation of a cytokine storm in the GI tract, but also in other organs including the liver, skin and thymus.

3.4 Chronic GVHD

3.4.1 Clinical presentation and risk factors

Chronic GVHD is a complication occurring in 30 to 50% of patients more than 100 days after allo-HSCT, despite the use of standard prophylaxis. Recipients who develop cGVHD experience reduced quality of life, increased long-term morbidity, and higher non-relapse mortality, due both to cGVHD complications and prolonged immunosuppressive therapy (199). cGVHD is similar to an autoimmune syndrome, characterized by immune dysregulation, persistent inflammation, and progressive tissue fibrosis. Nearly all organs may be affected, including skin, oral cavity, eyes, lungs, genital tissue and musculoskeletal system (200). A prior episode of aGVHD is a strong risk factor for subsequent development of cGVHD. Additional risk factors include the use of PBSCs as the graft source, HLA-disparities between donor and recipient, an unrelated donor, transplantation of female donor cells to male recipients, and older age of either donor or recipient (201).

Diagnosis and grading of cGVHD are based on criteria established by the National Institutes of Health (NIH) (200). Diagnosis requires either a diagnostic manifestation of cGVHD or a distinctive lesion confirmed by biopsy or other supporting tests. Disease severity is scored across 8 organ systems (skin, mouth, eyes, GI tract, liver, lungs, fasciae/joints, and genital tract), each assessed for the severity of involvement. The cumulative organ scores generate an overall severity classification (mild, moderate, or severe), which strongly correlates with OS (202).

3.4.2 Pathophysiology

The biological mechanisms underlying cGVHD are less well understood than those driving aGVHD. Current pathophysiological models integrate evidence from preclinical murine systems and clinical data, yet no single individual model fully recapitulates the wide clinical and immunological spectrum of cGVHD. The proposed model divides the pathophysiology of cGVHD into 3 interconnected phases (**Figure 11**) : (1) early inflammation caused by tissue injury, (2) thymic damage with dysregulation in T and B cells, and (3) aberrant tissue repair with fibrosis (203). Early tissue damage, differentiation of T cells in Th1/Tc1 and Th17/Tc17, and release of pro-inflammatory cytokines by innate immune cells following conditioning are shared initiating events in both aGVHD and cGVHD. This is supported by evidence of Th1 and Th17 transcriptional signature, and increased frequencies of IFN γ -producing CD8⁺ T cells in biopsies of cGVHD lesions (204).

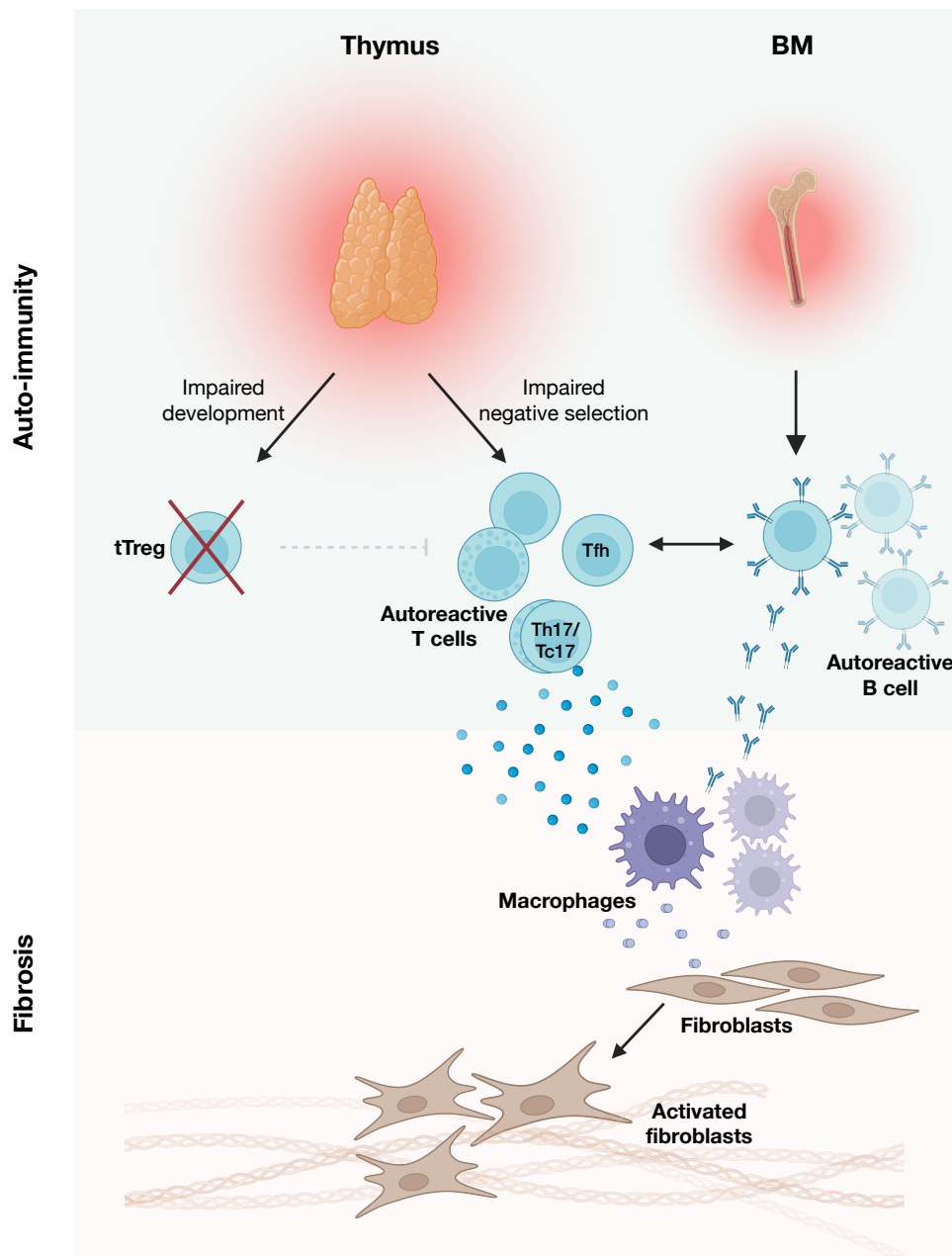


Figure 11. Pathophysiology of cGVHD

Thymic injury impairs negative selection and tTreg development, enabling autoreactive donor T cells to emerge. In parallel, defective B cell development and interaction with T follicular helper cells increase the production of autoantibodies. Activated by IL17 and autoantibodies, macrophages promote fibroblast differentiation and secretion of collagen, ultimately producing progressive tissue fibrosis.

Thymic dysfunction and failure in negative selection

The thymus is essential to establish tolerance through positive and negative selections that eliminate autoreactive T cells. Conditioning regimens and alloreactive T cell responses during aGVHD can damage the thymic microenvironment, leading to depletion of thymocytes, loss of thymic epithelial cell (TEC) integrity, and disruption of the medullary architecture required for effective negative selection and tTreg development.

The contribution of thymic dysfunction to cGVHD has been extensively studied in murine models. Preclinical studies demonstrated that MHC-mismatched transplantation leads to defective negative selection, enabling donor-derived autoreactive T cells to emerge and drive cGVHD manifestations (205, 206). The impact of thymic-dependent development of autoreactive T cells was highlighted by the absence of cGVHD manifestations in thymectomized recipients of allogeneic donor cells. Moreover, keratinocyte growth factor treatment enhanced TEC recovery and prevented the development of autoreactive T cells, demonstrating that preservation of thymic structure can mitigate cGVHD (206). However, experiments lacking donor BM progenitors failed to show thymic dependence, suggesting that cGVHD can also arise independently of negative selection, likely driven by chronic inflammation mediated by mature alloreactive donor T cells present in the graft (207, 208). These findings indicate that thymic involvement may be model-dependent, and is not universally required for disease induction, at least in mice. Mechanistic murine studies indicated that conditioning regimens upregulate Fas and death receptor 5 (DR5) on thymic stromal cells while reducing antiapoptotic regulators, rendering TECs vulnerable to killing by donor CD8⁺ alloreactive T cells (209, 210). In addition, syngeneic mouse models showed that medullary TECs (mTECs), including AIRE⁺ mTECs, exhibit prolonged dysfunction after allo-HSCT (211). These processes drive architectural destruction, and therefore impaired negative selection. Although effects on tTregs were not directly measured in mice and humans, the known dual role of mTECs in both negative deletion and tTreg development suggests concomitant impairment of the tTreg compartment (211).

Collectively, these preclinical models converge towards the concept that thymic damage, mediated by conditioning and alloreactive donor T cells, facilitates the emergence of autoreactive T cells that are not restrained by tTregs, thereby contributing to the loss of tolerance characteristic of cGVHD.

Autoreactive B cells

Thymic-driven T cell dysregulation can further enhance aberrant B cell activation and loss of tolerance, given that B cell functions depend on interaction with CD4⁺ T cells. Moreover, the development of donor-derived B cells, whose tolerance is established in the BM, is impaired by the destruction of BM components during aGVHD, allowing autoreactive B cells to escape deletion and contribute to autoimmune manifestations of cGVHD (212).

In GVHD, B cells exhibit an increased BCR responsiveness, leading to aberrant B cell activation and survival (213). Activated B cells participate in the formation of germinal center structures in collaboration with T follicular helper (Tfh) cells and cytokines, such as IL-17 and IL-21 secreted by Tfh and Th17 cells. Tfh cells are a subset of CD4⁺T cells characterized by expression of the transcription factor Bcl-6, high surface levels of CXCR5 and production of IL-21. They migrate into germinal centers where they provide critical help for B cell maturation, class switching and antibody

production (214). A major driver of B cell dysregulation in cGVHD is the elevation of circulating B cell activating factor (BAFF). High plasmatic BAFF concentrations have been strongly associated with autoreactive B cell survival, autoantibody production, and abnormal B cell activation in cGVHD (215-217). Elevated BAFF levels correlate also with active cGVHD and are considered as a potential biomarker of disease activity (217). Furthermore, regulatory B cells (Bregs) are profoundly disturbed in cGVHD. Bregs, characterized by their ability to secrete IL-10 and TGF- β 1, are reduced in frequency and exhibit impaired IL-10 production in patients with cGVHD, likely reflecting defective reconstitution of the memory B cell population from which many human Bregs arise (218).

Through Tfh-derived signals, activated B cells undergo somatic hypermutation and clonal expansion, ultimately producing high-affinity antibodies (219). In cGVHD, breakdown of B cell tolerance leads to the production of autoantibodies, including anti-double-stranded DNA antibodies, and antibodies directed against minor HAs such as Y chromosome-encoded proteins (220). These antibodies are detectable in approximately 50% of recipients within 4-12 months after female donor-to-male recipient allo-HSCT and have been correlated with clinical cGVHD. These antibodies contribute to tissue damage both through immune complex deposition and activation of macrophages.

Macrophages

In cGVHD, impairment of organ function is caused by aberrant tissue repair and progressive fibrosis. Characteristic lesions display excessive deposition of ECM components, mainly collagen, that progressively replace tissue architecture (221).

Macrophages are key players in the development of fibrosis by activating fibroblasts to produce collagen (222, 223). Macrophages also secrete metalloproteinases capable of degrading ECM components, thereby contributing to tissue remodeling. Histological analyses showed significant macrophage infiltration within fibrotic lesions of cGVHD (224). Both IL-17A and colony-stimulating factor 1 (CSF-1) contributed to macrophage recruitment and accumulation in affected tissues (111, 224). Inhibition of CSF-1 signaling using an anti-CSF1R blocking mAb showed reduced cutaneous and pulmonary cGVHD by depletion of circulating monocytes and decreased tissue infiltration by macrophages (224). Via secretion of PDGF and TGF- β 1, macrophages promote differentiation of fibroblasts into myofibroblasts, which secrete collagen (225). *In vivo* experiments in sclerodermatous cGVHD models showed that skin-infiltrating mononuclear cells express high levels of latent TGF- β 1 on the surface ; how this TGF- β 1 can be activated has not been demonstrated yet. However, administration of blocking TGF- β 1 mAbs from two weeks after transplantation decreased cutaneous cGVHD severity, suggesting a role of TGF- β 1 in the establishment of fibrosis in cGVHD mice (191, 224).

Macrophages are also activated by autoantibodies through their Fc receptors. It suggests a potential link between B cell activation and macrophage-mediated fibrosis. Supporting this notion, murine studies showed reduced fibrosis and GVHD severity in B cell deficient mice, or following B cell depletion by anti-CD20 mAbs (226). Together, these findings suggest a complex interplay between macrophages, T and B cells in cGVHD.

3.5 Targeting the immune system to prevent or treat GVHD

Multiple strategies have been developed to prevent GVHD, and their selection is influenced by the type of donor, stem cell source, underlying disease and comorbidities displayed by the recipient. Standard GVHD prophylaxis aims to limit donor T cell alloreactivity and typically combines an antimetabolite with a calcineurin inhibitor. In addition, anti-thymocyte globulin (ATG) is commonly administered in patients receiving grafts from unrelated donors or composed of PBSCs (227, 228). Over the past years, ATG has begun to be replaced by post-transplant cyclophosphamide (PTCy), particularly in the setting of mismatched transplantation.

Despite the widespread use of prophylactic regimens, 30-50% of patients still develop GVHD, a complication associated with considerable morbidity and mortality. Since the 1980s, GC has remained the first-line treatment for both aGVHD and cGVHD, with a systemic administration for diseases of severity grade $\geq$ II. However, approximately half of patients become GC-refractory, resulting in poor outcomes and a median survival of only 6 months. For more than 3 decades, no standard second-line therapy could be established, given the lack of robust large-scale randomized trials (228). Recently, four new agents have been approved by the FDA for adult steroid-refractory GVHD : ruxolitinib (JAK inhibitor) for a- and cGVHD, and ibrutinib (ITK/BTK inhibitor), belumosudil (ROCK2 inhibitor), and axatilimab (CSF1R inhibitor) for cGVHD. To date, only the ruxolitinib has received approval for the use in Europe from the European Medicines Agency (EMA). Although these therapies represent major advances, toxicities and treatment resistance remain important clinical challenges.

The following section of this introduction will describe current therapeutic approaches and promising strategies under clinical investigations (Table 1). It does not aim to provide an exhaustive overview of all treatments for GVHD but will instead focus on those that modulate the immune system to achieve effective GVHD control.

Prophylaxis	Treatment	
aGVHD & cGVHD	aGVHD	cGVHD
• <u>Standard prophylaxis</u> Antimetabolites (MTX, MMF) Anti-calcineurin (Cyclosporin A, Tacrolimus) <i>Sirolimus</i>	• <u>1st line</u> Glucocorticoids • <u>Steroid-refractory</u> JAK inh. (Ruxolitinib) Extracorporeal photopheresis Mesenchymal stromal cell infusion (only in children) <i>Regulatory T cell infusion</i> <i>Low-dose IL-2</i>	• <u>1st line</u> Glucocorticoids • <u>Steroid-refractory</u> JAK inh. (Ruxolitinib) BKT/ITK inh. (Ibrutinib) ROCK2 inh. (Belumosudil) CSF1R inh. (Axatilimab) Extracorporeal photopheresis <i>Regulatory T cell infusion</i> <i>Low-dose IL-2</i>
• <u>T cell depletion</u> <i>Ex vivo depletion</i> Anti-thymocyte globulin Alemtuzumab Post-transplant cyclophosphamide • <u>Costimulatory inhibitors</u> Abatacept <i>Siltagliptin</i>		

Table 1. Prophylaxis and treatment in aGVHD and cGVHD.

In **bold**, standard of care approved by FDA and EMA ; In regular, approved by the FDA but not by the EMA ; in *italics*, not approved yet and only available in clinical trials. MTX, methotrexate ; MMF, mycophenolate mofetil.

3.5.1 Limiting donor cell alloreactivity

3.5.1.1 Standard prophylaxis

3.5.1.1.1 *Antimetabolites*

Antimetabolites were the first agents used historically for GVHD prophylaxis. Methotrexate (MTX) and mycophenolate mofetil (MMF) decrease the proliferation of activated T cells by impairing purine metabolism. More specifically, MTX inhibits the dihydrofolate reductase, an enzyme required for purine and thymidine synthesis, whereas MMF blocks the inosine-5'-monophosphate-dehydrogenase, thereby inhibiting guanosine monophosphate synthesis. In addition, MMF and MTX could promote apoptosis and activation-induced cell death (AICD) in activated T cells (229).

Clinical trials demonstrated that combining MTX with a calcineurin inhibitor (CNI) is superior to MTX alone, resulting in lower rates of grade $\geq$ II aGVHD and improved long-term survival (230). However, MTX is associated with major toxicities in 30-60% of patients, including severe oral mucositis, hepatotoxicity, nephrotoxicity and delayed engraftment. In contrast, MMF generally exhibits a more favorable toxicity profile (231), although its prophylactic effectiveness is limited in some situations. A small phase II randomized single-center trial reported that MMF was less effective at preventing aGVHD in MAC allo-HSCT, particularly in MUD, without differences in relapse or mortality (232, 233). More recently, a large retrospective study found that MTX + CNI was associated with reduced overall mortality, non-relapse mortality (NRM), and aGVHD compared to MMF + CNI in patients transplanted for chronic myeloid malignancies or secondary AML (234). Accordingly, current European recommendations support MTX + CNI as the prophylaxis of choice for patients undergoing MAC (233, 235), while MMF + CNI is generally preferred in RIC or NMA regimens, based on clinical practice more than randomized trials (227, 228).

3.5.1.1.2 *Calcineurin inhibitors*

Cyclosporin A and tacrolimus inhibit the activity of the calcineurin-calmodulin-calcium complex, an important signaling pathway downstream of the TCR. CNIs prevent the dephosphorylation and nuclear translocation of NFAT, thereby suppressing the transcription of IL-2 and other cytokines required for activation and proliferation of T cells (236). However, CNIs also impair Treg functions, which similarly depend on IL-2 for proliferative and immunosuppressive activity (237).

Clinical studies have demonstrated that cyclosporin A combined with MTX is superior to cyclosporine A alone for the prevention of aGVHD and is associated with improved survival (238). Moreover, the combination of tacrolimus with MTX has also been shown to reduce the incidence of aGVHD more effectively than cyclosporin A + MTX, although without a survival benefit (239). CNIs are usually initiated 1-3 days before allo-HSCT and continued for approximately 3-6 months (228).

3.5.1.1.3 Sirolimus

Sirolimus, also known as rapamycin, is an inhibitor of the mammalian target of rapamycin (mTOR), a signaling pathway activated downstream of TCR, costimulatory and cytokine receptors. Inhibition of mTOR reduces the activation and proliferation of T cells, while promoting the expansion and function of Tregs. Given that the mTOR pathway is constitutively suppressed in Tregs, IL-2 signals through the STAT5 pathway to promote FOXP3 stability. Therefore, Tregs preferentially expand in the presence of sirolimus (240). Sirolimus also exerts broader immunosuppressive effects, including inhibition of antigen presentation and maturation of DCs to a tolerogenic phenotype (241).

Given that CNIs impair Treg expansion and function, replacing them by sirolimus, which supports Treg proliferation, represents a biologically rational strategy. Several phase III clinical trials have demonstrated that sirolimus is associated with reduced aGVHD severity, either when added to standard prophylaxis in NMA conditioning (242) or when used as replacement for tacrolimus in both MAC and NMA settings (243). However, improvement in OS was inconsistent across trials, and patients receiving sirolimus exhibited an increase in endothelial injury. Nevertheless, sirolimus has recently regained interest as an alternative to tacrolimus in haplo-HSCT using MMF + PTCy (244), but it is still only available in clinical trials.

3.5.1.2 T cell depletion

3.5.1.2.1 Ex vivo depletion

T cell depletion can be performed *ex vivo* using immunomagnetic beads applied to collected graft cells, either through positive selection of CD34⁺ HSCs or negative depletion of CD3⁺ or CD3⁺/CD19⁺ cells. T cell depletion is highly effective in preventing GVHD, with reported incidences of 7-40% in the absence of post-transplant immunosuppression. However, this strategy is associated with an important delay in adaptive immune reconstitution, resulting in increased risks of relapse, opportunistic infections and mortality (245, 246). Nonetheless, *ex vivo* T cell depletion remains a potential option in allo-HSCT performed for non-malignant diseases, particularly in children. In a recent matched-pair analysis, recipients of T cell-depleted grafts showed lower GVHD incidence but similar OS compared to unmanipulated BM transplantation in patients with aplastic anemia (247).

Over time, techniques have shifted from complete to selective T cell depletion, with the objective of preserving beneficial immune subsets such as NK cells, $\gamma\delta$ T cells and myeloid progenitors. These cell types contribute to improve immune reconstitution after allo-HSCT (248). Depletion of CD45RA⁺ naïve T cells reduces cGVHD, and the early peripheral reconstitution of memory T cells via HPE reduces infections and relapse. In contrast, depletion of $\alpha\beta$ T cells, although effective in preventing GVHD, is associated with a higher risk of viral infections. These selective approaches have been particularly explored in pediatric transplantation, where long-term immunosuppression carries substantial risks and should be avoided whenever possible (249). *Ex vivo* depletion remains only available during clinical trials.

3.5.1.2.2 Anti-thymocyte globulin

In vivo T cell depletion can be achieved by administration of anti-thymocyte globulin (ATG), alemtuzumab (anti-CD52), or post-transplant cyclophosphamide.

Rabbit ATG is polyclonal immunoglobulins produced after immunizing rabbits with human thymocytes or Jurkat T cells. These antibodies target multiple immune cell surface proteins, including CD2, CD3, CD4, CD8, MHC and adhesion molecules (250). ATG induces T cell depletion via complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). NK and B cells are relatively preserved, showing only a mild depletion. ATG has a long half-life, remaining in plasma for weeks after infusion. Consequently, when administered at the time of allo-HSCT, ATG depletes both host and donor T cells, therefore delaying adaptive immune reconstitution. Nowadays, given its high efficacy in preventing GVHD, ATG is currently recommended in European guidelines for MUD (251, 252) and MMUD allo-HSCT, as well as MSD transplanted with PBSCs (253-255).

3.5.1.2.3 Alemtuzumab

Alemtuzumab is a humanized anti-CD52 mAb that induces profound depletion of T cells, B cells, NK cells, monocytes, and dendritic cells. Compared to ATG, alemtuzumab causes broader and deeper immunosuppression, particularly a very prolonged lymphopenia. Alemtuzumab significantly reduces both acute and chronic GVHD, with incidences below 10%, substantially lower than conventional prophylaxis. Because of its deep and prolonged lymphodepletion, alemtuzumab is associated with multiple adverse outcomes including graft failure and potential increased relapse in malignant settings (256). For these reasons, alemtuzumab is not commonly utilized and, when it is, preferentially in allo-HSCT for non-malignant disease. It may also be considered as a second- or third-line treatment for GC-refractory cGVHD (227).

3.5.1.2.4 Post-transplant cyclophosphamide

In recent years, the clinical landscape of allo-HSCT has been transformed by the successful development of haploidentical HSCT, driven by the introduction of post-transplant cyclophosphamide (PTCy). Prior to the implementation of PTCy, haplo-HSCT was limited to specialized centers and relied on the infusion of high doses of *ex vivo* selected CD34⁺ progenitor cells following myeloablative conditioning (257). To preserve the GVL effect, donor NK cells were co-transfused (258). However, the long lasting immune T cell reconstitution resulted in high infectious-related morbidity and mortality (259). Administered on days +3 and +4, PTCy has enabled outcomes in haplo-HSCT comparable to those achieved with MSD, thereby making haploidentical donors a realistic and widely accessible option for virtually all patients (260). Cyclophosphamide, an alkylating agent, mitigates the immunological consequences of HLA disparity and significantly decreases GVHD incidence when administered in mismatched HSCT, including MUD and MMUD transplantation (261).

Multiple preclinical studies have investigated the mechanisms underlying PTCy-mediated GVHD prevention. While early work suggested that PTCy selectively depletes proliferative and alloreactive T cells in murine models (262), subsequent experiments demonstrated that some alloreactive T cells persisted after allo-HSCT but exhibited functional impairment. These cells were unable to induce clinical GVHD when reinfused into secondary mismatched recipients. Donor T cells from PTCy-treated GVHD mice failed to proliferate and secrete inflammatory cytokines upon alloantigen

restimulation *in vitro* (263). Moreover, a central component of PTCy therapy is the preservation of Tregs (263). Murine experiments have shown that donor Tregs are essential for PTCy efficacy, as their depletion before or shortly after PTCy abolishes GVHD protection (264). Human and murine functional Tregs were shown to persist after PTCy therapy in xGVHD or MHC-matched mouse models. This survival advantage is attributed to their high expression of aldehyde dehydrogenase (ALDH), an enzyme that metabolizes cyclophosphamide into inactive metabolites. Consequently, Tregs and HSCs, which also express high ALDH levels, are resistant to PTCy-mediated cytotoxicity. Surviving Tregs retain suppressive function and exhibit stable *FOXP3* demethylation. Collectively, the preclinical evidence suggests that PTCy not only eliminates alloreactive T cells, but also preserves Treg homeostasis.

The first clinical demonstration of PTCy efficacy was reported by Luznik and colleagues in 2008 in haploidentical HSCT (265). Since then, its use has expanded to HLA-matched and -mismatched allo-HSCT, supported by multiple prospective randomized trials (227, 266, 267). Despite its advantages in reducing GVHD, PTCy is associated with high susceptibility to infectious complications (268), which remain a leading cause of NRM, regardless of donor type (269). In particular, invasive bacterial infections and CMV reactivations are major concerns. Importantly, however, no significant increase in relapse has been observed with addition of PTCy to standard GVHD prophylaxis (266, 267).

3.5.1.3 Costimulatory inhibitors

3.5.1.3.1 *Abatacept*

Abatacept is a fusion protein composed of the extracellular domain of CTLA-4 linked to an IgG1 Fc fragment. By binding to CD80 and CD86 on APCs, abatacept blocks their interaction with CD28 and thereby prevents the co-stimulatory signals required for T cell activation. In this way, it mimics one of the functional mechanisms used by Tregs to maintain immune tolerance. In 2021, abatacept received FDA approval for the prophylaxis of aGVHD when administered in combination with MTX and CNI in matched or one-mismatched unrelated donor allo-HSCT (168). However, Abatacept is not approved by EMA for European use. Clinical studies have shown that adding abatacept to standard CNI+MTX prophylaxis significantly reduces the incidence of severe aGVHD and improves OS, achieving outcomes comparable to PTCy prophylaxis in MMUD allo-HSCT.

While abatacept effectively reduces T cell alloreactivity by blocking co-stimulation, its impact on Tregs is more complex. CD28 signaling is also crucial for Treg survival and function, therefore inhibiting this pathway could theoretically impair Treg homeostasis. Supporting this concern, abatacept treatment in systemic sclerosis was associated with reduced Treg counts (270), although some clinical studies in rheumatoid arthritis reported an increase of specific Treg subsets (271). To date, no mechanistic data are available in the context of GVHD, and the effects of abatacept on Treg biology in allo-HSCT remain unknown.

3.5.1.3.2 Inhibition of DPP-4

Dipeptidyl peptidase 4 (DPP-4) inhibitors have been used for many years in the management of type 2 diabetes mellitus (272). By inhibiting DPP-4, these agents prolong the half-life of glucagon-like peptide 1 (GLP-1), thereby increasing insulin secretion. Interestingly, leukocytes possess a surface protein with DPP-4 activity, named CD26 (273). Engagement of CD26 with its ligand on APCs induces upregulation of CD86 and promotes T cell activation (274). Preclinical studies in xGVHD mouse models showed that blocking CD26 on donor T cells with humanized anti-CD26 mAbs prevented aGVHD while preserving GVL effects (275).

In a phase II clinical trial, the addition of sitagliptin, a selective DPP-4 inhibitor, to tacrolimus and sirolimus markedly decreased the incidence of grade II-IV aGVHD, with only 2 of 36 patients developing the disease by day +100 after allo-HSCT (276). However, only two-thirds of the patients were able to take more than 80% of the planned oral doses, as sitagliptin was administered during the first 2 weeks after MAC, a period associated with severe mucositis, nausea and vomiting. Sitagliptin may also confer additional benefits in GVHD by maintaining intestinal homeostasis. Because GLP-1 is produced by intestinal L cells and rapidly degraded by DPP-4, its inhibition could preserve GLP-1 activity and contribute to gut integrity, an important factor in GVHD prevention. This promising approach warrants further evaluation in randomized clinical trials before being recommended in current practice. Sitagliptin could be particularly interesting in patients receiving RIC or NMA regimens, with reduced mucositis and likely better adherence to the full therapeutic course.

3.5.1.4 Kinase inhibitors

3.5.1.4.1 Ruxolitinib

Tissue damage associated with aGVHD is driven in part by inflammatory cytokines, whose effects are mediated through Janus kinases (JAK). Preclinical studies demonstrated that JAK inhibitors significantly decreased the severity of aGVHD. They suppress downstream signaling from multiple cytokine receptors, including those involved in T cell activation and infiltration into target organs (277). JAK inhibitors also impaired MHC-II expression on neutrophils and DCs, as well as the migration of neutrophils from ileum to mesenteric lymph nodes (278, 279). In murine models, ruxolitinib, a selective inhibitor of JAK1 and JAK2, appears more potent than JAK inhibitors that preferentially inhibit JAK2 instead of JAK1. Indeed, preferential inhibition of JAK2 can block pathways that are protective in GVHD (280), such as IL-12, which blocks alloreactive CD4⁺ T cell activation (278) or STAT3 signaling, which is important for the function of myeloid-derived suppressor cells (281). The therapeutic effect of ruxolitinib in mice was associated with reduced production of inflammatory cytokines, impaired T cell expansion and increased frequency of Tregs (282). The increased Tregs are explained by the inhibition of STAT3, which reduces pTreg differentiation (283).

The clinical efficacy of ruxolitinib has been demonstrated in two pivotal randomized trials. REACH2 and REACH3, conducted in patients with steroid-refractory aGVHD and cGVHD, respectively, both demonstrated that ruxolitinib significantly improved overall response rate (ORR) and failure-free survival, compared with best available therapy (284, 285). These results led to the approval of ruxolitinib as the first targeted therapy for GC-refractory acute and chronic GVHD in 2019 and 2021 by FDA and EMA.

3.5.1.4.2 Ibrutinib

Ibrutinib is an irreversible inhibitor of Bruton tyrosine kinase (BTK), therefore blocking downstream BCR signaling. Ibrutinib has demonstrated clinical efficacy in multiple B cell malignancies (286, 287). Interestingly, IL-2 inducible T cell kinase (ITK) shares high sequence and functional homology with BTK and, both *in silico* and *in vivo* studies have shown that ibrutinib also acts as an irreversible ITK inhibitor (287). By blocking ITK, ibrutinib suppresses the activation of NFκB, MAPK, and NFAT pathways, therefore limiting lymphocyte proliferation, differentiation and effector cytokine production. Given the central role of B and T cell dysregulation in the pathogenesis of cGVHD, BTK-ITK inhibition represents a rational therapeutic approach. In preclinical mouse models, treatment with ibrutinib significantly reduced cGVHD severity, including decreased pulmonary immunoglobulin deposition and fibrosis (288).

FDA approval of ibrutinib for the treatment of cGVHD after failure of at least one prior line of systemic therapy was supported by an open-label phase Ib/II study. Overall best response rate was 67% among 42 patients with steroid-dependent or steroid-refractory cGVHD (289). The most severe adverse events included pneumonia, fatigue and diarrhea, which tended to decrease in incidence after the first year of treatment (290). Real-world data further supported the efficacy of ibrutinib in decreasing cGVHD severity following first-line therapy failure, with a best ORR of 56%, most responses occurring within 1 to 3 months (291). However, first-line administration of ibrutinib in combination with GC did not significantly improve cGVHD severity compared with GC alone (292).

3.5.1.4.3 Belumosudil

Belumosudil is a selective inhibitor of rho-associated coiled-coil-containing protein kinase-2 (ROCK2), an enzyme implicated in the regulation of multiple cytokine pathways (including IL-17 and IL-21), growth factor receptor signaling and pro-inflammatory NFκB activation (293). In murine models of cGVHD, treatment with belumosudil resulted in reduced Tfh cell accumulation in the spleen, and decreased immunoglobulin and collagen deposition in the lungs, leading to improved pulmonary function (294). More specifically, inhibition of ROCK2 reduces differentiation of naïve CD4⁺ T cells into Th17 and Tfh subsets through the suppression of STAT3 activation and IL-21, IL-17 and IFN γ production. In addition, belumosudil promotes Treg expansion via upregulation of STAT5 signaling (294).

Phase I/II open-label and phase II randomized clinical trials both demonstrated an ORR around 65-75% after 6 cycles in patients with cGVHD refractory to ≥ 1 line of systemic therapy (295, 296). Two thirds of patients had severe cGVHD and had already received ibrutinib and/or ruxolitinib. Belumosudil provides a therapeutic approach targeting both immune dysregulation and fibrosis. FDA approved belumosudil in 2021 for the treatment of adult patients with cGVHD who have received ≥ 2 prior lines of systemic therapy.

3.5.2 Targeting macrophages

The first effective therapy specifically targeting macrophages in GVHD emerged in 2024 in FDA recommendations. Axatilimab is a humanized IgG4 mAb that blocks signaling of colony-stimulating factor 1 receptor (CSF1R). By inhibiting CSF1R, axatilimab decreases the differentiation, survival and function of CSF1R-expressing monocytes and macrophages, which are involved in inflammation and fibrosis in cGVHD (297, 298). In a sclerodermatous cGVHD mouse model, CSF1 administration increased macrophage infiltration in skin, whereas transplantation of donor cells from *Csf1r*^{-/-} mice significantly reduced cutaneous cGVHD, indicating that CSF1 signaling contributes to cutaneous damage. Consistently, administration of an anti-CSF1R mAb depleted tissue macrophages and decreased cutaneous and pulmonary cGVHD (224).

A phase II randomized trial in 240 patients with recurrent or refractory cGVH reported an ORR of 75% with axatilimab monotherapy, establishing it as the first effective therapy to target macrophages in GVHD (298). Biopsies of cutaneous cGVHD in patients treated with axatilimab showed that therapeutic response was associated with significant depletion in macrophages, but also an increase in FOXP3⁺ Tregs, suggesting a potential link between CSF1R-expressing macrophages and Tregs in cGVHD (299).

3.5.3 Promoting immune tolerance

In addition to traditional approaches, promoting immune tolerance has emerged as a central therapeutic objective in the management of GVHD. In addition to their capacity to limit donor T cell alloreactivity, several of the agents described above, including PTCy and sirolimus, also promote the re-establishment of a tolerant immune environment. Although extracorporeal photopheresis has been used for decades to treat cGVHD lesions, more recent years have seen the rapid development of cell-based therapies. These approaches aim to enhance or restore populations with intrinsic regulatory activity, notably Tregs and mesenchymal stromal cells (MSCs), with the goal of achieving durable immune tolerance rather than sustained immunosuppression.

3.5.3.1 Extracorporeal photopheresis

Extracorporeal photopheresis (ECP) is a photochemotherapy modality in which PBMCs collected by leukapheresis are exposed *ex vivo* to ultraviolet A radiation in the presence of the photosensitizing agent 8-methoxypsoralen (8-MOP). PBMCs are then reinfused into the patient. ECP exerts a broad range of immunomodulatory effects. Although activated 8-MOP induces DNA crosslinking and apoptosis of activated alloreactive T cells, lymphocyte apoptosis alone cannot account for all the therapeutic effects of ECP, since only 5-10% of circulating lymphocytes are treated during a session. Instead, ECP efficacy is thought to rely on the phagocytosis of apoptotic cells by APCs, promoting their differentiation into tolerogenic DCs. These DCs preferentially secrete IL-10 rather than pro-inflammatory cytokines and drive the differentiation and expansion of Tregs. ECP also favors restoration of the Th1/Th2 balance towards a Th2 phenotype (300, 301). In GVHD mouse models, infusion of ECP-treated cells reversed established GVHD, and mice showed increased donor-derived Tregs (302). Consistent with these observations, increased circulating Treg counts have also been observed in patients undergoing ECP (303).

ECP is effective in the treatment of steroid-refractory aGVHD and cGVHD, with particularly important responses in cutaneous GVHD, although benefits are also observed in other affected organs. However, ECP has limited efficacy when used as prophylaxis. Recent meta-analyses demonstrate improvement in ORR and OS in GC-refractory cGVHD, supporting the long-term effectiveness of this therapy (304). Clinical responses have been associated with high platelet counts at treatment initiation (305-307). ECP efficacy may be further enhanced through the addition of subcutaneous low-dose IL-2. This combination favors Treg and NK cell expansion, and therefore increases the Treg:Tconv ratio (308). ECP is characterized by an excellent safety and tolerability, but its use remains limited by logistical challenges. These include the need for venous access, which increases infection risk, as well as processing time, with treatment sessions lasting 1,5 to 3 hours. Standard regimens typically involve two consecutive days every 2-4 weeks for approximately six months.

3.5.3.2 Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) possess high plasticity, self-renewal capacity, and broad immunomodulatory properties. *In vitro*, MSCs are defined by their plastic adherence, expression of CD105, CD73 and CD90, and multipotent differentiation potential (309). MSCs exert immunomodulatory effects mostly through the expression of IDO, release of soluble factors, including TGF- β 1, and the secretion of extracellular vesicles containing messenger RNAs and microRNAs. Via these mechanisms, MSCs inhibit T, B and NK cell functions, induce T cell anergy, enhance Treg activity, promote generation of immature DCs, and favor differentiation of monocytes into M2 (anti-inflammatory) macrophages (310). Although the exact mechanisms underlying immunosuppressive effects of MSCs in GVHD remain elusive, several mouse models have demonstrated their capacity to decrease severe GVHD (311).

Remestemcel-L is the first MSC-based therapy approved by the FDA in 2024 for the treatment of GC-refractory aGVHD in children. MSCs are derived from the BM of healthy adult volunteer donors, expanded *ex vivo* and cryopreserved. In a phase III, prospective single-arm multicenter trial involving 54 pediatric patients treated twice weekly for four weeks, remestemcel-L achieved an ORR of 70% at day +100, including a complete response in 45% (312). However, a large, randomized trial including 260 pediatric and adult patients with steroid-refractory aGVHD found no significant benefit over placebo when *ex vivo* expanded adult MSCs were added to standard therapy, except in the pediatric subgroup and in patients with hepatic GVHD (313). These nuanced results have been reported in several clinical trials in adult patients. Variability in MSC therapeutic efficacy in GVHD has been attributed to differences in cell source, expansion protocols, priming conditions, and intrinsic functional properties such as the ability of MSCs to migrate to GVHD-affected tissues (314). Multiple clinical trials are currently ongoing to evaluate the efficacy of MSC therapy in adult patients with GC-refractory GVHD, using a standardized protocol.

3.5.3.3 Regulatory T cells

Among cellular therapies, adoptive Treg transfer is one of the most promising strategies for both prevention and treatment of GVHD. As discussed above, preclinical models have consistently demonstrated the important role of Tregs in suppressing alloreactive T cells and mitigating GVHD. Adoptive donor Treg transfer is most effective when administered at the time of allo-HSCT, whereas its efficacy is reduced once GVHD lesions are already established. Moreover, preclinical studies indicate that therapeutic benefit requires a Treg:T effector ratio as high as 1:1 or 1:2. The large numbers of Tregs required therefore impose substantial challenges for clinical translation.

The first clinical trial, conducted by Di Ianni and colleagues, provided compelling evidence of donor Treg-mediated GVHD protection in recipients. In this study, patients with high-risk hematological malignancies undergoing haplo-HSCT received freshly isolated donor Tregs 4 days before infusion of HSCs and conventional T cells (Treg:Tconv ratio 1:2) (315). Among 28 treated patients, only 2 developed grade $\geq$ II aGVHD, and none developed cGVHD, despite the absence of post-transplant immunosuppression. Importantly, GVL activity and immune reconstitution were preserved, although infection-related mortality remained high. Subsequent clinical studies in high-risk acute leukemia corroborated these positive results, without increasing relapse rates (316, 317). However, obtaining sufficient donor Tregs is associated with challenging procedures, therefore *ex vivo* expansion has been developed, either by CD3/CD28 stimulation with high amount of IL-2 or in presence of artificial APCs loaded with CD3 mAbs. Tregs derived from UCB and expanded *ex vivo* have also demonstrated safety and therapeutic efficacy. In double UCB transplantation, Tregs reduced both aGVHD and cGVHD without increasing infections, relapse or mortality (318, 319).

Preclinical studies on GVL have shown heterogeneous results, ranging from no impairment to complete suppression of the GVL effect, depending on the tumoral cell line, timing of Treg infusion, and mouse models (190, 316, 320, 321). However, most of the clinical trials showed relatively preserved GVL effects (315). A leading hypothesis is that donor Treg infusion reduces immune dysfunction associated with GVHD, allowing reduced thymic damage and more rapid immune reconstitution, thereby improving GVL efficacy.

The major challenges in Treg cellular therapy include the high cell doses required, often exceeding what can be isolated directly from peripheral blood, and the difficulties in *ex vivo* expansion which requires a high purity of Tregs to avoid excessive proliferation of effector T cells. Attempts to generate induced Tregs (iTregs) from naïve CD4⁺ T cells have been unsuccessful so far. Although iTregs express FOXP3 *in vitro*, they lose suppressive function and FOXP3 stability after infusion, and fail to protect mice from lethal GVHD, even at high Treg:Tconv ratios (321, 322). Nevertheless, their function could be partially stabilized by co-administration of sirolimus and low-dose IL-2, but clinical evidence remains limited (321). To reduce the number of Tregs required and enhance potency, next-generation strategies propose to generate antigen-specific Tregs. Compared to polyclonal Tregs, alloantigen-specific Tregs exhibit enhanced suppressive function and should require substantially lower cell numbers. Engineering approaches include CAR-Tregs recognizing HLA-A2 (323) and virus-specific Tregs engineered to target alloreactive T cells via β 2-microglobulin-TCR ζ construct (324).

Large-scale clinical trials of Treg infusion for the treatment of GVHD remain limited. Small case reports have reported promising responses in steroid-refractory GVHD, but robust evidence is still lacking (325). An alternative to adoptive Treg transfer is the *in vivo* expansion of donor-derived Tregs after allo-HSCT through administration of low-dose IL-2, which preferentially stimulates Tregs owing to their constitutively high expression of the high-affinity IL-2 receptor α -chain (CD25). Early phase I and II clinical trials in GC-refractory cGVHD have reported 50-60% partial response rates following daily low-dose IL-2 administered for 8-12 weeks (326, 327). Interestingly, IL-2 therapy was associated with a marked increase in Treg and NK cell counts, accompanied by enhanced STAT5 phosphorylation, while sparing conventional CD4⁺ and CD8⁺ T cells (328). Low-dose IL-2 has also shown promise as a prophylactic strategy, increasing circulating Tregs frequencies (329). In a recent study, the combination of low-dose IL-2 with adoptive Treg infusion was demonstrated to be safe and yielded partial responses in 20% of patients with GC-refractory GVHD (330).

Multiple clinical trials are currently evaluating Treg infusion for both prevention and treatment of GVHD, and combining adoptive Treg transfer with additional pro-Treg strategies may represent a particularly interesting therapeutic approach.

AIM OF THE STUDY

Research in our laboratory has established that the transmembrane protein GARP plays a key role in the activation of latent TGF- β 1 by regulatory T cells (Tregs). We previously generated GARP:TGF- β 1 mAbs that block TGF- β 1 activation by GARP-expressing cells. Structural studies using a crystal structure of the GARP:TGF- β 1 complex bound to a blocking Fab fragment revealed, for the first time, the architecture of latent TGF- β 1 presented by GARP and provided mechanistic insight into the mode of action of blocking GARP:TGF- β 1 mAbs. We demonstrated that blocking GARP:TGF- β 1 mAbs had therapeutic efficacy in solid tumor models resistant to anti-PD1 therapy and in myeloproliferative malignancies, leading to the initiation of clinical trials in patients with locally advanced or metastatic solid tumors. In a model of xenogeneic GVHD in mice, we showed that blocking GARP:TGF- β 1 mAbs inhibited the immunosuppression induced by human Tregs.

Recently, our laboratory developed antibodies that activate, rather than block, latent TGF- β 1 at the cell surface. Given the essential role of TGF- β 1 in maintaining immune homeostasis, we hypothesized that enhancing TGF- β 1 activity may represent a promising therapeutic strategy for autoimmune and alloimmune disorders. During my thesis project, I focused on GVHD, a frequent life-threatening complication after allo-HSCT.

The main objective of my work was to investigate the therapeutic potential of antibody-mediated TGF- β 1 activation in GVHD. The first article presented in the results section was published in Cell Reports (331). It describes how we succeeded in obtaining TGF- β 1 activating mAbs, the mechanistic insights we obtained on their mode of action from biochemical and structural analyses, and their therapeutic efficacy in a model of acute GVHD in mice. The second article presented in the results section is still in preparation and will be submitted for publication shortly. It describes the therapeutic efficacy of TGF- β 1 activating mAbs in a mouse model of chronic GVHD that is characterized by severe cutaneous damage.

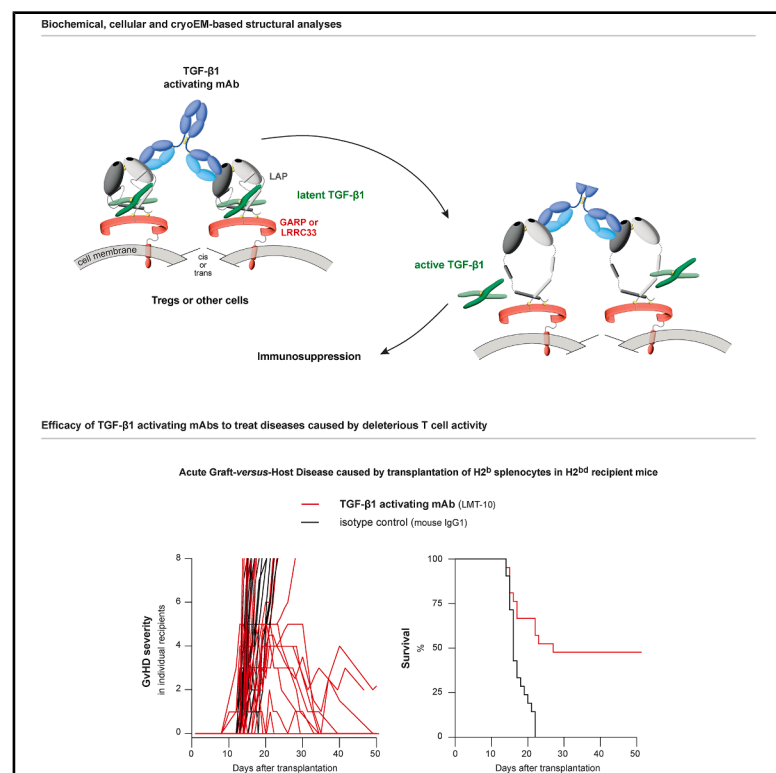
RESULTS

A first set of results, showing that TGF- β 1 activating mAbs increase TGF- β 1 activity to treat severe diseases characterized by excessive immune responses such as acute GVHD, was published as a research article in Cell Reports.

A second set of results is presented as a manuscript in preparation and shows that TGF- β 1 activating mAbs could also serve to treat chronic GVHD, by improving thymic function and controlling type 1 adaptive T cell responses and inflammation.

Antibody-mediated TGF- β 1 activation for the treatment of diseases caused by deleterious T cell activity

Graphical abstract



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In brief

Lambert et al. describe an antibody-based approach targeting the pleiotropic TGF- β 1 cytokine in a manner unexplored thus far. At variance with existing TGF- β 1-targeting strategies aiming to block TGF- β 1 immunosuppression in cancer or fibrosis, their antibodies augment TGF- β 1 activity to treat severe diseases characterized by excessive immune responses.

Highlights

- Latent TGF- β 1 on the surface of cells can be activated by monoclonal antibodies
- TGF- β 1 activation implies profound conformational changes and an active cytoskeleton
- Antibody-mediated TGF- β 1 activation exerts therapeutic effects in graft versus host disease



Article

Antibody-mediated TGF- β 1 activation for the treatment of diseases caused by deleterious T cell activity

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SUMMARY

Transforming growth factor β 1 (TGF- β 1) is an immunosuppressive cytokine produced as a latent homodimer, in which mature TGF- β 1 is encapsulated and kept inactive by the latency-associated peptide (LAP). The transmembrane protein GARP presents latent TGF- β 1 on the surface of regulatory T cells (Tregs) to enable activation and release of mature TGF- β 1 by integrins. Here, we derived monoclonal antibodies (mAbs) that activate latent TGF- β 1 anchored on cells by a transmembrane protein. Biochemical and structural studies by electron cryo-microscopy (cryo-EM) reveal that such mAb-mediated activation requires bivalent binding close to the LAP dimerization interface and crosslinking of two membrane-bound GARP:TGF- β 1 complexes on the same cell or across different cells. Administration of mAbs to mice with graft versus host disease reduced disease severity and increased survival. The therapeutic effect required Tregs. Collectively, our findings demonstrate that activation of membrane-bound TGF- β 1 *in vivo* is achievable with mAbs, introducing new immunotherapeutic options for allo- or autoimmune diseases characterized by deleterious T cell activity insufficiently controlled by Tregs.

INTRODUCTION

Due to the potent immunosuppression it exerts in mice and humans, production of transforming growth factor β 1 (TGF- β 1) is tightly regulated at a post-translation level, prior to its binding to a hetero-tetrameric receptor composed of T β RI and T β RII chains. Mice with a germline deletion of the *Tgfb1* gene or a T cell-specific deletion of *Tgfb2* die 3–4 weeks after birth from a lymphoproliferative multisystemic autoimmune syndrome, indicating that TGF- β 1 activity in T cells is required to maintain immune tolerance.^{1–3} Due to the widespread expression of the TGF- β receptor, TGF- β 1 also acts on most other if not all immune cell types, regulating their differentiation, proliferation, and activation.⁴ Owing to these pleiotropic functions, production of active TGF- β 1 is regulated at multiple levels.⁵ Translation of *TGFB1* mRNA leads to a precursor comprising a signal peptide followed by a 43 kDa amino acid (aa) sequence called LAP (latency-associated peptide) and a 12.5 kDa sequence corresponding to the mature TGF- β 1 cytokine. After signal peptide removal

and interchain disulfide bond formation, the pro-TGF- β 1 homodimer is cleaved by a furin convertase. The resulting LAP and mature TGF- β 1 homodimers remain non-covalently associated, forming a complex called “latent TGF- β 1” because LAP prevents binding of mature TGF- β 1 to its receptor. Structural studies by X-ray crystallography revealed that the N-terminal α 1 and α 2 helices and an intervening loop called the latency lasso act as a straitjacket embracing the extremities of the mature TGF- β 1 dimer that are needed for interaction with the TGF- β receptor.⁶ The C termini of the LAP monomers form rigid structures called the arms, overhung by a “bowtie” where the monomers are linked via interchain disulfide bonds.⁶ Whereas most cell types and virtually all immune cells produce latent TGF- β 1, only very few release mature TGF- β 1 from the LAP straitjacket. This process is referred to as “TGF- β 1 activation.” It is required to induce auto-crine or paracrine TGF- β 1 signaling and occurs via highly regulated cell type-specific mechanisms.

We and others previously showed that immunosuppressive lymphocytes known as Tregs (regulatory T cells) activate latent



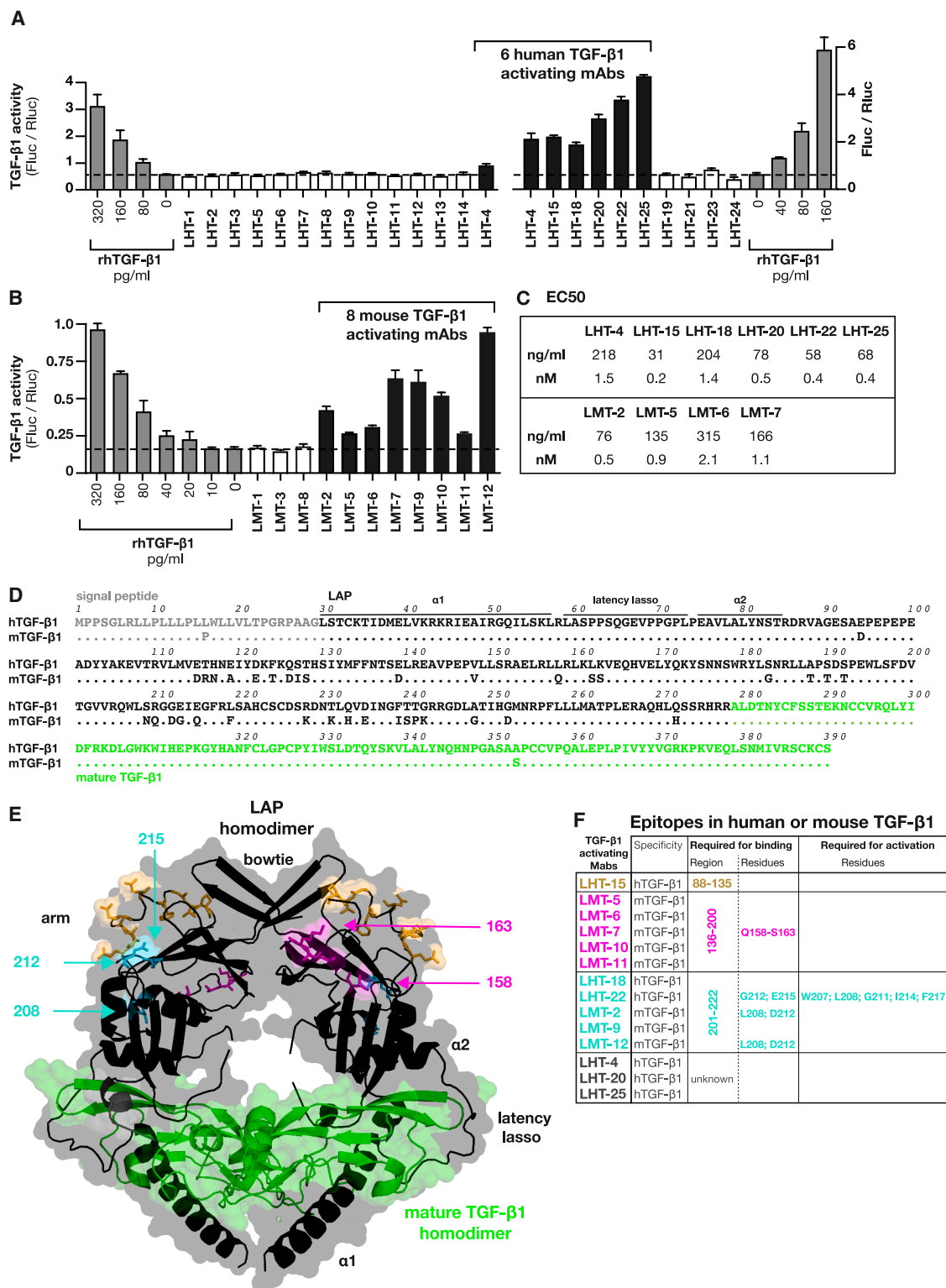


Figure 1. Human and mouse TGF-β1-activating mAbs bind close to the LAP dimerization interface

(A) HEK293T cells were co-transfected to express human GARP:TGF-β1 complexes, firefly luciferase (Fluc) under the control of a CAGA element as a reporter for TGF-β1 activity, and *Renilla* luciferase (Rluc) as a transfection control and then incubated with the indicated mAbs (20 μg/mL) or serial dilutions of active recombinant human TGF-β1 (rhTGF-β1). Bar graphs represent ratios of Fluc to Rluc activity (mean of triplicates + SD). Black bars highlight the 6 LHT activating

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TGF- β 1 by a mechanism that requires the transmembrane proteins GARP and integrin $\alpha_v\beta_8$.^{7–11} In the endoplasmic reticulum of Tregs, latent TGF- β 1 is covalently linked to the extracellular leucine-rich repeat (LRR) domain of GARP via formation of disulfide bonds between one cysteine in each LAP monomer and two cysteines in the GARP monomer. This results in presentation on the Treg surface of membrane-bound latent TGF- β 1, which can then bind integrin $\alpha_v\beta_8$ via an RGD motif in the arm region of LAP. T cell receptor (TCR) stimulation of Tregs induces surface expression of GARP:(latent)TGF- β 1 complexes and $\alpha_v\beta_8$, leading to TGF- β 1 activation and autocrine or paracrine signaling in Tregs and neighboring cells.^{7,8,10,11} Another LRR-containing transmembrane protein called LRRC33 presents latent TGF- β 1 on the surface of microglia (i.e., macrophages of the CNS) for activation by integrin $\alpha_v\beta_8$.¹²

The precise molecular mechanisms leading to activation of latent TGF- β 1 presented by GARP or LRRC33 to $\alpha_v\beta_8$ are not completely resolved. Most studies suggest exertion of mechanical forces on latent TGF- β 1 to release mature TGF- β 1. A major mechanistic hypothesis is that application of pulling forces on latent TGF- β 1 in opposing directions would unroll the LAP straitjacket and release mature TGF- β 1 in the extracellular milieu.^{13,14} Alternatively, it has been proposed that induction of modest conformational changes in the latency lasso could expose the extremities of mature TGF- β 1 for binding to the cytokine receptor.¹⁵

We previously derived anti-GARP:TGF- β 1 monoclonal antibodies (mAbs) that block TGF- β 1 activation and immunosuppression by Tregs and showed that the mAbs enhanced anti-tumor T cell responses and exerted therapeutic anti-tumor activity in mice with solid tumors or myeloproliferative neoplasms.^{11,14,16–18} Phase 1 and 2 trials are currently ongoing to test blocking anti-GARP:TGF- β 1 mAbs in patients suffering from advanced or metastatic solid tumors as a novel form of cancer immunotherapy (ClinicalTrials.gov: NCT03821935, NCT05822752, and NCT06109272).

Here, we sought to develop mAbs that enhance rather than block TGF- β 1 activation and Treg immunosuppression, with the objective to address diseases characterized by excessive T cell activity insufficiently controlled by Tregs. Integration of *in vitro* and *in vivo* experimental findings with structural insights obtained from complexes between GARP:TGF- β 1 and antibody fragments (Fabs) reveals that TGF- β 1 activation by a mAb requires crosslinking of at least two membrane-bound GARP:TGF- β 1 complexes on cells and a profound conformational change in latent TGF- β 1, shedding light on the molecular events that occur during TGF- β 1 activation by integrins *in vivo*.

RESULTS

mAbs that activate human or mouse TGF- β 1 recognize epitopes close to the LAP homodimerization interface

We screened 134 mAbs directed against human or mouse GARP, GARP:TGF- β 1, or latent TGF- β 1 for their ability to activate TGF- β 1 in a reporter assay. HEK293T cells were co-transfected to express surface human or mouse GARP:TGF- β 1 complexes, firefly luciferase under the control of a TGF- β 1-responsive promoter, and *Renilla* luciferase to normalize for transfection efficacy. Luciferase activity was measured in transfected cells 18 h after incubation with mAbs. Fourteen mAbs activated TGF- β 1 in the assay (Figures 1A and 1B): six are directed against human latent TGF- β 1 (LHT-4, -15, -18, -20, -22, and -25) and eight against mouse latent TGF- β 1 (LMT-2, -5, -6, -7, -9, -10, -11, and -12). None are directed against GARP or GARP:TGF- β 1 complexes, and none are cross-reactive against human and mouse latent TGF- β 1. Activation occurred at mAb concentrations in the nanomolar range (Figure 1C).

Because activating mAbs are not cross-reactive, we used human/mouse (h/m) latent TGF- β 1 chimeras to map their epitopes. Most amino acid (aa) differences between human and mouse latent TGF- β 1 are localized in LAP, downstream of a highly conserved region comprising helices α 1 and α 2 and the latency lasso (Figures 1D and 1E). We constructed plasmids encoding hemagglutinin (HA)-tagged h/m latent TGF- β 1 chimeras, in which aa from one segment of mouse LAP were replaced by the aa from the corresponding segment of human LAP. Constructs encoding HA-tagged human or mouse latent TGF- β 1 in which a single residue was replaced by alanine allowed us to further refine binding requirements. HEK293T cells were co-transfected to express wild-type, chimeric, or single-Ala mutant latent TGF- β 1 in complex with mouse GARP and analyzed for binding to mAbs by flow cytometry (Figures S1A–S1C). We also examined in the reporter assay whether some mAbs lost the ability to activate single-Ala mutants to which they could still bind (Figure S1D). Mapping analyses described in the supplemental information show that all activating mAbs bind loops close to the LAP dimerization interface, previously described as critical to maintain TGF- β 1 latency by keeping the LAP monomers welded together⁶ (Figure 1E). Amino acids 201–222 is the only region bound by anti-human and by anti-mouse latent TGF- β 1 mAbs, including LHT-22 and LMT-12. To activate human TGF- β 1, LHT-22 requires residues W207, L208, G211, G212, I214, E215, and F217; to activate mouse TGF- β 1, LMT-12 requires residues L208 and D212 (Figures 1F and S1D). LHT-22 and LMT-12 epitopes in human and mouse latent TGF- β 1, respectively, are very similar to each other and are not shared with non-activating mAbs.

mAbs (i.e., Fluc/Rluc in the presence of the mAb > Fluc/Rluc in the absence of rhTGF- β 1 in ≥ 3 independent experiments). The dotted line shows the FLuc/Rluc ratio in the absence of rhTGF- β 1 or mAb.

(B) Same as in (A), except that HEK293T cells were transfected with mouse GARP:TGF- β 1. Black bars highlight the 8 LMT activating mAbs.

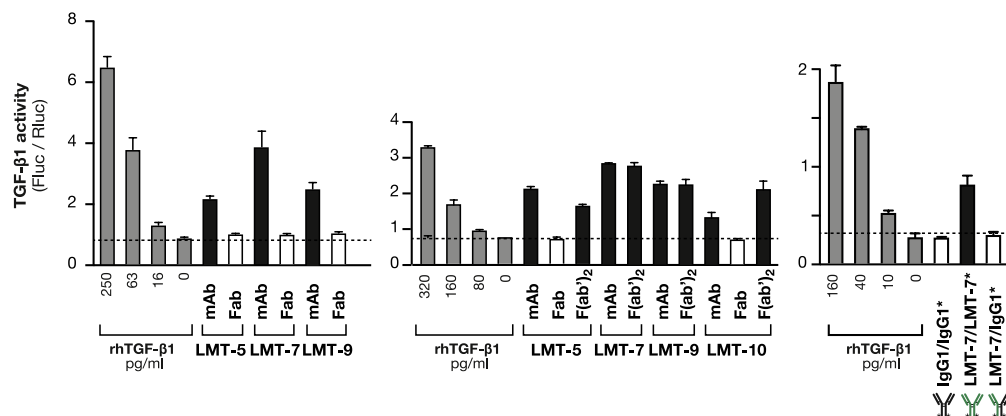
(C) Half-maximum effective concentration (EC₅₀) was measured by performing the reporter assay as in (A) or (B) with serial dilutions of the indicated mAbs.

(D) Annotated alignment of human (h) and mouse (m) latent TGF- β 1 sequences.

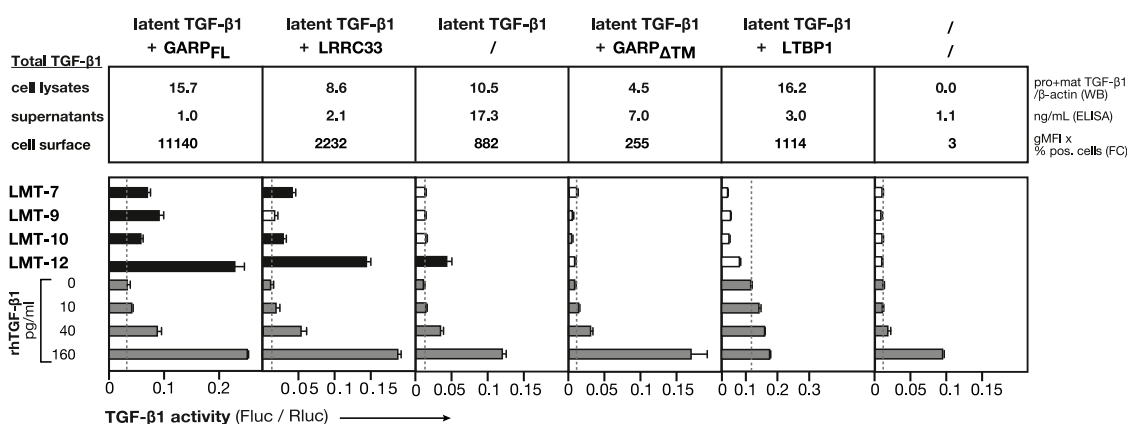
(E) Tridimensional structure of porcine latent TGF- β 1.⁶ Black, LAP; green, mature TGF- β 1; orange, magenta, and turquoise, important residues required for binding and activation by LMT or LHT mAbs.

(F) Summary of epitope mapping analyses shown in Figure S1, indicating the individual aa or aa stretches required for binding or activation by TGF- β 1-activating mAbs. Residues indicated as required for activation by LHT-22 are not required for binding.

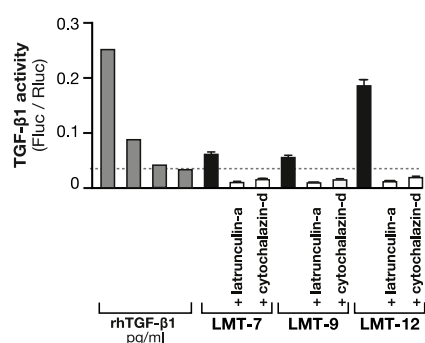
A Reporter 293T cells co-transfected to express mouse GARP and latent TGF- β 1



B Reporter 293T cells co-transfected to express mouse ...



C Reporter 293T cells co-transfected to express mouse GARP and latent TGF- β 1



D Latent TGF- β 1 activation on the surface of murine Tregs

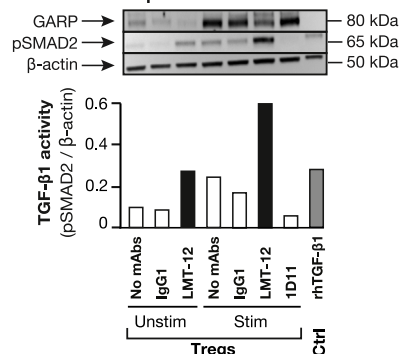


Figure 2. Bivalent binding, membrane anchorage, and actin polymerization are required for activation of latent TGF- β 1 on the cell surface by mAbs
(A) Reporter assay as in Figure 1B, except that transfected cells were incubated with LMT antibodies under full (mAb), F(ab')₂, or Fab formats (left and middle) or with antibody chimeras generated by Fab-arm exchange (right). IgG1/IgG1* (black): control chimera with two arms from an irrelevant mouse IgG1. LMT-7/LMT-7* (green): control chimera with two arms from LMT-7. LMT-7/IgG1* (black/green): chimera with one arm from LMT-7 and one arm from the IgG1 control. Bar graphs represent ratios of Fluc to Rluc activity (mean of triplicate + SD). Black bars highlight antibody formats that activate TGF- β 1. A dotted line shows Fluc/Rluc in the absence of rhTGF- β 1 or mAb.

(B) Reporter HEK293T *TGFB1* knockout (KO) cells transfected with CAGA-Fluc and Rluc were co-cultured at a 1:1 ratio with HEK293T *TGFB1* KO cells co-transfected to express latent TGF- β 1 and the indicated presenting molecules in the presence or absence of LMT mAbs or rhTGF- β 1. GARP_{FL}, full-length GARP; GARP Δ TM, GARP truncated from the C-terminal transmembrane and intracellular domains. Numbers above the bar graphs indicate levels of TGF- β 1 protein expression as detected by western blot (WB) in cell lysates (top), by ELISA in supernatants (middle), and by flow cytometry (FC) on the cell surface (bottom). Bar graphs represent ratios of Fluc to Rluc activity (mean of triplicate + SD). Black bars highlight conditions in which the mAbs activate latent TGF- β 1. A dotted line shows Fluc/Rluc in the absence of rhTGF- β 1 or mAb.

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Bivalent binding, anchorage of latent TGF- β 1 to the cell membrane, and actin polymerization are required for activation by mAbs

We tested 4 LMT antibodies as full length mAbs, F(ab')₂, or Fabs in the HEK293T reporter assay. All activated mouse TGF- β 1 when they were used as mAbs or F(ab')₂ but not as Fabs (Figure 2A). We also constructed chimeric antibodies by Fab-arm exchange. The LMT-7/immunoglobulin 1* (IgG1*) chimera comprises H and L chains from LMT-7 on one hand and H and L chains from a control mouse IgG1 on the other. LMT-7/IgG1* thus binds latent TGF- β 1 in a monovalent manner, with a half-maximum effective concentration (EC₅₀) similar to that of the bivalent LMT-7/LMT-7* control (Figure S2A). In contrast to LMT-7/LMT-7*, however, the LMT-7/IgG1* chimera does not activate latent TGF- β 1 (Figure 2A). Thus, bivalent binding is required to activate TGF- β 1 on the surface of cells *in vitro*.

Our screen to identify activating antibodies was performed in reporter cells transfected to express GARP, a transmembrane protein known to anchor and present latent TGF- β 1 on the Treg surface. We examined whether membrane anchorage was required for mAb-mediated activation (Figure 2B). Reporter cells were transfected to express mouse latent TGF- β 1 alone or in complex with full-length GARP (GARP_{FL}), GARP truncated from its transmembrane domain (GARP_{ΔTM}), or LRRC33, another LRR-containing transmembrane protein that covalently binds and presents latent TGF- β 1 on the surface of myeloid cells. We also tested latent TGF- β 1 binding protein 1 (LTBP-1), a secreted protein lacking a transmembrane domain, known to bind and deposit latent TGF- β 1 in the extracellular matrix. As expected, large amounts of TGF- β 1 (proTGF- β 1 + mature TGF- β 1) were found in the lysates of all HEK293T cells transfected to express latent TGF- β 1. Large amounts of latent TGF- β 1 were secreted in the supernatants of cells transfected with TGF- β 1 alone or with GARP_{ΔTM}, whereas high levels were found on the surface of cells co-transfected with TGF- β 1 and GARP_{FL} or LRRC33. Surface TGF- β 1 was also detected, albeit at lower levels, on cells transfected with TGF- β 1 alone, or with GARP_{ΔTM} or LTBP-1, indicating that molecule(s) other than LRRC33 or GARP_{FL} can retain some latent TGF- β 1 on the HEK293T cell surface. The GRP78 chaperone, for example, has been reported to present latent TGF- β 1 on cells.¹⁹ All mAbs tested (LMT-7, -9, -10, and -12) bound surface TGF- β 1 whether it was presented by GARP, LRRC33, or the unidentified molecule(s) expressed in HEK293T cells. All mAbs activated TGF- β 1 presented by GARP_{FL}, as expected, and most (LMT-7, -9, and 12) activated TGF- β 1 presented by LRRC33. In contrast, none activated TGF- β 1 expressed alone, with GARP_{ΔTM}, or with LTBP-1 (Figure 2B). These results show that mAbs activate latent TGF- β 1 presented on the cell surface by a protein anchored in the membrane via a transmembrane domain.

We tested two inhibitors of actin polymerization in the reporter assay. Latrunculin-a and cytochalasin-d blocked TGF- β 1 activation by LMT-7, LMT-9, and LMT-12 without reducing GARP:

TGF- β 1 levels on the cell surface (Figures 2C and S2B). This indicates that an active cytoskeleton is required for mAb-mediated activation of latent TGF- β 1 on the surface of HEK293T cells.

mAbs can activate latent TGF- β 1 presented on the surface of Tregs

We verified that the mAbs can also activate latent TGF- β 1 presented on the surface of cells that naturally express surface GARP:TGF- β 1. As expected, whereas resting (unstimulated) murine Tregs expressed low amounts of GARP and did not activate TGF- β 1, TCR stimulation increased GARP levels and induced TGF- β 1 activation^{20,21} (Figure 2D). Incubation with LMT-12 was sufficient to induce TGF- β 1 activation by resting Tregs and increased TGF- β 1 activation by TCR-stimulated Tregs (Figure 2D).

Molecular and structural mechanism of TGF- β 1 activation by mAbs

To obtain insights into the structural basis and mechanism of TGF- β 1 activation by mAbs, we sought to determine the structure of human and mouse GARP:TGF- β 1 in complex with Fabs derived from activating mAbs LHT-22 and LMT-12, respectively, by single-particle electron cryo-microscopy (cryo-EM). Initial attempts were hampered by extreme anisotropy observed in the obtained 3D reconstructions due to preferred particle orientations and the absence of top views. To overcome this, additional data were collected by applying 16° and 35° tilts for the human GARP:TGF- β 1:LHT-22 complex and a 20° tilt for the mouse GARP:TGF- β 1:LMT-12 complex. The acquisition of tilt data combined with balancing of sets of particles extracted from datasets obtained with and without tilting protocols resulted in isotropic maps for human and mouse GARP:TGF- β 1:Fab complexes to a nominal resolution of 2.9 and 3.0 Å, respectively (Figures S3 and S4; Table 1). The cryo-EM maps and corresponding structural models reveal a 1:1:2 stoichiometry in the complexes, comprising one GARP, one latent TGF- β 1 (i.e., one LAP_A/LAP_B and one mature TGF- β 1_A/TGF- β 1_B dimer), and two LHT-22 or LMT-12 Fabs (Figures 3A and 3B). Each Fab copy binds one of the two LAP subunits to a region adjacent to the RGD motif, a structural and functional determinant for the binding of integrins to enable release of mature TGF- β 1. A structural alignment of integrins $\alpha_V\beta_6$ (PDB: 5FFO)¹³ and $\alpha_V\beta_8$ (PDB: 6UJA)¹⁵ onto the human GARP:TGF- β 1:LHT-22 complex demonstrates that integrin binding does not clash with LHT-22 and can occur when LHT-22 is bound to GARP:TGF- β 1 (Figure S5). In both human and mouse GARP:TGF- β 1:Fab complexes, the C termini of the two Fabs are too far apart to be accommodated in the context of a single, full-length mAb molecule (Figure S6).

Structural superposition of one of the two Fabs from a complete IgG molecule in either the T (PDB: 1IGT) or Y conformation (PDB: 1IGY) to one of the two individual LHT-22 Fabs binding to the human GARP:TGF- β 1 complex reveals that >150 Å separate

(C) As in (B), except that co-cultures of transfected cells were also incubated with latrunculin-a (5 μ g/mL) or cytochalasin-d (0.5 μ M) where indicated.

(D) Mouse cells enriched in Tregs (MACS-sorted CD25⁺ cells containing ~50% CD4⁺FOXP3⁺ cells) were incubated for 18 h with anti-CD3/CD28 beads (Stim) or left resting in medium alone (Unstim) in the presence of the indicated antibodies (20 μ g/mL). 1D11, neutralizing anti-mature TGF- β 1,2,3 mAb. Cell lysates were analyzed by western blot with antibodies against GARP, pSMAD2, and β -actin. Bar graphs show quantification of ECL signals. Splenocytes incubated with rhTGF- β 1 (1 ng/mL) served as positive controls (Ctrl) for pSMAD2 signals. Results are representative of a total of 6 independent experiments.

Table 1. Cryo-EM data collection, refinement, and validation statistics

	hGARP: TGF- β 1:Fab LHT-22 complex EMDB: EMD-19110 (PDB: 8REW)	mGARP: TGF- β 1:Fab LMT-12 complex EMDB: EMD-19111 (PDB: 8REX)
Data collection and processing		
Magnification	60,000	60,000
Voltage, kV	300	300
Electron exposure, e ⁻ /Å ²	61.8	61.8
Defocus range, μ m	0.8–2.0	0.8–2.0
Pixel size, Å	0.72	0.72
Symmetry imposed	C1	C1
Initial particle images, no.	4,585,815	4,293,248
Final particle images, no.	320,551	288,887
Map resolution, Å	2.89	3.00
FSC threshold	0.143	0.143
Map resolution range, Å	1.53–40.23	2.60–48.97
Refinement		
Initial model used, PDB code/AlphaFold entry	6GFF	AF-G3XA59-F1 AF-P04202-F1
Model resolution, Å	3.20	3.50
FSC threshold	0.5	0.5
Model resolution range, Å	∞ –3.20	∞ –3.50
Map sharpening <i>B</i> factor, Å ²	–87.4	–84.0
Model composition		
Non-hydrogen atoms	12,426	12,355
Protein residues	1,609	1,588
Ligands	NAG: 11 BMA: 2 MAN: 2	NAG: 8
<i>B</i> factors, Å²		
Protein	122	138
Ligand	93	99
RMSD		
Bond lengths, Å	0.004	0.004
Bond angles, °	0.792	0.821
Validation		
MolProbity score	1.68	1.87
Clashscore	7.81	8.42
Poor rotamers, %	0.15	0.07
CaBLAM outliers, %	1.23	3.02
CC (mask/volume)	0.80/0.79	0.73/0.73
EMRinger, sharpened/unsharpened	3.41/3.26	2.07/1.72
Ramachandran plot		
Favored (%)	96.25	93.83
Allowed (%)	3.75	6.17
Disallowed (%)	0.00	0.00

Table 1. Continued

	hGARP: TGF- β 1:Fab LHT-22 complex EMDB: EMD-19110 (PDB: 8REW)	mGARP: TGF- β 1:Fab LMT-12 complex EMDB: EMD-19111 (PDB: 8REX)
Ramachandran Z score		
Whole	–1.22	–1.71
Helix	–1.38	–1.54
Sheet	–0.70	–0.59
Loop	–0.72	–1.34

the second Fab of the IgG molecule from the other epitope in the same GARP:TGF- β 1 complex (Figure S6A). Since the maximum distance between the ends of Fab domains in IgG1 mAbs is shorter than 110 Å, as shown by structural studies of mAbs in solution by small-angle X-ray and neutron scattering experiments,²² or 120 Å as shown for IgG1 by electron tomography,²³ such a distance is not compatible with bivalent binding of one IgG1 molecule to a single GARP:TGF- β 1. The same holds true for LMT-22 binding to mouse GARP:TGF- β 1 (Figure S6B). To address possible bending of the Fab domains at the elbow angle and hinge region, we also performed a structural superposition of IgG Fabs with tilts around the elbow angle ranging from 127° to 220°²⁴ onto the hGARP:TGF- β 1:LHT-22 complex. This analysis results in distances between the Fab C termini of over 135 Å, which is far more than twice the length of the linker between the IgG Fab and Fc domains at the hinge region (Figure S6C).

To confirm this experimentally, we performed size-exclusion chromatography-multi angle laser light scattering analyses after incubation of equimolar amounts of LHT-22 with GARP:TGF- β 1 for either 1 h at 20°C or up to 18 h at 37°C (Figure S7). Under both conditions, megadalton complexes comprising GARP, LAP, mature TGF- β 1, and L and H chains of LHT-22 were observed, likely corresponding to long filaments of GARP:TGF- β 1:LHT-22 in which each bivalent LHT-22 binds 2 distinct GARP:TGF- β 1, and each GARP:TGF- β 1 is bound by 2 distinct LHT-22. Importantly, no peak corresponding to complexes comprising one GARP:TGF- β 1 and one LHT-22 was observed at all.

Therefore, our combined structural and biophysical analyses indicate that a single GARP:TGF- β 1 complex can only be bound by two distinct mAbs when both binding epitopes are occupied, while a single mAb can only bind to two distinct GARP:TGF- β 1 complexes (Figure 4).

The elucidated structural epitopes agree closely with our epitope mapping data (Figures S1C and S1D) so that LHT-12 and LMT-22 address highly similar and overlapping epitopes on human and mouse LAP, respectively. The epicenter of the binding epitopes involves LAP residues 210–215 and helix α 4 (136–142) and is situated $\sim$ 20 Å away from the RGD motif that mediates recruitment of integrin α v β 8. Interestingly, the positions of the heavy (V_H) and light (V_L) chain variable domains are swapped in the two complexes; while human LAP G212 and E215 form hydrogen bonds with LHT-22 V_L (Y837) and V_H (Y125), respectively, mouse LAP D212 and Q215 interact with LMT-12 V_H (S123) and V_L (Y836), respectively (Figure 3; Table S1 and S2).

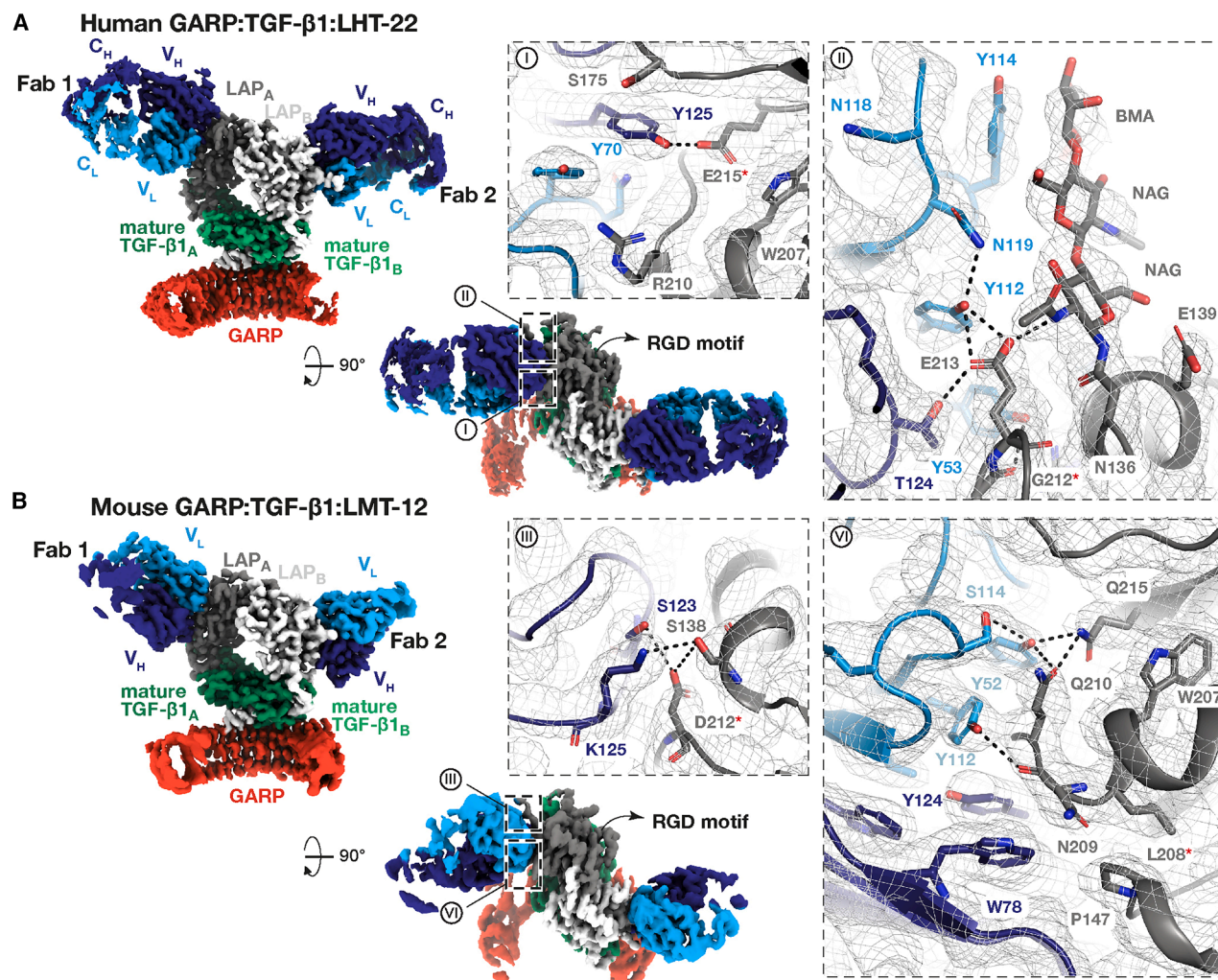


Figure 3. Cryo-EM structure of human and mouse GARP:TGF-β1 bound to Fabs from TGF-β1-activating mAbs

Shown are annotated side and top views of cryo-EM maps of GARP:TGF-β1:Fab complexes. Insets show magnifications of the Fab binding epitopes, with cryo-EM maps shown as a grey mesh. Structural models are shown as cartoons, with annotated residues shown as sticks, and hydrogen bonds are depicted as black dotted lines. Red asterisks indicate residues identified as binding hotspots using TGF-β1 single Ala mutants (Figure S1).

(A) Human GARP:TGF-β1:LHT-22 complex.

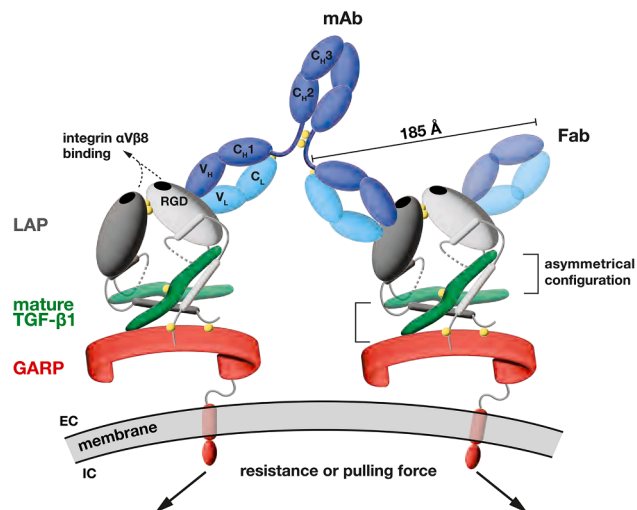
(B) Mouse GARP:TGF-β1:LMT-12 complex.

Structural superpositions of latent TGF-β1 in the GARP:TGF-β1:LHT-22 and GARP:TGF-β1:LMT-12 complexes with latent TGF-β1 in its complex with GARP and a Fab MHG-8 that reinforces latency (PDB: 6GFF)¹⁴ revealed no major structural differences between latent TGF-β1 in the 3 complexes (root-mean-square deviation [RMSD] = 0.667 Å) (Figure S8). In particular, we note high structural similarity of helices α1 and the latency lassos of the LAP monomers, which mask residues in mature TGF-β1 and prevent its interaction with the TβRII receptor chain. Similarly, no structural differences were observed in helices α3 and 3₁₀ (η1) helices, or “thumbs,” of the mature TGF-β1 monomers, which are fixed by the α1 helices in LAP into positions incompatible with binding to the TβRI receptor chain. Thus, TGF-β1 presented by GARP and bound by two LHT-22 or two LMT-12 Fabs is latent and would be unable to interact with

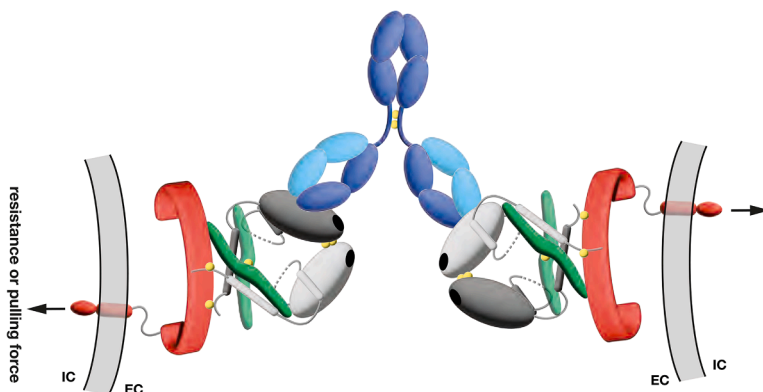
the TGF-β receptor to output signaling activity. Of note, the asymmetrical mode of binding of dimeric latent TGF-β1 inclining upon monomeric GARP in the context of the TGF-β1:MHG-8 complex¹⁴ is also observed here in the GARP:TGF-β1:LHT-22 and GARP:TGF-β1:LMT-12 complexes (Figure 4A), indicating that this is the intrinsic interaction mode of GARP with latent TGF-β1.

Our structural data and analyses of antibody requirements for binding and activity lead us to conclude that TGF-β1 activation cannot be achieved when the LAP dimerization region close to the RGD motifs is bound by two individual Fab molecules. Activation requires binding of one bivalent mAb molecule to two latent TGF-β1 presented by GARP or another transmembrane protein on the cell surface. Whether the two GARP:TGF-β1 complexes need to be on the same cell (in *cis*; Figure 4A) or on

A One mAb - Two GARP:TGF- β 1 in *cis*



B One mAb - Two GARP:TGF- β 1 in *trans*



C TGF- β 1 activation mechanism

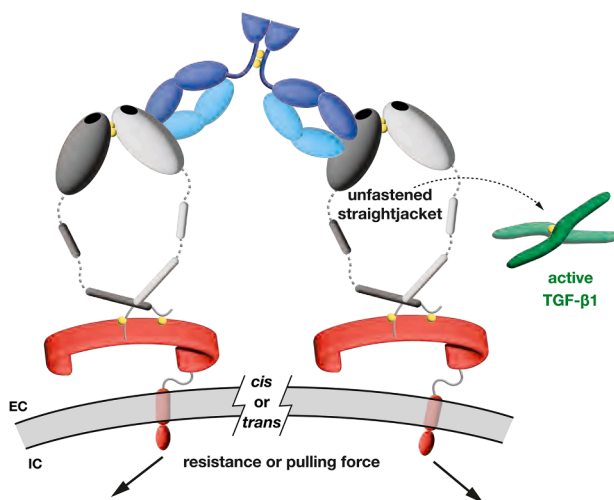


Figure 4. Molecular mechanism of TGF- β 1 activation by mAbs

(A and B) Schematic of one activating mAb crosslinking two membrane-anchored GARP:TGF- β 1 complexes located on the same cell (*cis*) or two adjacent cells (*trans*).

(C) Resistance or pulling force exerted on two membrane-bound GARP:TGF- β 1 complexes crosslinked by one mAb results in unfastening of the LAP straitjacket and release of active TGF- β 1.

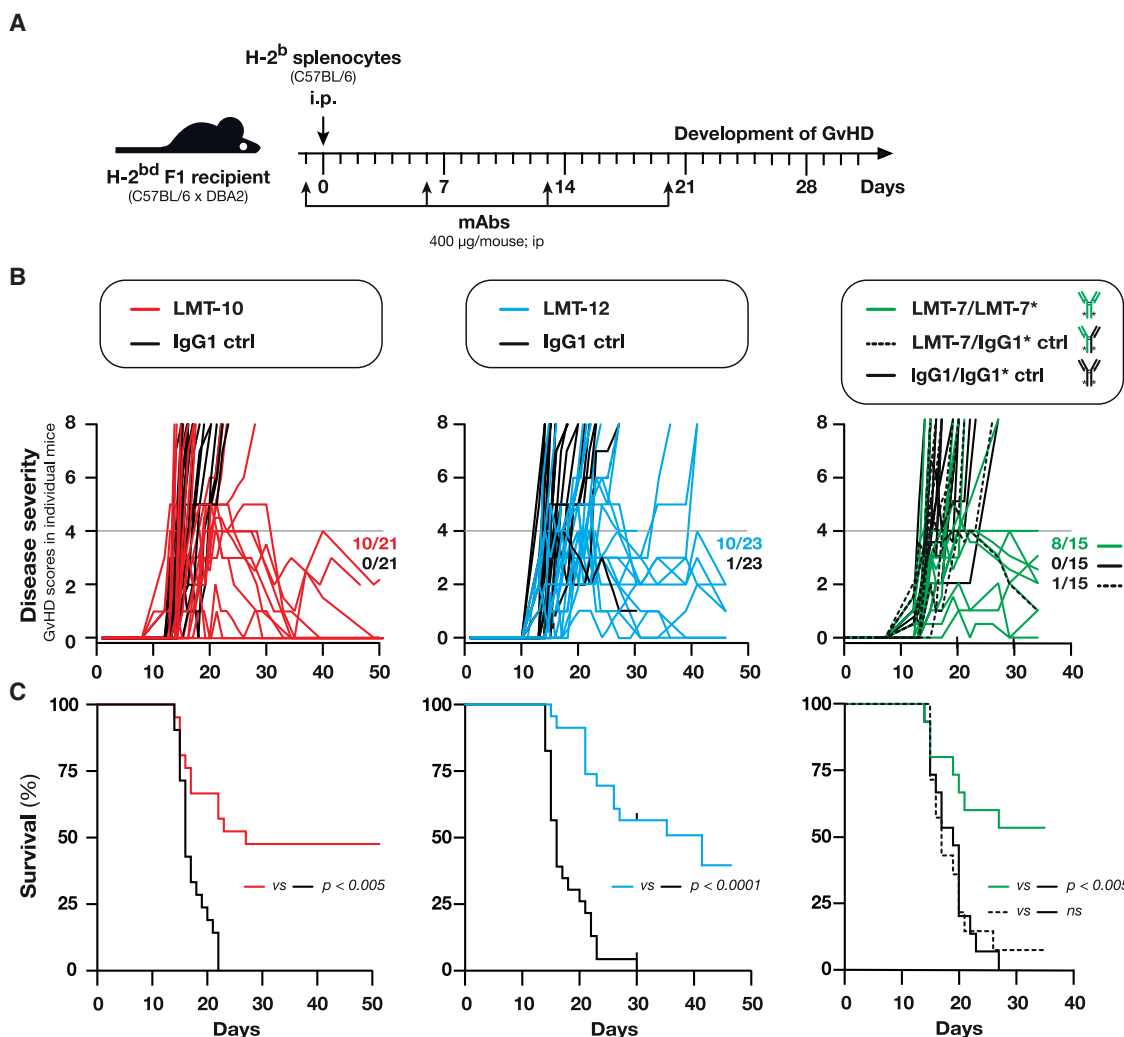


Figure 5. TGF- β 1-activating antibodies reduce disease severity and increase survival in a mouse model of GvHD

(A) Schematic of the experimental design. H-2^{bd} F1 mice transplanted with H-2^b splenocytes ($70\text{--}100 \times 10^6$) on day 0 were injected with TGF- β 1 activating antibodies (mAbs or chimeras) or isotype controls once a week for 4 weeks, starting on day -1 . Mice were scored for symptoms of GvHD, as indicated in the [STAR Methods](#) (maximum score, 8; mice were sacrificed when reaching a score of 6).

(B) Evolution of GvHD scores in individual mice. Each line represents one mouse. Ratios below the gray line indicate the proportion of mice in the corresponding group that were alive 30–50 days after transplantation.

(C) Kaplan-Meier plots representing the proportion of mice alive at the indicated days after transplantation. p values were calculated using a Gehan-Breslow Wilcoxon test. Data were pooled from 2–3 independent experiments for each of the LMT antibodies or chimeras and their respective controls. Vertical mark: censored data.

different cells (in *trans*; [Figure 4B](#)) is not known. Finally, steric constraints imposed by the LAP α 1 helix are poised to prevent binding of mature TGF- β 1 to the T β RI receptor chain unless a profound conformational change in LAP and mature TGF- β 1 occurs to unwind the LAP straitjacket ([Figure 4C](#)). The plethora of structural data at hand support this mechanistic scenario.

mAb-mediated TGF- β 1 activation *in vivo* reduces disease severity and increases survival in mice with graft versus host disease

Given that TCR-stimulated Tregs in humans and mice are known to express GARP:TGF- β 1 complexes at their surface, we sought

to determine whether the mAbs presented here could increase TGF- β 1 activation and immunosuppression in a mouse model of a disease characterized by excessive T cell activity that is insufficiently controlled by Tregs. Graft versus host disease (GvHD) is a life-threatening side effect of allogeneic hematopoietic stem cell transplantation caused by donor T cells reacting against allogeneic antigens on recipient cells. We induced GvHD in mice by transplanting H-2^b (C57BL/6) splenocytes in H-2^{bd} F1 (C57BL/6 $\times$ DBA/2) recipients and treated the mice with TGF- β 1-activating antibodies or controls ([Figure 5A](#)). Antibodies were administered once a week for 4 weeks, starting 1 day before cell transfer. H-2^b donor-derived T cells induced

GvHD-like symptoms (GvHD score ≥ 1) within 10–20 days after transplantation in all recipient mice receiving control antibodies, leading to death of nearly all mice (72 of 74) by day 25 (Figure 5B). In contrast, approximately half of the mice treated with the activating LMT-10 or LMT-12 mAbs or the LMT-7/LMT-7* chimera experienced slower disease progression, reduced disease severity (GvHD score < 4), or complete disease regression and long-term survival (Figure 5B). Notably, all mice treated with the activating antibodies developed GvHD-like symptoms early after cell transfer (days 9–20), indicating that donor alloreactive T cells had engrafted and that the therapeutic activity of the antibodies at later time points was not due to inhibition of donor T cell engraftment. Altogether, treatment with LMT-10, LMT-12, or LMT-7/LMT-7* significantly improved survival by comparison to IgG1, IgG1/IgG1*, and LMT-7/IgG1* controls (Figure 5C). Long-term survival induced by LMT-7/LMT-7* but not LMT-7/IgG1* indicates that, similar to TGF- β 1 activation *in vitro* (Figure 2A), bivalent binding by mAbs is also required for their therapeutic activity *in vivo*.

Increased TGF- β 1 activity and Treg numbers in spleens of mice treated with TGF- β 1-activating mAbs

To search for signs of increased TGF- β 1 activity *in vivo*, we repeated GvHD experiments that we interrupted on day 9 after transplantation to collect organs from recipient mice.

As a first readout, we measured levels of phosphorylated SMAD2 (pSMAD2) by western blot and expression of TGF- β 1 target genes (*Smad7*, *Serpine1*, and *Col1a1*) by RT-qPCR. We observed significantly increased pSMAD2 and TGF- β 1 target gene expression in the spleen after treatment with activating mAbs (Figure 6A). No such increase was observed in other organs (Figures S9A and S9B). No increase in active TGF- β 1 levels was measured in the plasma of C57BL/6 mice 2, 6, or 24 h after administration of activating mAbs (Figure S9C). This suggests that mAb-mediated TGF- β 1 activation occurs locally in the spleen but not in circulating blood.

As a second readout for increased TGF- β 1 activity, we measured Treg numbers across various tissues. TGF- β 1 signaling in secondary lymphoid organs such as the spleen is indeed known to drive the differentiation of activated naive CD4⁺ T cells into peripheral Tregs (pTregs), which can then circulate to inflamed tissues. We observed a 1.5- to 2-fold increase in numbers of donor- and recipient-derived Tregs in the spleen of mice treated with activating mAbs, although the increase did not reach statistical significance for recipient-derived Tregs (Figure 6B). In a repeat experiment, similar trends for increased donor and recipient Tregs were observed in the spleen, mesenteric lymph nodes, and other organs, such as the liver (Figure S10). In line with increased Treg numbers in various organs, *Foxp3* mRNA levels were significantly increased in spleens and lungs after treatment with activating mAbs (Figure S11). In contrast to Tregs, numbers of CD4⁺ or CD8⁺ T cells and those of other immune cells were similar in all organs from mice receiving TGF- β 1-activating or control mAbs (Figures 6B and S10).

GvHD is known to be associated with type 1 immune responses. As expected, expression of genes characteristic of Th1 and CTL activity (*Tbx21*, *Ifng*, *Gzmb*, or *Prf1*) was increased in most organs in mice with GvHD by comparison to C57BL/6

controls, whereas that of genes associated with Th2 and Th17 responses was not. In line with the observed reduction in GvHD severity, and although numbers of CD4⁺ and CD8⁺ T cells were not reduced, expression of Th1/CTL genes tended to decrease in mice receiving TGF- β 1-activating mAbs (Figure S11).

Our observations suggest that, in mice with GvHD, activating mAbs can trigger TGF- β 1 activation, at least in the spleen. This would favor local differentiation of naive CD4⁺ T cells into pTregs, which would then circulate and accumulate in inflamed tissues to inhibit pathogenic Th1 and CTL activity without reducing the expansion of allo-reactive conventional T cells.

Pre-existing Tregs are required for TGF- β 1-activating mAbs to exert therapeutic activity in mouse GvHD

Whereas on one hand increased TGF- β 1 activity and consequent differentiation of CD4⁺ T cells into Tregs may result in increased Treg numbers 9 days after treatment initiation, on the other hand, Tregs existing before transplantation in the graft or in recipients may serve as a source of latent TGF- β 1 that can be activated by the mAbs.

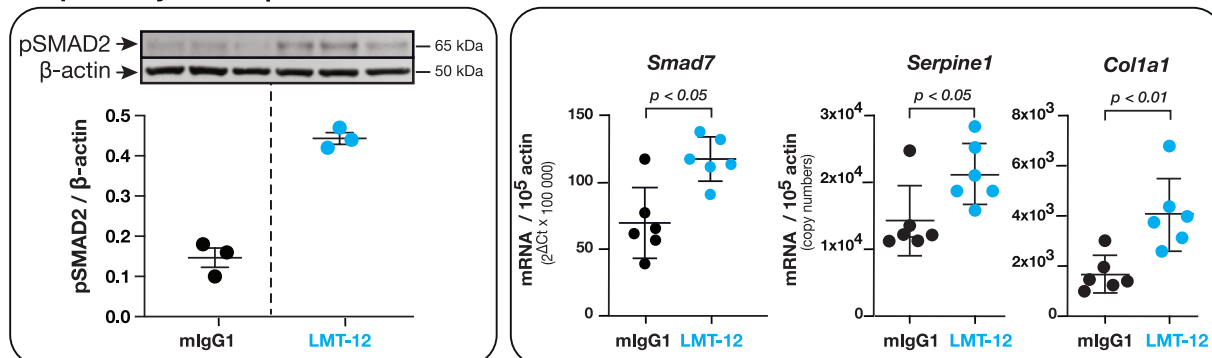
To test whether pre-existing Tregs are required to mediate the therapeutic effects of TGF- β 1-activating mAbs, we performed transplantation experiments using *H-2^{b/b} Foxp3^{DTR/DTR}* or *Foxp3^{WT/WT}* females as splenocyte donors and *H-2^{b/d} Foxp3^{DTR}* or *Foxp3^{WT}* males as recipients. We injected diphtheria toxin (DTx) at the time of transplantation to deplete pre-existing Tregs from donor, recipient, or both origins (Figures 7 and S12).²⁵ In all experiments, as expected, donor splenocytes induced GvHD, and LMT-12 increased survival when Tregs were not depleted (Figure 7, no DTx). In contrast, when both donor and recipient Tregs were depleted by DTx, the therapeutic activity of LMT-12 was lost, indicating that pre-existing Tregs are required to mediate this effect (Figure 7A). Loss of therapeutic activity was also observed when recipient Tregs, but not donor Tregs, were depleted by DTx (Figures 7B and 7C). This could indicate that pre-existing Tregs in recipients, but not donor Tregs present in the graft, are required for the therapeutic activity of the mAbs in mouse GvHD. Alternatively, it might reflect the fact that, at the time of transplantation, recipient Tregs vastly outnumber donor Tregs ($\sim 1.5 \times 10^6$ per graft) in recipient mice, which are not irradiated in this model. Pre-existing recipient Tregs thus represent a significantly larger reservoir of membrane-bound latent TGF- β 1 than donor Tregs. Our results suggest that the latent TGF- β 1 reservoir on the surface of pre-existing Tregs is required for activating mAbs to exert therapeutic activity in GvHD. TGF- β 1 activity elicited by the mAbs *in vivo* may, in turn, drive differentiation of naive CD4⁺ T cells into pTregs in secondary lymphoid organs, explaining the increased splenic Treg numbers observed 9 days after transplantation and treatment initiation (Figure 6B).

From the above, we conclude that TGF- β 1 activation can be triggered by mAbs *in vivo* and could serve to treat diseases caused by excessive T cell activity. In mouse GvHD, the therapeutic effect requires the presence of Tregs.

DISCUSSION

The ubiquitous expression of TGF- β receptors across all cells and the pleiotropic nature of signaling mediated by TGF- β 1

A TGF- β activity in the spleen



B H-2^b donor-derived cells in spleen H-2^{bd} recipient-derived cells in spleen

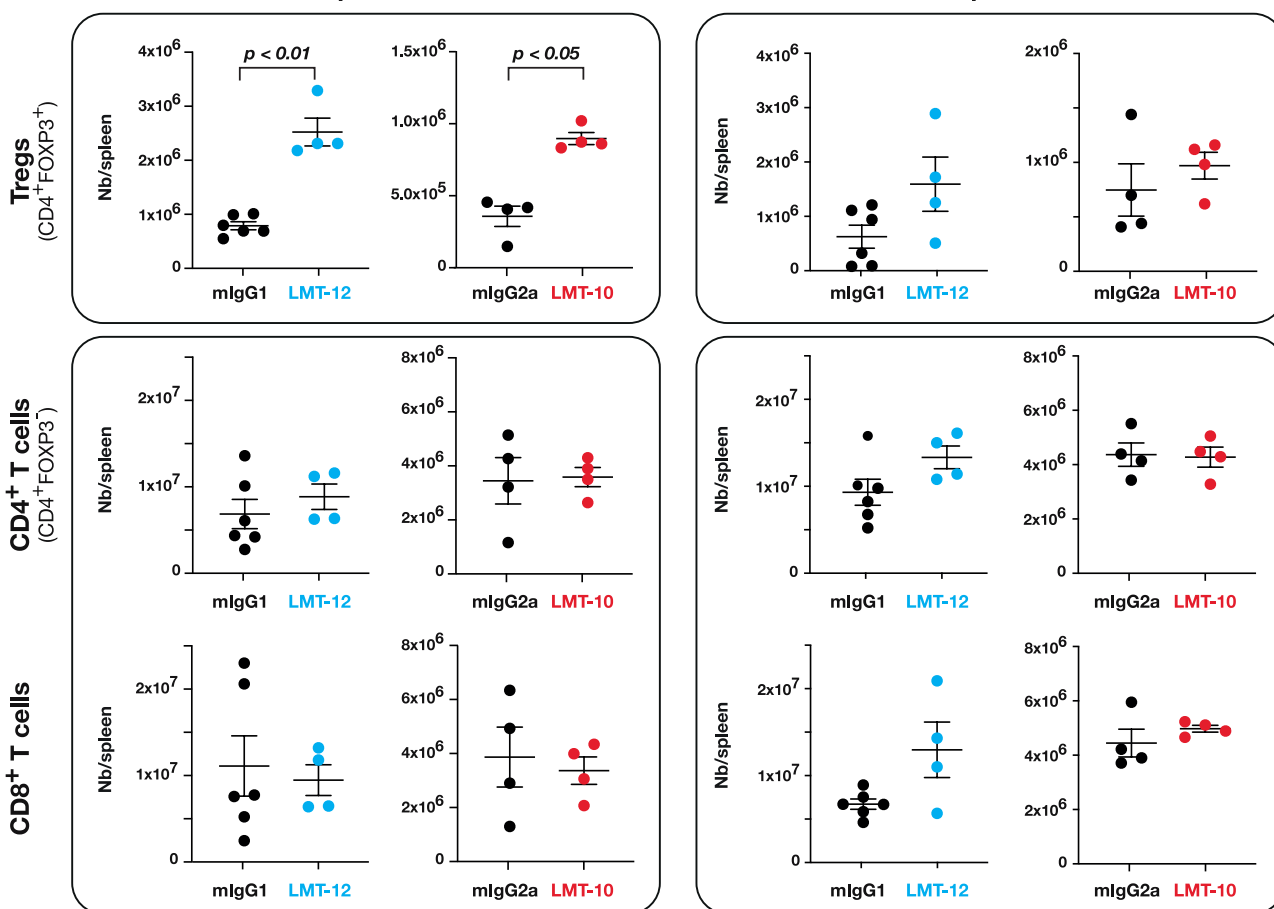


Figure 6. Increased TGF- β activity and Treg numbers in spleen of mice treated with TGF- β 1-activating mAbs

GvHD was induced as indicated in Figure 5, except that recipient mice were euthanized 9 days after transplantation to collect spleens. Results from two independent experiments are shown in (A) and (B), respectively. Data points represent values measured in one individual mouse. Horizontal lines represent mean per group $\pm$ sem. Statistical analyses of p values were performed using an unpaired two-tailed Mann-Whitney test.

(A) Left: TGF- β signaling was analyzed by western blot with antibodies against pSMAD2 and β -actin. ECL signals were quantified to establish pSMAD2/ β -actin ratios. Right: expression of TGF- β 1 target genes was measured by RT-qPCR. Each lane in the western blot and data point in the graphs represent values measured in one individual mouse.

(B) Splenocytes were counted and analyzed by FC with antibodies against CD4, CD8, FOXP3, H-2D^d, and H-2K^b/H-2D^b to evaluate numbers of Tregs and CD8⁺ and CD4⁺ T cells of donor (H-2^b) and recipient (H-2^{bd}) origin.

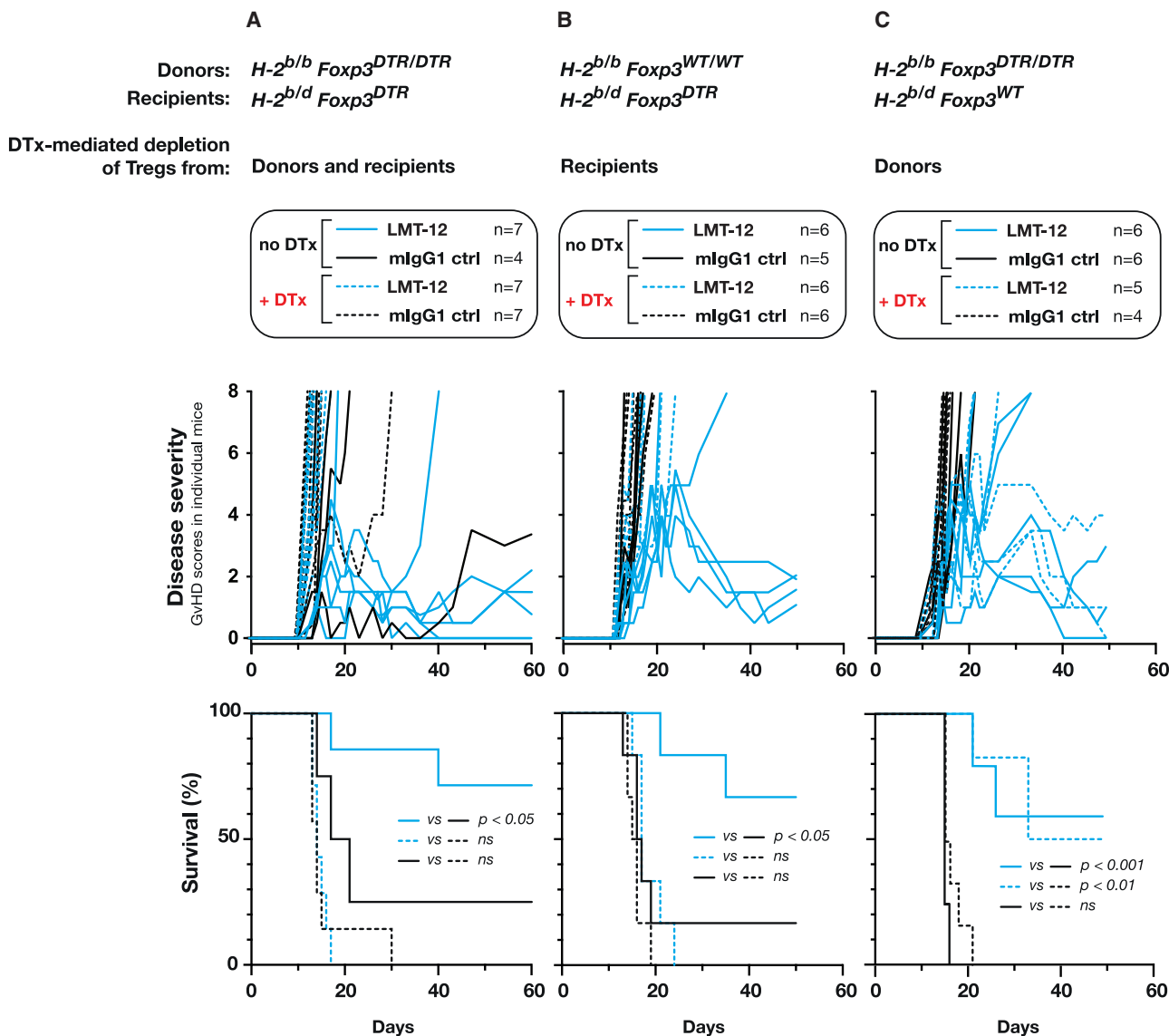


Figure 7. Tregs are required for the therapeutic effect of TGF- β 1-activating mAbs

GvHD was induced in three independent experiments as described in Figure 5 by transplanting splenocytes from $H-2^{b/b} Foxp3^{DTR/DTR}$ or $Foxp3^{WT/WT}$ female donors to $H-2^{b/d} Foxp3^{DTR}$ or $Foxp3^{WT}$ male recipients on day 0.

DTx was injected into donors, recipients, or both between day -1 and +1 to deplete Tregs of donor, recipient, or both origins, as illustrated in Figure S12. Graphs at the top show evolution of GvHD scores in individual mice. Graphs at the bottom show Kaplan-Meier plots representing the proportion of mice alive on the indicated days after transplantation. p values were calculated using a Gehan-Breslow Wilcoxon test.

imposes tightly regulated maintenance of TGF- β 1 latency under homeostatic conditions. To date, all mAbs developed to therapeutically target TGF- β 1 aim at blocking its immunosuppressive, pro-tumoral, and pro-fibrotic activities in the context of cancer or fibrosis. The mAbs described here do the opposite: they activate latent TGF- β 1 presented by a transmembrane protein on the cell surface, providing a targeted pharmacological approach to increase immunosuppression in the context of inflammatory or immune-related diseases.

Our biochemical and structural analyses of TGF- β 1 binding and activation by mAbs, Fabs, and chimeric antibodies have

not only shed light on the pathological context in which the mAbs could exert therapeutic activity but also on the physiological mechanism of TGF- β 1 activation by integrins in mammals. Three important observations need to be considered: (1) in contrast to the bivalent activating mAbs and chimeras, the corresponding monovalent Fabs and chimeras cannot activate latent TGF- β 1, (2) TGF- β 1 activation by mAbs requires an active cytoskeleton and anchorage of latent TGF- β 1 at the cell surface via a transmembrane protein such as GARP or LRRC33, and (3) the binding mode of two Fabs derived from an activating mAb to one GARP:TGF- β 1 complex sterically excludes the possibility

that the two Fabs can originate from the same mAb molecule *in vivo*. Therefore, a mAb can only activate TGF- β 1 *in vivo* by crosslinking two distinct latent TGF- β 1 molecules presented at the cell surface by transmembrane proteins such as GARP or LRRC33.

A major implication of these observations for translation into the clinic is that such activating mAbs are not expected to activate soluble latent TGF- β 1 circulating in the blood or other biological fluids but will only activate TGF- β 1 presented at a high density on the surface of single cells or in tissues abundantly infiltrated by spatially connected cells. This allows for specific targeting and activation of latent TGF- β 1 presented by GARP or LRRC33 on the surface of immune cells such as TCR-stimulated Tregs or myeloid cells, respectively. Even though endothelial cells also express GARP:TGF- β 1 on their surface and might serve as TGF- β 1 reservoirs amenable to activation by therapeutic mAbs, activation of latent TGF- β 1 by mAbs on the surface of endothelial cells was not sufficient to exert therapeutic activity in the absence of Tregs in mouse GvHD experiments. Furthermore, our *in vitro* experiments indicate that cell-free reservoirs of latent TGF- β 1 deposited in the extracellular matrix via covalent binding to LTBP would not be amenable to mAb-mediated activation. Altogether, the data presented here suggest that activating mAbs can exert potent immunosuppressive therapeutic activity *in vivo* in a Treg-dependent manner.

Another implication of our observations pertains to the molecular machinery of TGF- β 1 activation: high-affinity binding to the two LAP monomers from a single latent TGF- β 1 molecule is not sufficient to activate the cytokine, even when binding occurs close to the RGD motifs and dimerization interface. Activation requires profound conformational changes to allow access of the mature TGF- β 1 dimer to the β RI: β RII heterotetrameric receptor. These changes include not only unmasking mature TGF- β 1 residues from the LAP latency lasso to allow interaction with β RII but also modification of the mature TGF- β 1 helix α 3 fixed by LAP α 1 in a position precluding interaction with β RI. Recently, elucidation of the cryo-EM structure of one α ν β 8 ectodomain bound to a latent TGF- β 1 dimer led to the suggestion that flexibility in TGF- β 1 and integrin molecules in the complex is sufficient to expose mature TGF- β 1 to a minimal TGF- β receptor comprising a single β RI: β RII heterodimer instead of the conventional β RI: β RII heterotetramer without complete dissociation of mature TGF- β 1 from LAP. The authors suggested that this would result in a minimal TGF- β 1 signaling cascade.¹⁵ However, helix α 3 of mature TGF- β 1 in that structural analysis is in the same position as that observed here and in previously reported structures of latent TGF- β 1, free or bound to GARP, LRRC33, or a Fab.^{6,14,26} Therefore, this conformation is grossly incompatible with the proposed interaction between helix α 3 in mature TGF- β 1 and a β RI receptor chain upon binding by a single α ν β 8 due to severe steric clashes with helix α 1 of LAP. Thus, we propose that TGF- β 1 activation requires crosslinking of two membrane-bound latent TGF- β 1 by one mAb or, alternatively, crosslinking of two membrane-bound integrins by one latent TGF- β 1. This would allow the exertion of cellular forces in opposite directions, leading to unwinding of LAP and profoundly modify the conformation of TGF- β 1, as suggested previously.¹³ Our proposed model is

compatible with recent work by Duan et al., who reported a more efficient activation of TGF- β 1 presented by GARP or LRRC33 upon binding of two α ν β 8 molecules rather than one.²⁶ Our mechanistic proposal also implies that latent TGF- β 1 reservoirs deposited in cell-free extracellular matrix would not be activated by soluble activating mAbs, avoiding massive release of active TGF- β 1 in tissues that are not inflamed or infiltrated by immune cells.

Our data from a murine model of GvHD indicate that TGF- β 1-activating mAbs may serve to treat patients with severe alloreactive immune responses observed during allogeneic hematopoietic stem cell or solid organ transplantation. Several severe autoimmune or inflammatory diseases for which efficient treatments are still lacking, including systemic lupus erythematosus or inflammatory bowel disease, could also benefit from this novel therapeutic approach. Autoimmune diseases driven by Th17 cells, such as multiple sclerosis, would not qualify for these types of treatments *a priori* due to the role played by TGF- β 1 in Th17 differentiation. Due attention will also need to be given to the development of fibrosis, which often accompanies chronic inflammatory diseases and could be favored by persistent TGF- β 1 activity.

Collectively, our findings pave the way for clinical trials with TGF- β 1-activating mAbs to investigate a targeted immunosuppressive therapy for diseases characterized by excessive immune activity insufficiently controlled by Tregs or TGF- β 1.

Limitations of the study

We did not observe therapeutic activity with TGF- β 1-activating mAbs in three murine models of autoimmunity. The activating mAbs did not improve autoimmune colitis induced by the transfer of Treg-depleted naive CD4⁺ T cells in *Rag1*^{-/-} mice. When Tregs were co-transferred with naive T cells at a 1 Treg to 10 naive T cell ratio, Tregs were still completely protective against colitis, leaving no room to observe an additive therapeutic effect of the activating mAbs. We also tested the mAbs in lupus-prone mice (*B6.Sle1.2.3*^{27,28}), but long-term (>8 months) treatment did not delay or reduce development of glomerulonephritis or appearance of anti-dsDNA auto-antibodies. Activating mAbs also did not exert therapeutic activity in experimental autoimmune encephalitis (EAE). Interestingly however, the mAbs did not exacerbate inflammation or disease in any of these pro-inflammatory settings, including in EAE. It should also be noted that GvHD, in which we did observe therapeutic activity of the mAbs, shares significant features with autoimmune diseases. Nevertheless, we are pursuing our search for a model of autoimmune disease in which the TGF- β 1-activating mAbs would exert therapeutic activity.

RESOURCE AVAILABILITY

Lead contact

Requests for further information, resources, and reagents should be directed to and will be fulfilled by the lead contact, Sophie Lucas (sophie.lucas@uclouvain.be).

Materials availability

Unique/stable reagents generated in this study are available from the lead contact upon signature of a material transfer agreement.

Data and code availability

- All data associated with this study are available in the main text or the supplemental information. Cryo-EM maps of human and mouse GARP:TGF- β 1:Fab complexes and corresponding structural models were deposited in the EMDB/PDB with accession codes EMD-19110/PDB: 8REW and EMD-19111/PDB: 8REX, respectively.
- No code was generated.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

S.W., F.L., and S.L. conceived and designed the overall project. L.M., M.L., and B.v.d.W. derived mAbs by phage display and designed constructs to express and purify recombinant protein complexes. F.L. developed the screening assay to identify activating mAbs and performed detailed epitope mapping analyses. F.L., S.W., C.M., and M.J. identified the requirements for mAb-mediated activation *in vitro*. F.L., S.W., C.M., M.G., and E.D. designed and performed the murine GvHD experiments *in vivo*. J.F. and S.N.S. performed cryo-EM (grid preparation, specimen screening, and data collection) and analyzed the data (image processing and model building and refinement). S.W., F.L., J.F., S.N.S., and S.L. wrote the manuscript with input from all authors.

DECLARATION OF INTERESTS

L.M., B.v.d.W., and M.L. are full-time employees of argenx. Patents pertaining to activating antibodies against TGF- β 1 have been filed under the Patent Cooperation Treaty (international application number PCT/EP2023/056082) with S.L., F.L., L.M., and B.v.d.W. as inventors and UCLouvain as applicant.

STAR★METHODS

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 - Cryo-electron microscopy (cryo-EM)

- Cryo-EM image processing
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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2025.116061>.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
LHT-1 to 24: Llama anti-human latent TGF- β 1	This paper	N/A
LMT-1 to 14: Llama anti-mouse latent TGF- β 1	This paper	N/A
IgG1/IgG1*: chimeric antibody	This paper	N/A
LMT-7/IgG1*: chimeric antibody	This paper	N/A
LMT-7/LMT-7*: chimeric antibody	This paper	N/A
1D11: anti- TGF- β 1,2,3	R&D System	Cat#MAB1835; RRID: AB_357931
mIgG1 isotype control (Motavizumab)	ImmunoPrecise Antibodies Ltd	N/A
AF700 rat anti-mouse CD45, clone 30-F11	Biolegend	Cat#103127; RRID: AB_493714
BV711 rat anti-mouse CD3, clone 17A2	Biolegend	Cat#100241; RRID: AB_2563945
BV510 rat anti-mouse CD4, clone RM4-5	Biolegend	Cat#100559; RRID: AB_2561388
PerCP rat anti-mouse CD8a, clone 53-6.7	BD Pharmigen	Cat#553036; RRID: AB_394573
APC rat anti-mouse Foxp3, clone FJK-16s	eBioscience	Cat#17-5773-82; RRID: AB_469457
APC rat IgG2a isotype control, clone eBR2a	ThermoFisher Scientific	Cat#17-4321-81; RRID: AB_470181
FITC mouse anti-mouse H-2Dd, clone 34-2-12	Biolegend	Cat#110606; RRID: AB_313487
PE mouse anti-mouse H-2Kb/H-2Db, clone 28-8-6	Biolegend	Cat#114607; RRID: AB_313598
PE/Cyanine 7 rat anti-mouse CD49b, clone DX5	Biolegend	Cat#108922; RRID: AB_2561459
BV785 hamster anti-mouse CD11c, clone N418	Biolegend	Cat#117335; RRID: AB_11219204
BV650 rat anti-mouse/human CD45R/B220, clone RA3-6B2	Biolegend	Cat#103241; RRID: AB_11204069
BV421 rat anti-mouse CD19, clone 6D5	Biolegend	Cat#115538; RRID: AB_10895761
Rat anti-mouse CD16/CD32 (TruStain FcX), clone 93	Biolegend	Cat#101320; RRID: AB_1574973
Rabbit anti-phospho-SMAD2, clone 138D4	Cell Signaling	Cat#3108; RRID: AB_10117720
Rabbit anti- β -actin, clone D6A8	Cell Signaling	Cat#8457; RRID: AB_10950489
Sheep anti-mouse GARP, polyclonal	R&D System	Cat#AF6229
HRP donkey anti-sheep IgG, polyclonal	R&D System	Cat#HAF016; RRID: AB_562591
HRP goat anti-rabbit IgG, polyclonal	Cell Signaling	Cat#7074; RRID: AB_2099233
HRP goat anti-mouse IgG-light-chain, polyclonal	Jackson	Cat#115-035-174; RRID: AB_2338512
HRP goat anti-rat IgG, polyclonal	Cell Signaling	Cat#7077; RRID: AB_10694715
HRP goat anti-mouse IgG, polyclonal	R&D System	Cat#HAF007; RRID: AB_357234
Mouse anti-HA, clone 16B12	Eurogentec	Cat#MMS-101R; RRID: AB_2820200
Mouse anti-HA, clone GG8-1F3.3.1	Miltenyi	Cat#130-092-257; RRID: AB_871939
Rat anti-mouse/human mature TGF- β 1, clone A75-2	BD Bioscience	Cat#555052; RRID: AB_395673
PE goat anti-mouse IgG, polyclonal	Biolegend	Cat#405307; RRID: AB_315010
APC anti-mouse LAP, clone TW7-16B4	Biolegend	Cat#141406; RRID: AB_10898159
PE anti-mouse GARP, clone YGIC86	eBioscience	Cat#12-9891-82; RRID: AB_1963598
Chemicals, peptides, and recombinant proteins		
Penicillin/Streptomycin	Sigma Aldrich	Cat#P4333
Diphtheria Toxin	Sigma Aldrich	Cat#D0654
Human recombinant TGF- β 1	R&D System	Cat#240-B
DMEM	Gibco	Cat#10938
IMDM	Gibco	Cat#21980
X-vivo	Lonza	Cat#04-380Q
Fetal Bovine Serum	Cytiva	Cat#SV30160.03
Glutamax	Gibco	Cat#35050
Opti-MEM	Gibco	Cat#51985

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dual-Glo System	Promega	Cat#E2940
Acridine Orange/Propidium Iodide Stain	Logos Biosystem	Cat#F23001
Dynabeads™ mouse T-activator CD3/CD28	ThermoFisher Scientific	Cat#11452D
StrepTactin Sepharose High Performance beads	GE Healthcare	Cat#28-9355-99
Foxp3/Transcription factor/fixation/Permeabilization concentrate and diluate	eBioscience	Cat#00552100
CountBright Absolute Counting beads	Invitrogen	Cat#C36950
Latrunculin-a	Merck Life Science	Cat#428021
Cytochalasin-d	Merck Life Science	Cat#C2618
Fixable Viability Dye eFluor™	eBioscience	Cat#65-0865-14
TransIT-LT1	Mirus Bio	Cat#MIR2360
StrepTactin Sepharose High Performance beads	GE Healthcare	Cat#28-9355-99
Takyon™ Low ROX Probe 2x Master mix dTTP blue	Eurogentec	Cat#UF-LPMT-B0710
SuperSignal West Pico PLUS Chemiluminescent Substrate	ThermoFisher Scientific	Cat#34578
SuperSignal West Femto Maximum Sensitivity Substrate	ThermoFisher Scientific	Cat#34096
Bolt LDS sample buffer	ThermoFisher Scientific	Cat#B0007
Bolt reducing Agent	ThermoFisher Scientific	Cat#B0009
Red Blood Cell lysing Buffer (10X)	Invitrogen	Cat#00-4300-54
Krebs-Ringer buffer	ThermoFisher Scientific	Cat#J67795-K2
Collagenase IV	ThermoFisher Scientific	Cat#17104019
Dnase I	Roche	Cat#10104159001
HBBS (CaCl2 MgCl2)	ThermoFisher Scientific	Cat#15266355
DTT	Sigma Aldrich	Cat#D0632
Percoll	GE Healthcare	Cat#GE17-0891-02
Laemmli buffer	Bio-Rad	Cat#161-0737
2-mercapto-ethanol	Bio-Rad	Cat#161-0710
Orthovanadate	Biolab	Cat#P0758
Halt protease inhibitor cocktail	ThermoFisher Scientific	Cat#1861278
PMSF	ThermoFisher Scientific	Cat#36978
Tween 20	Sigma Aldrich	Cat#P2287
Trypsin 0,25%-EDTA in HBSS	Biowest	Cat#L0931-100
UltraPure 0.5M EDTA	Life technologies	Cat#15575
RNA later	Invitrogen	Cat#AM7021

Critical commercial assays

Q5 Site-Directed Mutagenesis kit	NEB	N/A
Human/Mouse TGF-β1 uncoated ELISA kit	Invitrogen	Cat#88-8350-88
NucleoSpin RNA, Mini kit for RNA purification	Macherey Nagel	Cat#740955
Maxima First Strand cDNA Synthesis Kit	ThermoFisher Scientific	Cat#K1672
Miltenyi MACS CD25 MicroBeads kit	Miltenyi	Cat#130-091-072

Experimental models: Cell lines

293T <i>TGFB1</i> KO cells	This paper	N/A
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Experimental models: Organisms/strains

Mouse: C57BL/6: C57BL/6J Ola Hsd	Harlan Laboratories	MGI ID: 4459305
Mouse: DBA/2: DBA/2 Ola Hsd	Harlan Laboratories	MGI ID: 3510418
Mouse: C57BL/6 <i>Foxp3</i> ^{DTR} : B6.129(Cg)- <i>Foxp3</i> ^{tm3(Hbegf/GFP)Ayr/J} strain	Jackson Laboratory	MGI ID: 3698131
Mouse: F1: C57BL/6 x DBA/2	This paper	N/A
Mouse: F1 <i>Foxp3</i> ^{DTR} : C57BL/6 <i>Foxp3</i> ^{DTR/DTR} x DBA/2	This paper	N/A
Mouse: <i>Foxp3</i> ^{YFP-Cre} (B6.129(Cg)- <i>Foxp3</i> ^{tm4(YFP/cre)Ayr/J})	Jackson Laboratory	RRID: IMSR_JAX:016959

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
pCAGA-12-Fluc ¹¹	Kindly given by Aris Moustakas	N/A
pRL-TK	Promega	Cat#E2241
pDisplay	Kindly given by Laure Dumoutier	N/A
pcDNA3.1/V5-His-TOPO	Kindly given by Thomas Michiels	N/A
Latent TGF- β 1: pEF-BOS-puro-mTgfb1(cDNA)	This paper	N/A
GARP _{FL} : pDisplay-HA-mGarp(21–663)	This paper	N/A
GARP _{ΔTM} : pcDNA3-Garp _{ΔTM}	This paper	N/A
LRRC33: pDisplay-HA-mLrrc33(ORF)	This paper	N/A
LTBP: pcDNA3-mLtp1(cDNA)	VectorBuilder	N/A
Software and algorithms		
GraphPad Prism version 10	GraphPad Software	https://www.graphpad.com/
FlowJo v10	Tree Star	https://www.flowjo.com/
Adobe Illustrator 2022, 26.5	Adobe, inc	https://www.adobe.com
Design and analysis software for qPCR	ThermoFisher Scientific	https://apps.thermofisher.com/apps/da2/#/home/welcome
Bio1D software	Vilber-Lourmat	N/A
MacVector Software	MacVector, inc	N/A
Fusion Capt Advance Solo 4S	Vilber-Lourmat	N/A
BD FACSDiva TM Software	BD Biosciences	https://www.bdbiosciences.com/
Other		
LS column	Miltenyi	Cat#130-042-401
Superdex 200 HiLoad 16/600 column	GE Healthcare	Cat#GE28-9893-35
LUNA-FL TM Dual Fluorescence Cell Counter	Logos biosystems	Cat#L20001
NanoDrop 2000c	ThermoFisher Scientific	Cat#ND-2000C
Microson Ultrasonic cell disruptor	Misonix	N/A
Bolt 4–12% Bis-tris gels	ThermoFisher Scientific	Cat#NW04122BOX
iBlot TM 2 Transfer Stacks, nitrocellulose, regular size	ThermoFisher Scientific	Cat#IB23001X3
Fusion solo s	Vilber-Lourmat	N/A
gentleMACS TM C Tubes	Miltenyi Biotec	Cat#130-096-334
gentleMACS TM Dissociator	Miltenyi Biotec	Cat#130-093-235

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

293T and 293T-TGFB1 KO cells

The HEK 293T adherent cell line (referred to herein as 293T cells) was cultured at 37°C, 8% CO₂ in DMEM (Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Cytiva), Glutamax 1% (Gibco) and antibiotics (Penicillin 100 U/ml and Streptomycin 0.1 mg/mL, Sigma Aldrich). When reaching 70–90% confluency, cells were detached with 0.05% Trypsin (Gibco), neutralized with culture medium and centrifuged to remove supernatant, then resuspended in fresh culture medium and passaged at a 1:20 dilution. A cell clone deleted for the *TGFB1* gene (293T-*TGFB1* KO) was generated by CRISPR-Cas9, by transiently transfecting 293T cells with a *TGFB1*-targeting construct encoding GFP [Addgene plasmid # 48138; <http://n2t.net/addgene:48138>; RRID:Addgene_48138; corresponding to plasmid pSpCas9(BB)-2A-GFP (PX458), gift from Feng Zhang,²⁹ GFP⁺ cells were cloned by FACS, and deletion of *TGFB1* was validated by Western Blot. The 293T-*TGFB1* KO clone was cultured like 293T cells.

Murine Tregs

CD25⁺ splenocytes from Foxp3^{YFP-Cre} mice were isolated using the Miltenyi MACS CD25 MicroBeads kit (Miltenyi) according to the manufacturer's protocol. Briefly, spleens were collected and mechanically dissociated using a sterile syringe plunger in IMDM medium +10% FCS. After red blood cells lysis (RBC lysis buffer 1X, Biolegend), cells were resuspended in degassed MACS buffer (PBS + EDTA 2mM + 1% FCS, filter-sterilized) and incubated with CD25 MicroBeads. Cells were then subjected to magnetic separation using an LS column (Miltenyi) and a MACS Separator (Miltenyi). The isolated CD25⁺ fraction was assessed by flow cytometry to measure enrichment in CD4⁺YFP⁺ Tregs. Isolated cells were used immediately in *in vitro* assays.

Mice

C57BL/6 (C57BL/6J Ola Hsd), DBA2 (DBA/2 Ola Hsd), *Foxp3*^{DTR} (B6.129(Cg)-*Foxp3*^{tm3(Hbegfl/GFP)Ayr/J} strain), F1 (C57BL/6 x DBA/2), F1 *Foxp3*^{DTR} (C57BL/6 *Foxp3*^{DTR/DTR} x DBA/2), and *Foxp3*^{YFP-Cre} (B6.129(Cg)-*Foxp3*^{tm4(YFP/cre)Ayr/J}) mice were bred and maintained at the SPF animal facility of the UCLouvain (LAF facility with approval number LA2230397). As *Foxp3* is located on the X chromosome, F1 *H-2^b/day Foxp3*^{DTR} males were obtained by crossing C57BL/6 *Foxp3*^{DTR/DTR} females to DBA/2 *Foxp3*^{WT} males. The facility is controlled to maintain the temperature between 20°C and 24°C; humidity rate between 40 and 65% and day–night cycles of 12 h–12 h. All animal studies were performed in accordance with national and institutional guidelines for animal care, under permit numbers 2017/UCL/MD/19, 2019/UCL/MD/032 and 2023/UCL/MD/37 at the UCLouvain. Female and male mice of 8–12 weeks of age were used as donors and/or recipients, respectively, to induce graft-versus-host disease. We only used females as donors and males as recipients to avoid graft rejection due to H-Y minor histocompatibility antigens.

METHOD DETAILS

mAbs directed against human and mouse latent TGF-β1

mAbs against GARP and GARP:TGF-β1 complexes were derived and described previously.^{11,20} mAbs against latent TGF-β1 were obtained following immunization of llamas with plasmids encoding human or mouse TGF-β1, construction of V_H/V_K and V_H/V_λ Fab cDNA libraries and selection by phage display. Fab-coding regions were subcloned into plasmids encoding human (hlgG1) or mouse (mlgG1, or mlgG2a_{DANA}) IgGs. mlgG1 and mlgG2a mlgG2a_{DANA} exert few or no FcγR-dependent activities.³⁰ Chimeric antibodies (LMT-7/LMT-7*, LMT-7/IgG1* and IgG1/IgG1*) were constructed by and purchased from ImmunoPrecise Antibodies Ltd by controlled Fab-arm Exchange.³¹ Contamination of antibody preparations by active TGF-β1 was verified by Western Blot (Figure S13), by ELISA and in reporter assays. All mAb solutions used herein contained less than 0.8 pg of active TGF-β1 per μg of mAb.

TGF-β1 reporter assays

In Figures 1 and 2A and supplemental S1, 293T cells were plated one day before transfection in 96w plates (±2.10⁴ cells/w). Cells were co-transfected using TransIT-LT1 (Mirus Bio) with plasmids encoding human or mouse GARP_{FL} (50 ng/well), human or mouse latent TGF-β1 (10 ng/well), Firefly luciferase under the control of a TGF-β1-responsive promoter (*pCAGA-12-Fluc*,³² 50 ng/well, Promega), and Renilla luciferase under a constitutive promoter (*pRL-TK*, 1 ng/well, Promega). Four to six hours after transfection, mAbs (20 μg/mL) or serial dilutions of active recombinant human TGF-β1 (rhTGF-β1, R&D System) were added to the culture medium. Firefly and Renilla luciferase activities were measured 24 h after transfection using the Dual-Glo System (Promega). EC50s were calculated with GraphPad Prism software based on results obtained with serial dilutions of the mAbs.

In Figures 2B and 2C and S2, 293T *TGFB1* KO cells were plated in 6w plates (±1.10⁶/w) and co-transfected on the same day with a plasmid encoding mouse latent TGF-β1 (0.5 μg/well) and a plasmid encoding mouse GARP_{FL}, GARP_{ΔTM}, LRRC33, or LTBP-1 (2 μg/well). Another batch of 293T *TGFB1* KO cells were co-transfected with plasmids encoding Firefly luciferase under the control of a TGF-β1-responsive promoter (*pCAGA-12-Fluc*,³² 2 μg/well, Promega), and Renilla luciferase under a constitutive promoter (*pRL-TK*, 50 ng/well, Promega). One day after transfection, reporter cells (CAGA-Fluc) cells and cells expressing latent TGF-β1 alone or with a presenting molecule (GARP_{FL}, GARP_{ΔTM}, LRRC33 or LTBP-1) were collected and counted, then mixed in co-cultures in 96w plates at a 1 reporter cell to 1 TGF-β1 expressing-cell ratio (2.10⁴ + 2.10⁴ cells/w). Co-cultures of transfected cells were incubated in the presence of mAbs (20 μg/mL), serial dilutions of active recombinant human TGF-β1 (rhTGF-β1, R&D System) or Latrunculin-a (0.5 μg/mL, Merck Life Science) or Cytochalasin-d (0.5 μM, Merck Life Science) during 24 h. Firefly and Renilla luciferase activities were measured using the Dual-Glo System (Promega).

Short-term TCR-stimulation of murine tregs in vitro

MACS-sorted CD25⁺ splenocytes enriched in Tregs were seeded in 200 μL X-Vivo (Lonza) plus 1% Glutamax (Gibco) in the presence (Stim) or absence (Unstim) of murine CD3/CD28 Dynabeads (ThermoFisher Scientific) at a 2 beads: 1 cell ratio (10⁶ cells/w in 48w plates). As indicated in Figure 2D, some wells were pre-coated with a control IgG1 or the LMT-12 mAb (20 μg/mL PBS) during 2 h at room temperature. Active rhTGF-β1 (1 ng/mL, R&D System) was added to unstimulated total splenocytes, as a positive control for TGF-β1 activity, and antibody 1D11 (anti-TGF-β1,2,3) was added to TCR-stimulated Tregs to verify the specificity of TGF-β signals. Cells were incubated during 16 h at 37°C in a humidified atmosphere containing 5% CO₂, then collected and analyzed by Western Blot with anti-phosphorylated SMAD2 antibodies.

Western blot analyses

Dry pelleted HEK 293T cells or murine Tregs (enriched CD25⁺ murine cells) were counted and lysed in Laemmli buffer (Bio-Rad) supplemented with 5% of 2-mercaptoethanol (Bio-Rad). Lysis of HEK 293T cells was facilitated using a 23G needle, and that of murine Tregs by sonication during 15 s using a Microson Ultrasonic cell disruptor (Misonix). Cell lysates were then denatured by boiling at 100°C for 5 min, and clarified by centrifugation at 12,000 rpm for 5 min at 4°C. Spleen or tissue fragments were snap-frozen, immersed in NP40 lysis buffer (1% NP40, 0.05M Tris-HCl Ph8, 0.15M NaCl, 1mM EDTA, 15% Glycerol, 100μM orthovanadate, 1X halt protease inhibitor and 100μM PMSF in H₂O) and lysed using TissueLyser (Qiagen) for 4 min at 50Hz. Proteins were quantified using the Pierce BCA protein assay kit (ThermoFisher).

For electrophoretic separation, equivalents of 2.5×10^4 HEK 293T or 2×10^5 murine Treg cells or 5 μ g of proteins for tissues were diluted in Bolt LDS sample buffer and Bolt reducing Agent (ThermoFisher Scientific), then loaded on Bolt 4–12% Bis-tris gels (ThermoFisher Scientific). After migration, proteins were transferred to nitrocellulose membranes using the iBlot system (ThermoFisher Scientific). After blocking, membranes were incubated with primary antibodies directed against GARP (R&D), HA (Eurogentec), mature TGF- β 1 (clone A75-2, BD Pharmingen), pSMAD2 (clone 138D4, Cell Signaling Technology), or β -actin (clone D6A8, Cell Signaling Technology), then with a relevant secondary HRP-coupled antibody [anti-sheep IgG-HRP (R&D), anti-rabbit IgG-HRP (Cell Signaling), anti-mouse-IgG-light-chain-HRP (Jackson), anti-rat-IgG-HRP (Cell Signaling) or anti-mouse IgG-HRP (R&D System)]. and revealed with using Pierce SuperSignal chemiluminescent substrates (SuperSignal WEST pico or femto; Thermo Fisher Scientific) on a Fusion Solo 4S system (Vilber-Lourmat). Quantifications were done with the Bio1D software (Vilber-Lourmat).

Epitope mapping

Alignment of mouse and human protein sequences was performed using MacVector software. DNA sequences encoding the h/m TGF- β 1 chimeras and single Ala-mutants were obtained from IDT and cloned in vector *pDisplay* to add an N-ter HA-tag or in *pcDNA3* (for Ala-mutant). Other mutants were generated by site-directed mutagenesis with the Q5 Site-Directed Mutagenesis kit (NEB). HEK 293T cells (5×10^5) were transfected in 6 well plates with plasmids encoding mouse GARP (1250 ng/well) and h/m TGF- β 1 chimeras or single-Ala TGF- β 1 mutants (1250 ng/well). 24 h after transfection, cells were incubated with mAbs (5 μ g/mL), washed, and incubated with secondary antibodies coupled to a fluorochrome or to streptavidin (+ biotin coupled to a fluorochrome), and analyzed by flow cytometry on a FACS Verse flow cytometer (BD Biosciences). The expression level of each mutant was measured by staining with an anti-HA antibody (Eurogentec) or an antibody against GARP:TGF- β 1 complexes,^{11,20} and used to normalize binding results. Flow cytometry data were computed using the FlowJo software (Tree Star). For each mAb, residual binding represents percentage of binding to a given chimera or single-Ala mutant by comparison to corresponding human or mouse wild type TGF- β 1.

Recombinant protein expression and purification

To enable isolation and purification of soluble GARP:TGF- β 1 complexes, coding regions of the GARP extracellular domain (hGARP_{ECD} M1 to I628, mGARP_{ECD} M1 -I629) were fused to sequences encoding a C-terminal TEV-cleavable Strep3x tag (EAAENLYFQG AAGG GSGGGG WSHPQFEKGA AWSHPQFEKG AAWSHPQFEK GAA) and cloned in *pcDNA3.1*. Sequences coding for h and m TGF- β 1 were cloned in *pcDNA3.1*. One day prior to transfection, 293EBNA cells were seeded at an optimal density to transiently transfect the cells using 25 μ g total DNA/50 mL of cell culture. Complexes were produced by co-transfecting TGF- β 1 and GARP-ECD-TEV-Strep-encoding plasmids at a 2-to-1 ratio, to ensure that all GARP was in complex with TGF- β 1. Six days after transfection, cells were centrifugated and supernatants were transferred to tubes containing StrepTactin Sepharose High Performance beads (GE Healthcare). After a minimal 2 h incubation period, allowing the beads to capture the complexes via the streptavidin tag on GARP, the beads were transferred to a column. Unbound material was washed away by PBS, and the complexes were competitively eluted with 2.5 nM desthiobiotin. The proteins were then buffer exchanged into PBS, followed by TEV digestion to remove the streptavidin tag. A second StrepTactin purification was done to remove free streptavidin tag, undigested complex and TEV-protease. Protein containing fractions of the StrepTactin purification flow-through were pooled, concentrated and further purified by gel filtration using a Superdex200 16/600 column (GE Healthcare) that had been equilibrated in 20 mM HEPES 150 mM NaCl pH 7.4. These protein containing fractions were analyzed by LabChip capillary electrophoresis. Recombinant human and mouse soluble GARP-TGF- β 1 complexes were produced and purified at UProtein Express (UPE, Utrecht, The Netherlands).

Purified human and mouse GARP:TGF- β 1 complexes were mixed with a 6x molar excess of purified Fab (derived from LHT-22 and LMT-12, respectively) prior to injecting the mixture onto a Superdex 200 HiLoad 16/600 column (GE Healthcare) equilibrated in HEPES-buffered saline buffer (HBS, 25 mM HEPES, pH 7.5, 150 mM NaCl). Peak fractions after size-exclusion chromatography (SEC) were collected and flash-cooled in liquid nitrogen for further use.

Cryo-electron microscopy (cryo-EM)

For both the human and mouse GARP:TGF- β 1:Fab complexes, 4 μ L of sample (at a conc. of 0.13 and 0.12 mg/mL respectively) was applied to glow-discharged Quantifoil R 0.6/1 300 mesh gold grids coated with graphene (PUXANO), blotted for 1 s at a blot force of 1 under 100% humidity at 22°C and plunged into liquid ethane using a FEI Vitrobot Mark IV device. Grid screening was performed on a JEOL 1400 Plus TEM (BECM, Brussels), and data was acquired on a JEOL cryoARM 300 microscope (BECM, Brussels) equipped with an Omega filter (JEOL) and a 6k $\times$ 4k K3 direct detector (Gatan) operated in correlated double-sampling (CDS) mode. For each sample, 60-frame movies were collected with a total exposure of 3.37 s, a total dose of 61.8 e⁻/Å², an energy filter slit width of 20 eV and a pixel size of 0.72 Å/pixel at the specimen level. For the human GARP:TGF- β 1:Fab complex, a total of 6678 untilted movies were collected with a defocus of 0.8–2 μ m. Following untilted data collection, 4020 movies were collected at 16° tilt and 3200 movies were collected at 35° tilt, all using similar microscope settings. For the mouse GARP:TGF- β 1:Fab complex, a total of 6605 untilted movies were collected followed by 3508 movies at 20° tilt. Optimal tilts for tilted data collection were acquired using cryoEF.³³

Cryo-EM image processing

Processing of uncorrected movies was performed in CryoSPARC v4.2.1.³⁴ Recorded movies were motion corrected and dose weighted using Patch motion correction, and ensuing CTF estimation was performed using Patch CTF estimation. An initial particle

stack was acquired using Blob picker with a minimum and maximum particle diameter of 100 and 200 Å respectively. Inspected particle picks were extracted using a box size of 480 pixels down-sampled to 240 pixels, corresponding to a pixel size of 1.44 Å/pixel. Extracted particle stacks were cleaned using several rounds of iterative 2D classification and subsequent 2D class selection to remove junk, and cleaned particle stacks were used for *Ab Initio* model generation with 3 classes as input. To obtain the best results in terms of map isotropy, different amounts of highest resolution 2D classes were mixed from untilted and tilted datasets to obtain following ratios: 0° tilt: 35%, 16° tilt: 45%, 35° tilt: 20% (human GARP:TGF-β1:Fab complex) and 0° tilt: 70%, 20° tilt: 30% (mouse GARP:TGF-β1:Fab complex). After *Ab Initio* model generation, a heterorefinement was performed using all 3 *Ab Initio* models as input for the human GARP:TGF-β1:Fab complex, and using 2 out of 3 *Ab Initio* models for the mouse GARP:TGF-β1:Fab complex. Particles corresponding to heterorefinement Class 1 and 2 of the human GARP:TGF-β1:Fab complex were grouped for subsequent homo- and Non-Uniform refinement jobs, while for the mouse GARP:TGF-β1:Fab complex only particles corresponding to heterorefinement Class 1 were used. For the human GARP:TGF-β1:Fab complex, the particle stack was used for a final round of 2D classification and class selection, resulting in a slightly trimmed final particle stack. For both complexes, a final Non-Uniform refinement run was performed using unbinned data (0.72 Å/pixel). Post-processing was performed using regular sharpening (using B-factors of −30 Å and −70 Å for human and mouse GARP:TGF-β1:Fab complex maps respectively) to obtain maps for subsequent model refinement in Phenix,³⁵ and using deepEMhancer³⁶ to obtain maps for model building in Coot³⁷ and visualization in ChimeraX³⁸ and Pymol. A schematic overview of cryo-EM image processing can be found in [Figures S3](#) and [S4](#).

Cryo-EM model building and refinement

For the human GARP:TGF-β1:Fab complex, the structure of human GARP:TGF-β1 was extracted from PDB 6gff³⁴ and rigid-body fitted in the deepEMhancer sharpened map using ChimeraX, together with extracted V_H and V_L chains of an hFAB LHT-22 model generated via ESMFold.³⁹ The resulting model was used as an input for automatic molecular dynamics flexible fitting using the NAMDINATOR webserver.⁴⁰ Next, the flexibly fitted model was used for several cycles of iterative manual model building in Coot guided by the deepEMhancer sharpened map, and real-space refinement in Phenix using the regularly sharpened map. Settings during refinement in Phenix were: enabled global minimization, local grid search, ADP refinement, secondary structure and Ramachandran restraints, and a non-bonded weight parameter of 100. A final refinement was performed using identical settings, but with a non-bonded weight parameter of 300. For the mouse GARP:TGF-β1:Fab complex the same strategy was used as described for the human complex, with the exception that the initial models for mouse GARP and mouse TGF-β1 were obtained from the AlphaFold Protein Structure database (AF-G3XA59-F1 and AF-P04202-F1), after trimming of regions with low per-residue confidence score (pLDDT<50). A summary of cryo-EM data collection, refinement and validation statistics can be found in [Table 1](#).

Cryo-EM Data availability

Cryo-EM maps of human and mouse GARP:TGF-β1:Fab complexes and corresponding structural models are deposited in the EMDB/PDB with accession codes EMDB: EMD-19110/PDB: 8REW and EMDB: EMD-19111/PDB: 8REX, respectively.

Induction and scoring of murine graft-versus-host disease

Splenocytes (70–110.10⁶) collected from C57BL/6 donor mice were injected i.p. in F1 (C57BL/6 x DBA/2) male recipients to induce GVHD. Starting one day before splenocyte transplantation, recipients received weekly i.p. injections of mAbs (LMT-10, LMT-12, LMT-7/LMT-7*, LMT-7/IgG1*, IgG1/IgG1* or mIgG1 isotype control; 400 µg/mouse). In experiments shown in [Figures 7](#) and [S12](#), diphtheria toxin (DTx, 0.5 µg/mouse, i.e., ±25 µg/kg) was administrated to *FOXP3*^{DTR/DTR} donor females and/or *FOXP3*^{DTR} recipient males to deplete pre-existing Tregs. Mice were evaluated for the development of GvHD symptoms twice a week starting on the day of transplantation until the development of a first symptom in one of the mice (usually around day 13), then daily thereafter (from days 13–20). GvHD scores were established based on weight loss (+1 or +2), posture (+1 or +2), reduced mobility (+1 or +2), and ruffled fur (+1 or +2). Mice were euthanized when they reached a score of 6, or at the end of the experiment (between day 30 and 50). Maximum score (death): 8. In some experiments, a pre-defined group of mice were euthanized on day 9 to collect organs for analyses.

Flow cytometry

Mouse organs were harvested immediately after euthanasia. Spleens and mLN's were mechanically dissociated, homogenates were clarified through 70 µm filters and red blood cells (RBC) were lysed using a standard RBC lysis buffer. Livers were washed in Krebs-Ringer buffer, transferred in C tubes (Miltenyi) containing prewarmed dissociation medium (Collagenase IV, DnaseI and HBSS supplemented with CaCl₂ and MgCl₂), and mechanical dissociation was performed using the gentleMax Tissue Dissociator following manufacturer instructions. Liver cell suspensions were filtered through 70 µm strainers, and immune cells were isolated using a 30% Percoll gradient (GE Healthcare) followed by RBC lysis. Ileums were cleaned of fat and Peyer's patches. Cell fractions from the epithelium and the lamina propria were separated, and immune cells from both fractions were isolated using a 30% Percoll gradient (GE Healthcare). All single cell suspensions were counted on a Luna cell counter with a live-dead cell marker (Acridine Orange/Propidium Iodide Stain, Logos Biosystem), centrifuged, resuspended in FACS Buffer (2 mM EDTA and 1% FCS in PBS, filter-sterilized) and clarified through 40µm filters prior to incubation with antibodies coupled to fluorochromes. 293T *TGFB1* KO cells were resuspended in FACS Buffer prior to incubation with antibodies coupled to fluorochromes.

All antibodies used in flow cytometry experiments are listed in the Key Resources Table and indicated in the figure legends. Cells were sequentially incubated with primary and secondary antibodies coupled to fluorochromes during 20 min at 4°Cs. After staining for surface markers, splenocytes were washed, fixed and permeabilized with the Foxp3/Transcription Factor staining buffer set (eBioscience), then incubated with an anti-Foxp3 antibody (clone FJK-16s – eBioscience) in the presence of anti-CD16/32 to block Fcγ receptors (Biolegend).

Cells were analyzed on a FACS LSR Fortessa flow cytometer (BD Biosciences) and data were computed using the FlowJo software (Tree Star).

Real-time quantitative polymerase chain reaction

Mouse spleens and tissues were collected, weighted and immersed in RNA later (Invitrogen) for 24 h before being stored at –80°C until processing. Tissue disruption was performed using the TissueLyser (Qiagen) for 5 min at 50Hz. Total RNA was extracted using NucleoSpin Mini Columns (Macherey Nagel) and RNA concentration was determined using the NanoDrop spectrophotometer (Thermo Scientific). Reverse transcription was carried out with the Maxima First Strand cDNA Synthesis Kit (ThermoFisher). For real-time qPCR, a master mix was prepared containing Takyon Master Mix (0.025 U/μL, Eurogentec), primers (300nM of each), Takyon probe (100nM) and 1.25 μL of cDNA. Reactions were performed on a QuantStudio3 device (ThermoFisher) under either standard conditions (95°C for 3 min; 45 cycles of 95°C for 10 s and 60°C for 60 s) or fast conditions (95°C for 3 min; 95°C for 10 s and 60°C for 30 s) depending on amplicon size. Primers and probes sequences are listed in Table S3. Data analysis was conducted using the ThermoFisher Design and analysis software. Expression of genes of interest are expressed as a ratio of mRNA/10⁵ β-actin (house-keeping gene), calculated either as $10^{5 \times 2^{-\Delta Ct}}$ (where $\Delta Ct = Ct \text{ for gene of interest} - Ct \text{ for } \beta\text{-actin}$), or as a ratio of mRNA copy numbers evaluated using standard curves with recombinant DNA.

Enzyme-linked immunosorbant assay ELISA

Human/Mouse TGF-β1 uncoated ELISA kit (Invitrogen) was used following manufacturer's instructions to measure active and total (i. e., active + latent) TGF-β1 in untreated or acidified samples, respectively. For acidification, samples were treated with HCl 1N (1:5 v/v for 10 min) then neutralized with NaOH 1.2N/HEPES 0.5M (1:6 v/v).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism software (version 9).

Comparisons of measurements made on samples from mice receiving TGF-β1 activating mAbs or control antibodies were tested using an unpaired two-tailed Mann-Whitney test. For survival analyses, we used Kaplan Meier plots representing the proportion of mice alive at the indicated days after transfer of splenocytes, with *p* values calculated using a Gehan-Breslow Wilcoxon test. Numbers of experiments and numbers of mice (*n*) in the various experimental groups are indicated in the corresponding figure legend. Threshold for statistical significance: 0.05.

Supplemental information

**Antibody-mediated TGF- β 1 activation
for the treatment of diseases caused
by deleterious T cell activity**

Fanny Lambert, Jan Felix, Séverine Wautier, Emilie Dupré, Mathieu Jamez, Camille Michiels, Mélanie Gaignage, Lore Mariën, Manon Lesage, Bas van der Woning, Savvas N. Savvides, and Sophie Lucas

SUPPLEMENTAL FIGURE TITLES AND LEGENDS

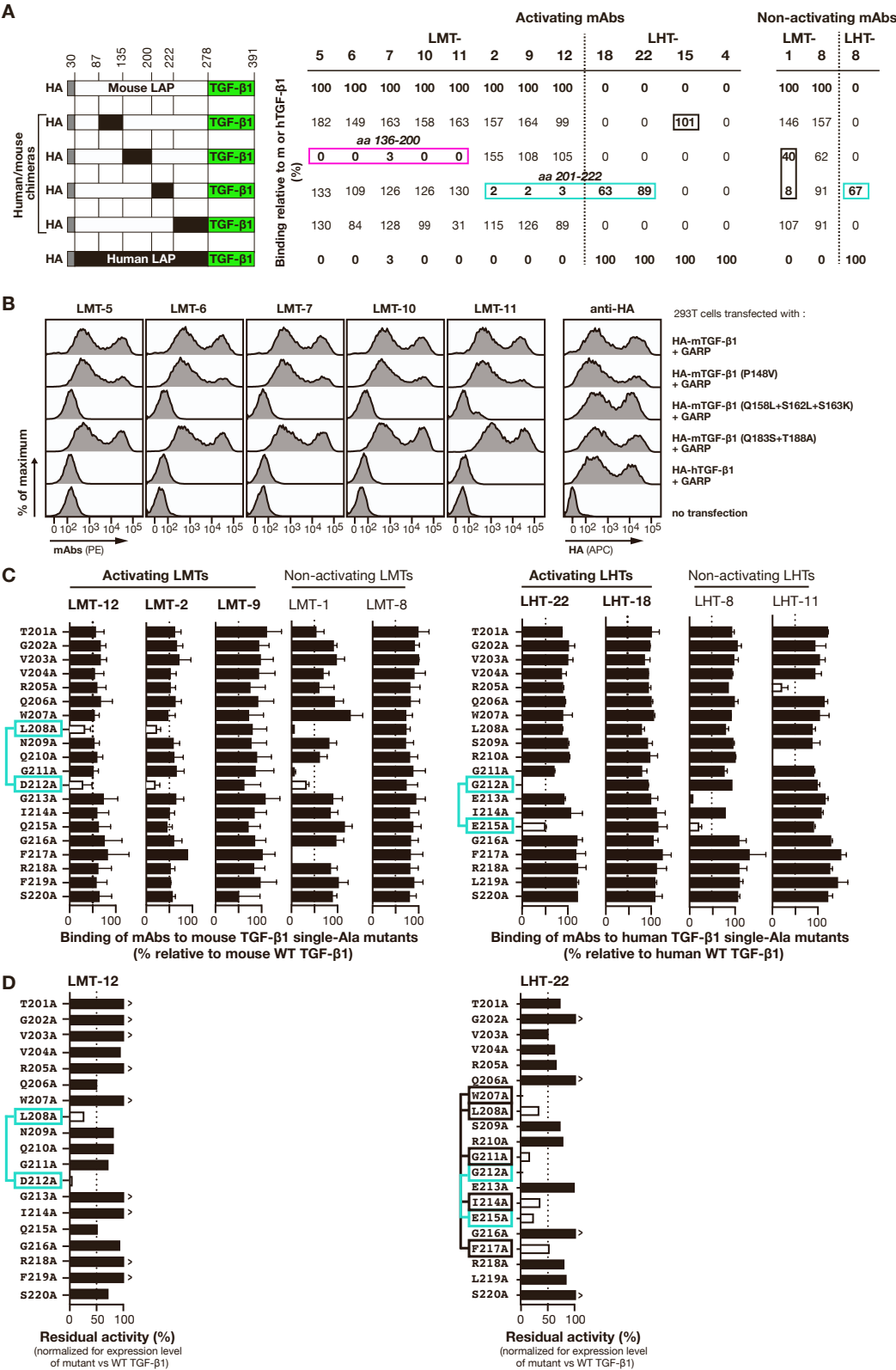


Figure S1. Mapping of epitopes recognized by TGF- β 1 activating mAbs

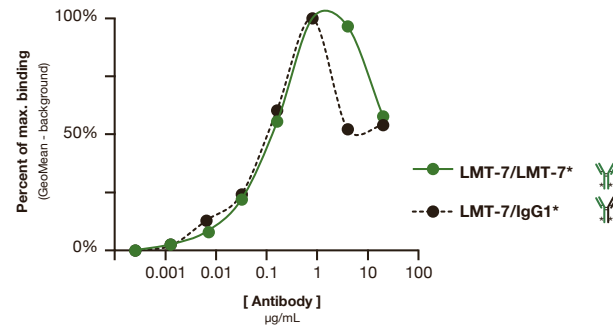
(A) Left: Schematic representation of h/m latent TGF- β 1 chimeras used to map regions required for binding by LMT and LHT mAbs. In each chimera, aa from one segment of mouse LAP were replaced by aa from the corresponding segment of human LAP. Right: 293T cells were co-transfected to express mouse GARP in complex with the HA-tagged wild type (WT) or h/m latent TGF- β 1 chimera indicated on the left and analyzed for binding to mAbs by flow cytometry. Results are expressed as percent binding to the indicated h/m latent TGF- β 1 chimera relative to mouse WT TGF- β 1 for LMT mAbs, or human WT TGF- β 1 for LHT mAbs. Magenta box: binding by 5 mouse TGF- β 1 activating mAbs (LMT-5, 6, 7, 10 and 11) was lost when region 136-200 of mouse latent TGF- β 1 was replaced by that of human latent TGF- β 1. Turquoise box: binding by 3 other mouse TGF- β 1 activating mAbs (LMT-2, 9 and 12) required aa from region 201-222. The same region was required for binding by 2 human TGF- β 1 activating mAbs (LHT-18 and 22), as these were not able to bind h/m latent TGF- β 1 chimeras unless region 201-222 was replaced by that of human latent TGF- β 1. Black box: human TGF- β 1 activating LHT-15 required region 87-135. No region required for binding could be identified for LHT-4, 20, 25.

(B) 293T cells were co-transfected to express mouse GARP in complex with the additional HA-tagged h/m chimeras indicated on the right and analyzed for binding to the indicated mAbs by flow cytometry. Histogram are gated on live cells. This allowed to refine the binding requirement of 5 mouse TGF- β 1 activating mAbs (LMT-5, 6, 7, 10 and 11) to aa 158-163.

(C) 293T cells were co-transfected to express human or mouse GARP with the indicated HA-tagged human or mouse latent TGF- β 1 single-Ala mutants, respectively, and analyzed for binding to mAbs by flow cytometry. Bar graphs indicate percent binding to each single-Ala mutant relative to WT TGF- β 1 (mean of 2 to 4 experiments + SD). White bars: < 50% binding. Binding by LMT-2 and LMT-12 was lost only when mouse latent TGF- β 1 L208 or D212 were mutated to A, and binding by LHT-22 was lost only when human latent TGF- β 1 G212 or E215 were mutated to A (turquoise boxes). No other LMT or LHT required these residues but no other for binding.

(D) Reporter assay as in Figure 1A, with 293T cells co-transfected to express GARP in complex with the indicated HA-tagged latent TGF- β 1 single-Ala mutants or WT controls. As expected, the mAbs lost the ability to activate mutants to which they do not bind (turquoise boxes). Interestingly, 5 residues that are not required for binding (W207, L208, G211, I214, and F217) are nevertheless required for LHT-22 to activate human TGF- β 1 (black boxes). > : activity on the indicated latent TGF- β 1 single-Ala mutant is superior to that on the corresponding WT TGF- β 1.

A 293T cells co-transfected to express mouse GARP and latent TGF- β 1



B 293T cells transfected with :

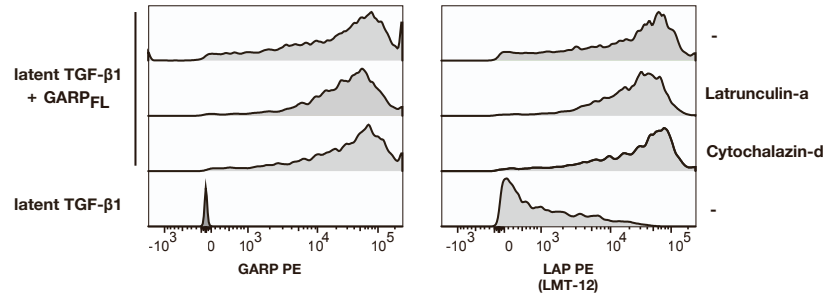


Figure S2. GARP:TGF- β 1 on the cell surface is bound with similar affinities by LMT-7/LMT-7* and LMT-7/IgG1* chimeras, and is not decreased by treatment with inhibitors of actin polymerization

(A) 293T cells transfected as in Fig. 2A were incubated with LMT-7/IgG1*, LMT-7/LMT-7* or control IgG1/IgG1* at the indicated concentrations, then with a secondary goat anti-mouse IgG coupled to PE, and analyzed by flow cytometry. Background: geomMean fluorescence intensity in the presence of IgG1/IgG1* (no binding).

(B) 293T cells transfected as in Fig. 2A were incubated with Latrunculin-a, Cytochalazin-d or medium alone for 24h, then incubated with anti-GARP coupled to PE, or LMT-12 and a secondary anti-mouse IgG coupled to PE prior to analysis by flow cytometry.

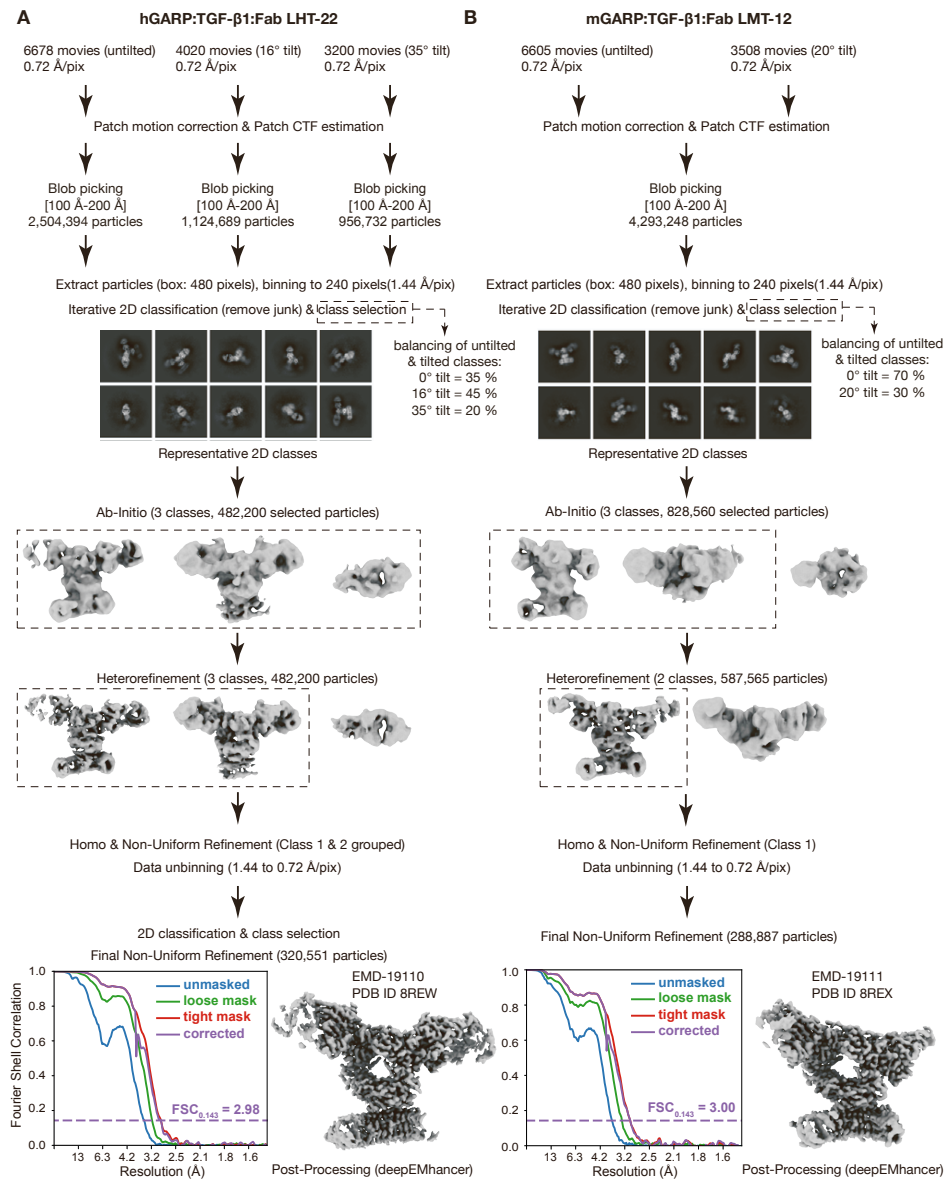


Figure S3. Cryo-EM data processing workflow of human (A) and mouse (B) GARP:TGF-β1:Fab complexes.

Data processing was performed using CryoSPARC v4.2.1 [S1]. Gold-standard Fourier Shell Correlation (FSC) curves are shown (A and B, bottom) after applying either no mask (blue curve), a loose mask (green curve), or a tight mask (red curve) to both half maps before calculating FSC curves. The purple curve depicts the corrected FSC calculated using an auto-tightened mask with correction by noise substitution [S2]. The estimated resolution at FSC = 0.143 (dotted purple lines) is shown for the corrected FSC (purple curve).

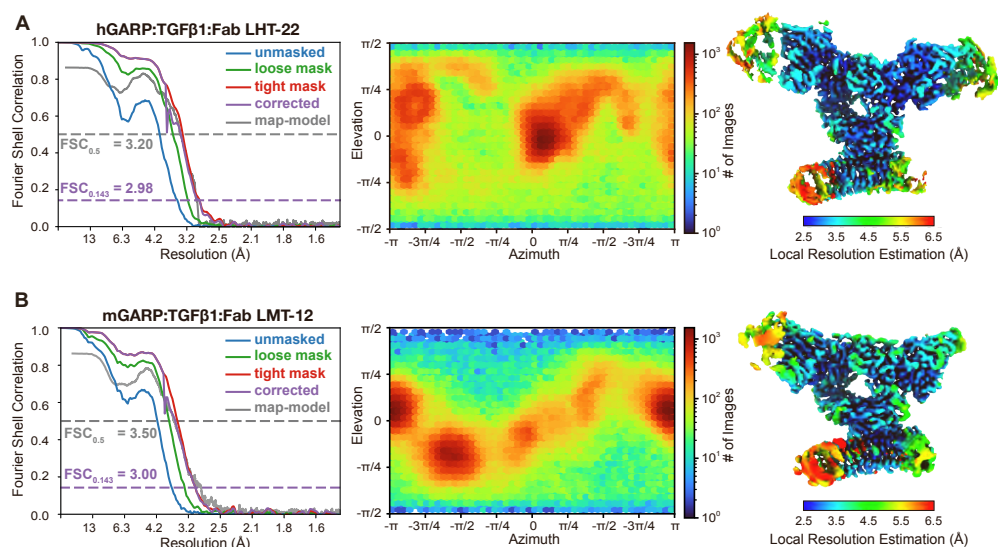


Figure S4. Gold-standard Fourier Shell Correlation (FSC) curves, particle orientation distribution plots and local resolution estimation of human (A) and mouse (B) GARP:TGF-β1:Fab complexes. FSC curves are calculated after applying either no mask (blue curve), a loose mask (green curve), or a tight mask (red curve) to both half maps. The purple curve depicts the corrected FSC calculated using an auto-tightened mask with correction by noise substitution [S2]. The estimated resolution at FSC = 0.143 (dotted purple lines) is shown for the corrected FSC (purple curve). Map-to-model FSC curves are shown as grey curves, and the estimated resolution at FSC = 0.5 is shown as a grey dotted line. To the right of each FSC curve, a particle orientation distribution plot is shown along with the corresponding map colored to local resolution.

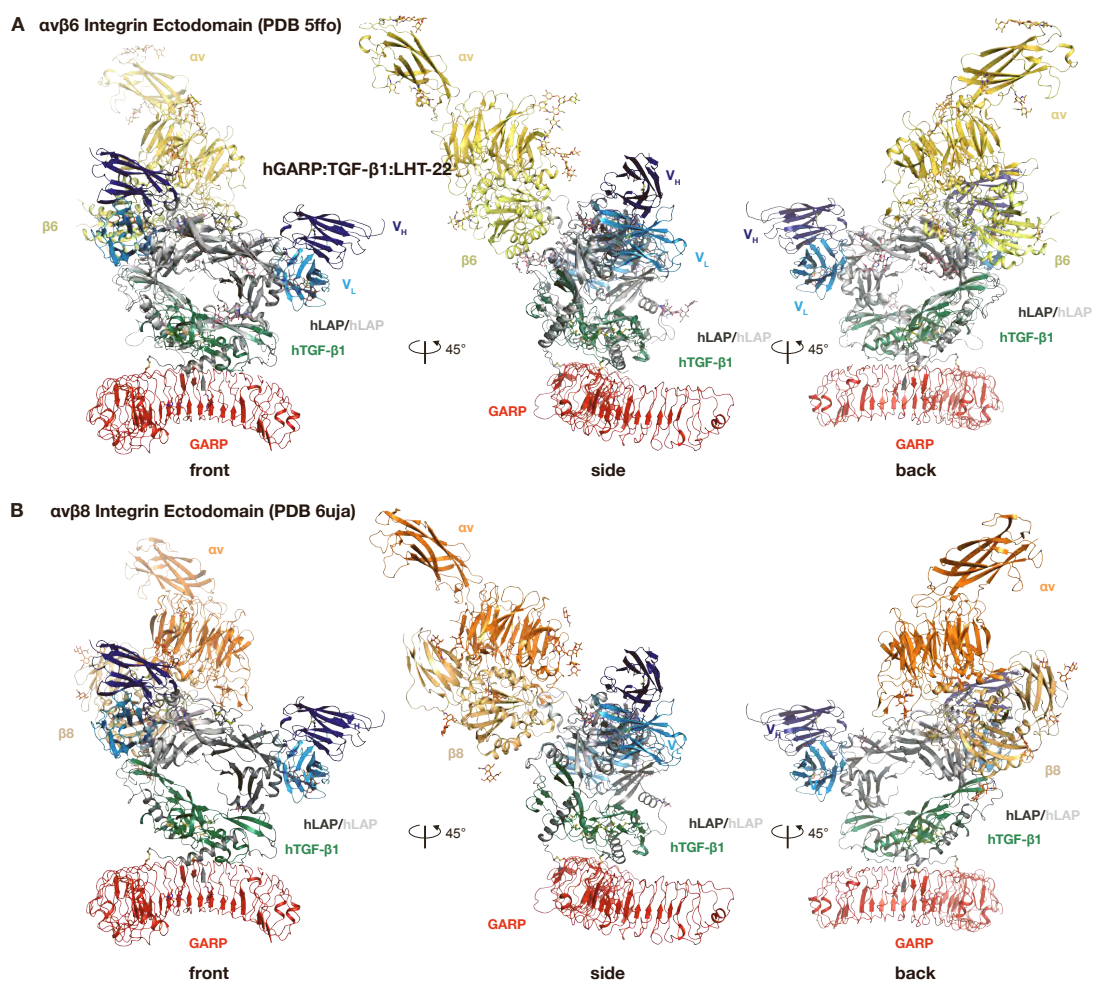
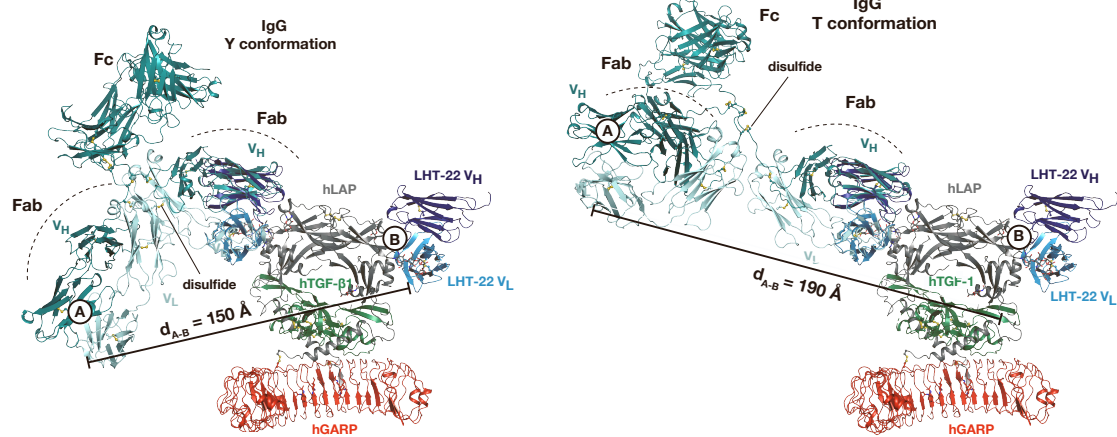


Figure S5. Structural alignment of $\alpha\beta6$ and $\alpha\beta8$ integrin complexes onto the human GARP:TGF- $\beta1$:LHT-22 complex.

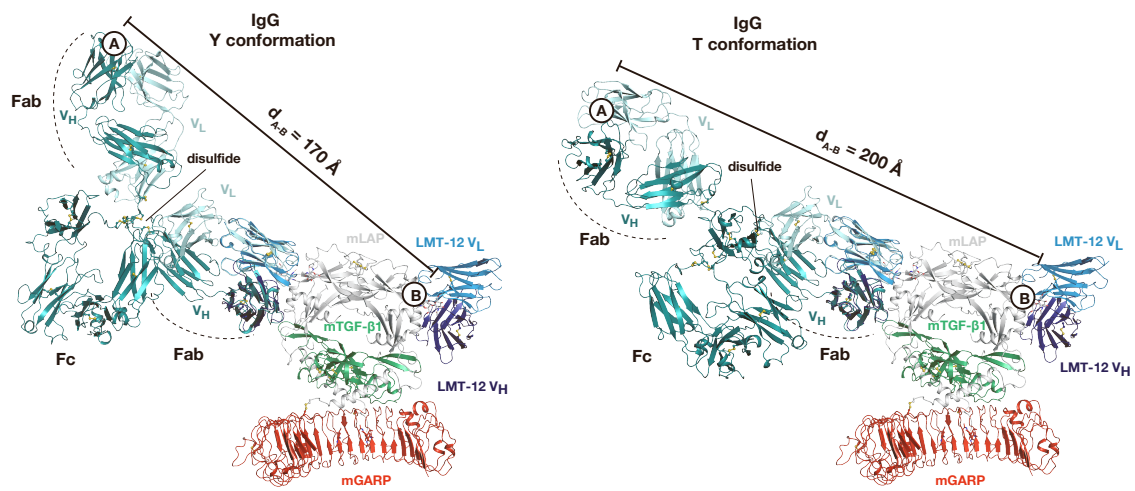
(A) Front (left), side (middle) and back (right) views of the integrin $\alpha\beta6$:TGF- $\beta1$ complex (PDB 5FFO [S3], colored yellow/gold for $\alpha\beta6$ subunits and light grey for TGF- $\beta1$), aligned onto the human GARP:TGF- $\beta1$:LHT-22 Fab complex (hGARP: red, hTGF- $\beta1$: green, hLAP: grey, LHT-22 Fab V_L : light blue, LHT-22 Fab V_H : dark blue).

(B) Front (left), side (middle) and back (right) views of the Integrin $\alpha\beta8$:ITGF- $\beta1$ complex (PDB 6UJA [S4], colored light orange/dark orange for $\alpha\beta6$ subunits and light grey for ITGF- $\beta1$), aligned onto the human GARP:TGF- $\beta1$:LHT-22 Fab complex (hGARP: red, hTGF- $\beta1$: green, hLAP: grey, LHT-22 Fab V_L : light blue, LHT-22 Fab V_H : dark blue).

A Alignment of IgG on a human GARP:TGF- β 1:LHT-22 Fab complex



B Alignment of IgG on a mouse GARP:TGF- β 1:LMT-12 Fab complex



C Alignment of IgG Fab regions with different bends on a human GARP:TGF- β 1:LHT-22 Fab complex

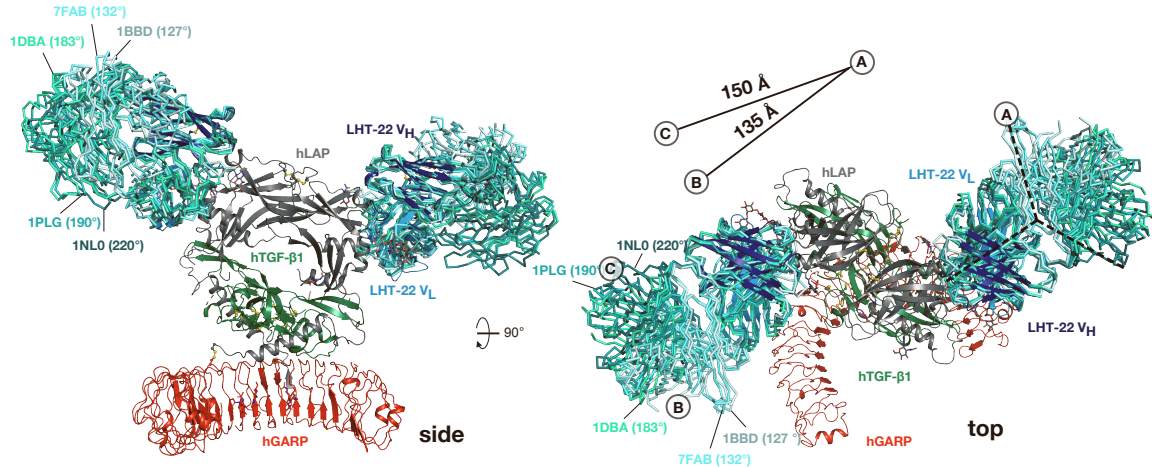


Figure S6.

(A-B) Structural alignment of IgG in Y and T conformations onto human GARP:TGF- β 1:LHT-22 and mouse GARP:TGF- β 1:LMT-12 complexes. Structural alignment of IgG (colored cyan) in the Y (left, PDB 1IGY [S5]) and T (right, PDB 1IGT [S5]) conformations onto LHT-22 or LMT-12 Fabs in the human (top) or mouse (bottom) GARP:TGF- β 1:Fab complexes (human/mouse GARP: red,

human/mouse TGF- β 1: dark/light green, human/mouse LAP: dark/light grey, LHT-22/LMT-12 Fab V_L: light blue, LHT-22/LMT-12 Fab V_H: dark blue). The distances d_{A-B} between the unoccupied epitopes on IgG (A) and the interfaces between human/mouse LAP and LHT-22/LMT-12 Fab (B) are annotated as black lines.

(C) Structural alignment of IgG1 Fab structures with different bends on the human GARP:TGF- β :LHT-22 Fab complex. Side (left) and top (right) views are shown of the human GARP:TGF- β 1:LHT-22 Fab complex (hGARP: red, hTGF- β 1: green, hLAP: grey, LHT-22 Fab V_L: light blue, LHT-22 Fab V_H: dark blue) with a set of Fab structures (colored light to dark cyan) aligned onto LHT-22 Fab. The aligned Fab structures display a wide range of tilts around the elbow angle, ranging from 127° to 220° [S6]: 1BBD: 127°, 7FAB: 132°, 1DBA: 183°, 1PLG: 190°, 1NL0: 220°. The distance between the extremities of the 127° (least) and 220° (most) bend Fabs is annotated as 135 Å (distance B-C) and 150 Å (distance A-C) respectively.

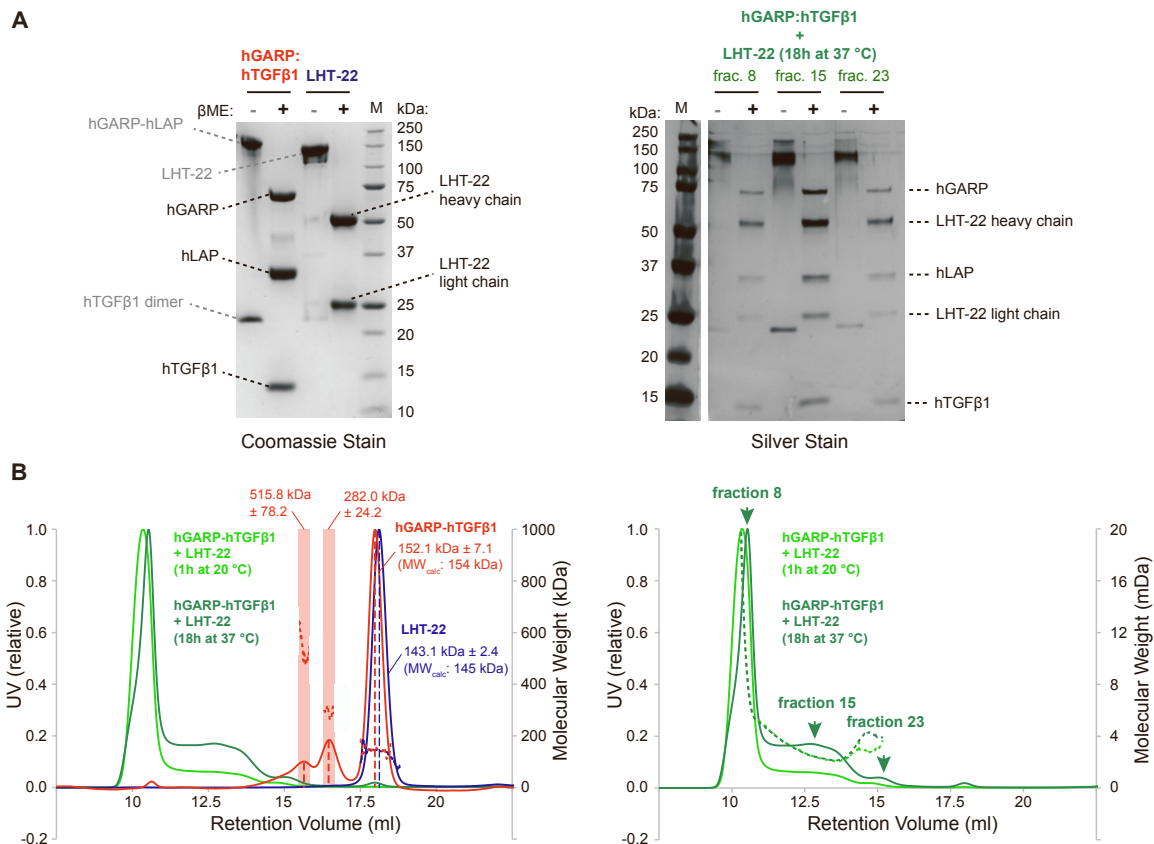


Figure S7. Size Exclusion Chromatography coupled to Multi-Angle Laser Light Scattering (SEC-MALLS) of human GARP:TGF- β 1 mixed with LHT-22 mAb in equimolar amounts, after incubating for either 1 hour at 20 °C or 18 hours at 37 °C.

(A) SDS-PAGE gels of the main peak fractions of SEC-MALLS experiments annotated in B (top red peak: hGARP:ITGF- β 1, top blue peak: LHT-22 mAb, green peak fractions 8, 15 and 23: hGARP: ITGF- β 1 + LHT-22 mAb (1:1 ratio, 18 hours incubation at 37° C). Annotated +/- β ME corresponds to samples with or without added 2-Mercaptoethanol. Bands on the SDS-PAGE gel corresponding to disulfide linked hTGF- β 1 dimer, hGARP-hLAP, or LHT-22 mAb in lanes without β ME are annotated with grey dotted lines. Bands on the SDS-PAGE gel corresponding to hTGF- β 1, hGARP, hLAP, LHT-22 mAb light chain and LHT-22 mAb heavy chain in lanes with added β ME are annotated with black dotted lines.

(B) Molecular weight determination of hGARP:ITGF- β 1 (red curve), LHT-22 mAb (blue curve), hGARP:ITGF- β 1 + LHT-22 mAb (1:1 ratio) after one hour incubation at 20° C (light green curves) and hGARP:ITGF- β 1 + LHT-22 mAb (1:1 ratio) after 18 hour incubation at 37° C (dark green curves) by SEC-MALLS. The determined molecular weight (right axis, dashed curves) is plotted along with the relative UV signal (light axis, full curves). The right plot (light and green curves) displays the same data as the left plot, but with added determined molecular weight (dashed curves) and annotated fractions used in SDS-PAGE gels for clarity.

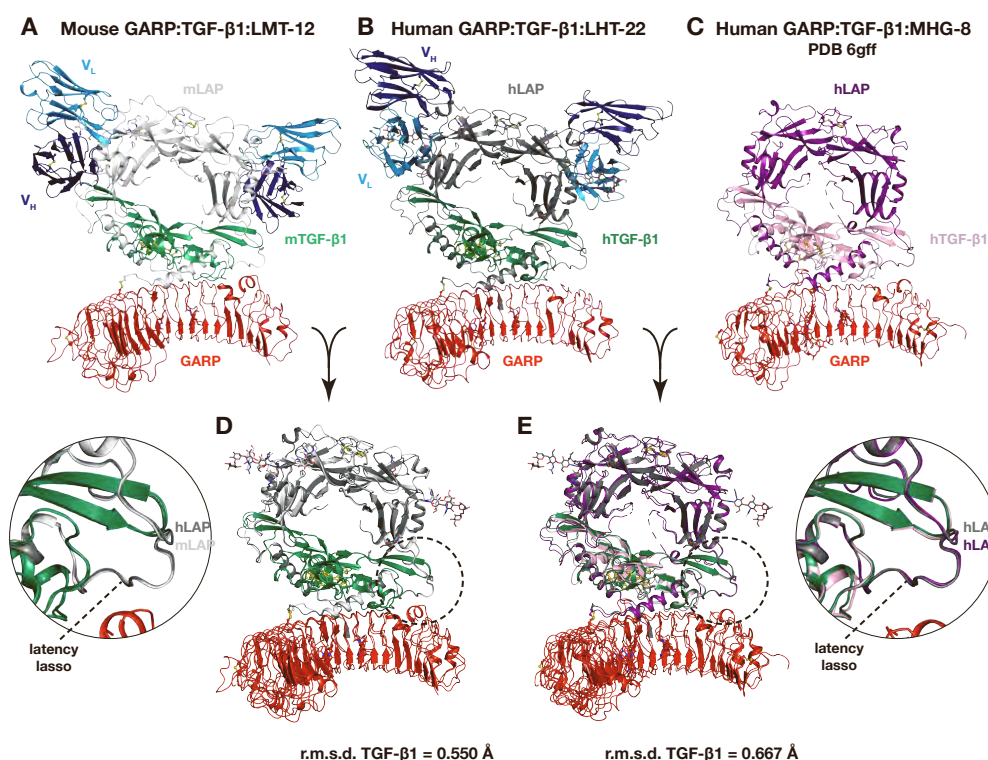


Figure S8. Structural alignment of GARP:TGF-β1:LHT-22, GARP:TGF-β1:LMT-12, and GARP:TGF-β1:MHG-8 complexes.

(A-C) Cartoon representation of the mouse GARP:TGF-β1:LMT-12 complex (A), the human GARP:TGF-β1:LHT-22 complex (B) and the human GARP:TGF-β1 extracted from PDB 6GFF (C).

(D) Structural alignment of TGF-β1 from mouse GARP:TGF-β1:LMT-12 and human GARP:TGF-β1:LHT-22 complexes.

(E) Structural alignment of TGF-β1 from human GARP:TGF-β1:LHT-22 and GARP:TGF-β1:MHG-8 complexes.

R.m.s.d. values depicted under each structural alignment were calculated in PyMOL. For the r.m.s.d. calculation between mouse GARP:TGF-β1:LMT-12 and human GARP:TGF-β1:LHT-22, 518 out of 615 Cα atoms were aligned, while for the r.m.s.d. calculation between human GARP:TGF-β1:LHT-22 and human GARP:TGF-β1:MHG-8, 536 out of 629 Cα atoms were aligned. Alignment of all 615 or 629 Cα atoms results in r.m.s.d. values of 1.594 Å and 1.224 Å respectively. Insets show a zoomed view of the latency lasso region of the 'straitjacket' domain of LAP.

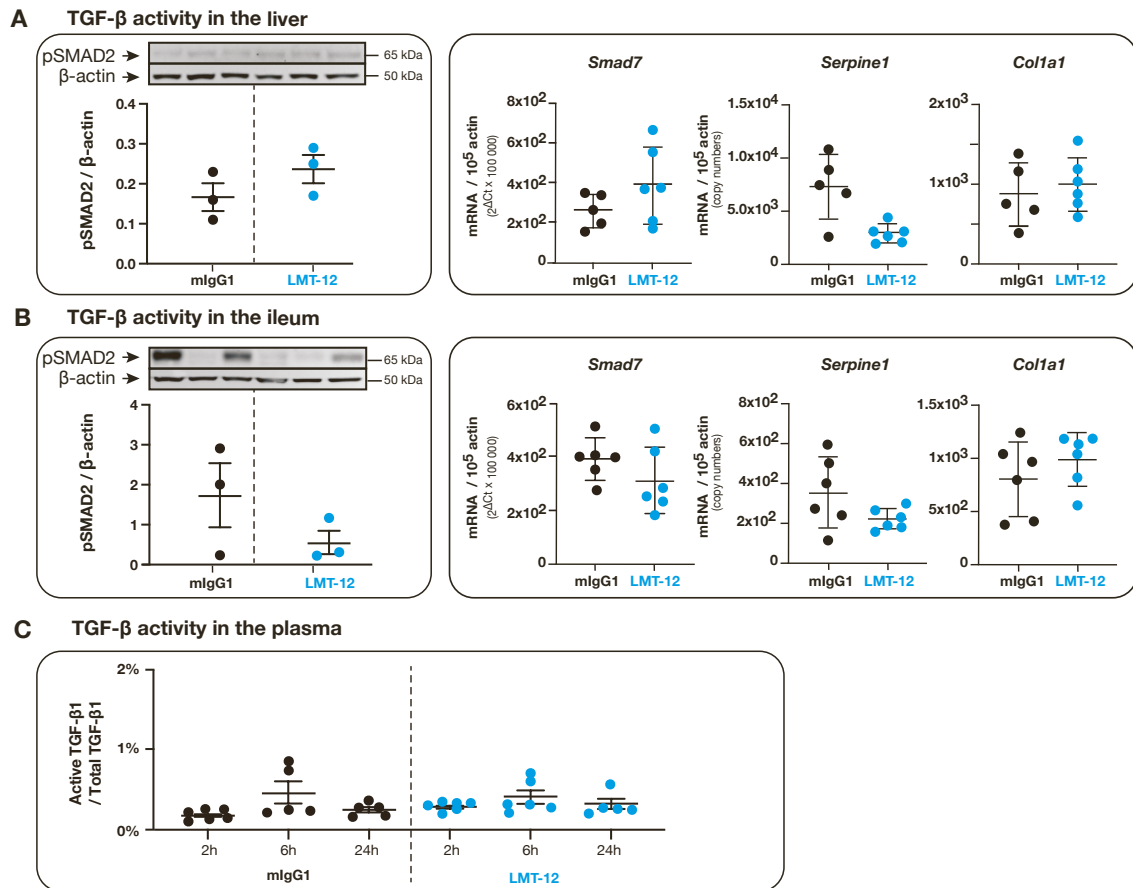


Figure S9. Treatment with LMT-12 does not increase TGF- β 1 activity in liver and ileum in mice with GVHD and does not increase active TGF- β 1 levels in the circulation of C57BL/6 mice.

(A-B) GvHD was induced as indicated in Figure 5, except that recipient mice were euthanized to collect various organs on day 9 after transplantation. Left panels: organs collected from recipient mice on day 9 after transplantation were lysed and analyzed by Western Blot with antibodies against pSMAD2 and β -actin. Each lane in the Western blots and data point in the graphs show analysis of lysate from one individual mouse. ECL signals were quantified to establish pSMAD2/ β -actin ratios. Right panels: expression of TGF- β 1 target genes measured by RT-qPCR. Each data point represents value measured in one individual mouse. Organs were collected from the same mice shown in Figure 6A (spleens). **(C)** Plasma of healthy C57BL/B6 mice was collected at the indicated timepoints after i.p. injection of LMT-12 or a mouse IgG1 control mAb. Plasmatic concentrations of active and total (i.e. active + latent) TGF- β 1 were measured by ELISA. Limit of detection: 16 pg/mL of active TGF- β 1. TGF- β 1 activity is represented as a proportion of active TGF- β 1 in total TGF- β 1.

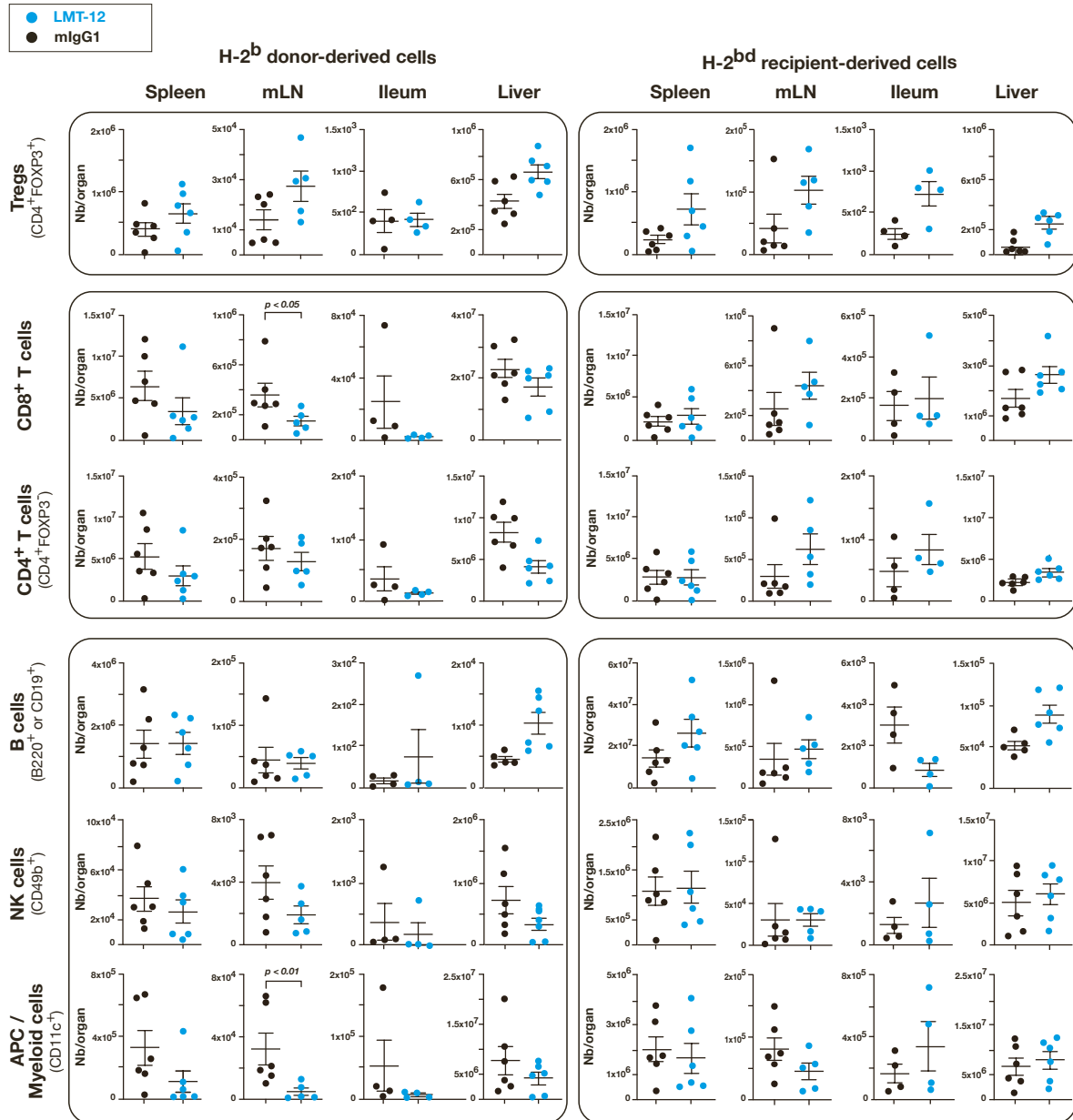


Figure S10. Treatment with LMT-12 does not modify most immune cell populations in spleen, mLN, ileum and liver.

Spleen, mesenteric lymph nodes (mLN), ileum and liver were collected 9 days after transplantation from the same recipient mice shown in Figure S9A-B. Single cell suspensions were prepared on Percoll density gradients for liver and ileum epithelia. Cells were counted and analyzed by flow cytometry with antibodies against CD4, CD8, B220, CD19, CD49b, CD11b, FOXP3, H-2D^d and H-2K^b/H-2D^b to evaluate numbers of cells from donor (H-2^b) and recipient (H-2^{bd}) origin. Statistical analysis was performed using an unpaired two-tailed Mann-Whitney test.

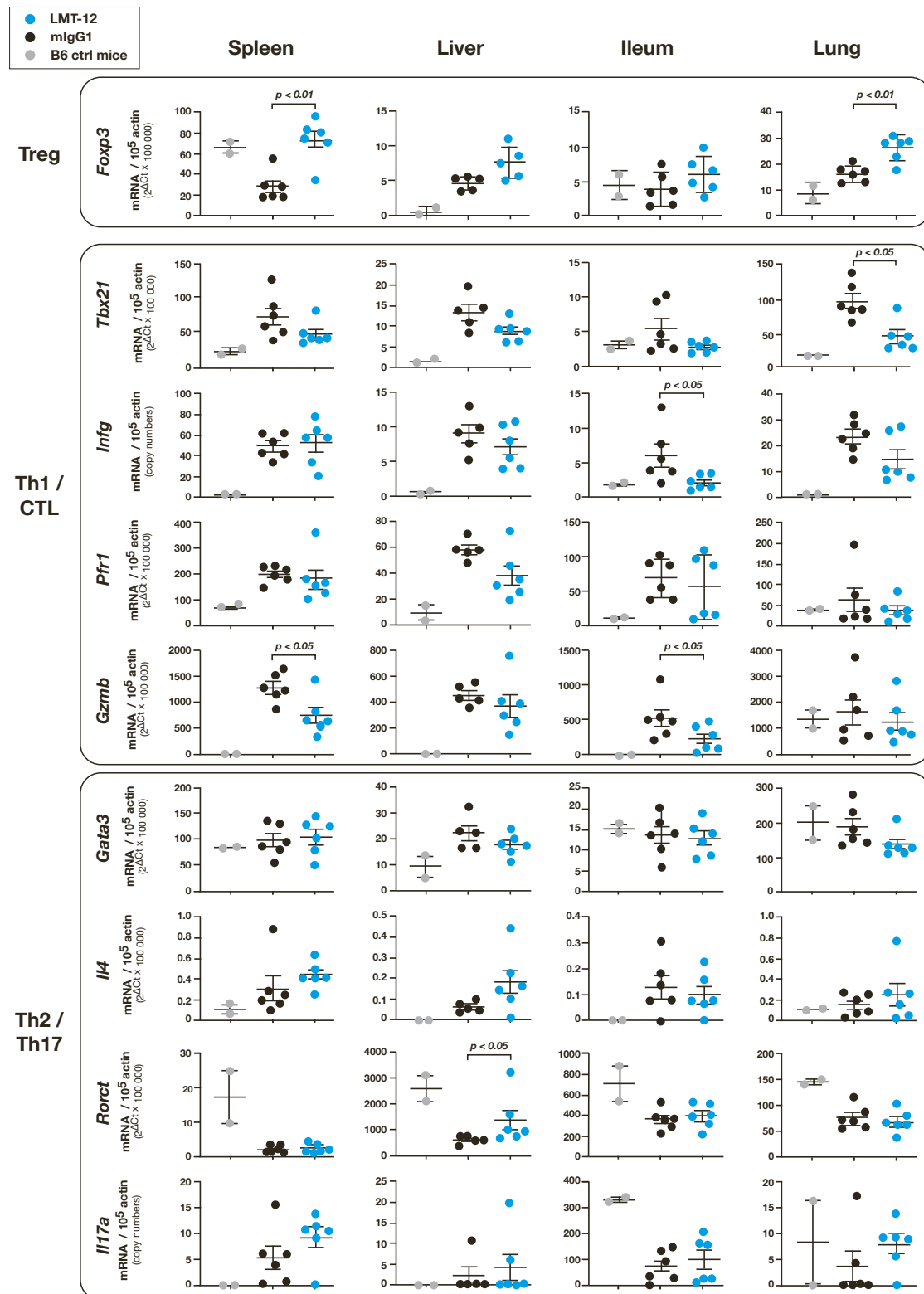


Figure S11. Expression of Th1/CTL genes tend to decrease in mice treated with TGF- β 1 activating mAbs.

RT-qPCR analyses on organs collected 9 days after transplantation from the same recipient mice shown in Figure S9A-B and S10, or from healthy B6D2 F1 mice as controls. Each data point represents expression level in one individual mouse. Statistical analysis was performed using an unpaired two-tailed Mann-Whitney test.

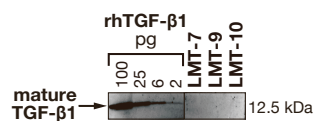


Figure S13. Absence of contaminating active TGF-β1 in antibody preparations

Western Blot analysis of mature TGF-β1 content in 25 μg of the indicated mAb preparations. The indicated amounts of rhTGF-β1 were used as references.

SUPPLEMENTAL TABLE 1

Interaction between human latent TGF- β 1 and a Fab derived from LHT-22
Analysis performed with PISA server (<https://www.ebi.ac.uk/pdbe/pisa/>)

LAP - LHT-22 V_L interaction

Hydrogen bonds (pairwise interactions, chain A: LAP, chain F: LHT-22 V_L)

	Chain F (LHT-22 V _L)	Dist. (Å)	Chain A (LAP)
1	F:ARG 50 [NH1]	2.57	A:SER 209 [O]
2	F:ASN 51 [ND2]	3.30	A:SER 138 [O]
3	F:ASN 51 [ND2]	2.99	A:GLU 142 [OE2]
4	F:TYR 53 [OH]	2.95	A:GLY 212 [O]
5	F:TYR 53 [OH]	3.27	A:SER 138 [OG]
6	F:TYR 53 [OH]	2.72	A:ARG 141 [NH1]
7	F:ASN 71 [ND2]	2.98	A:ARG 210 [O]
8	F:TYR 112 [OH]	3.08	A:GLU 213 [OE1]
9	F:TYR 112 [OH]	3.05	A:GLU 213 [OE2]

Full list of interfacing residues (not pairwise, chain A: LAP, chain F: LHT-22 V_L)

Chain F (LHT-22 V _L)	Chain A (LAP)
1 F:ARG 50 (H*)	1 A:SER 138 (H)
2 F:ASN 51 (H)	2 A:ARG 141 (H)
3 F:TYR 53 (H)	3 A:GLU 142
4 F:TYR 70	4 A:PRO 145
5 F:ASN 71 (H)	5 A:GLU 146
6 F:VAL 73	6 A:PRO 147
7 F:ALA 74	7 A:LEU 208
8 F:ARG 75	8 A:SER 209 (H)
9 F:TYR 112 (H)	9 A:ARG 210 (H)
10 F:ASN 119	10 A:GLY 211
	11 A:GLY 212 (H)
	12 A:GLU 213 (H)
	13 A:GLU 215

*H: Hydrogen Bond

LAP - LHT-22 V_H interaction

Hydrogen bonds (pairwise interactions, chain A: LAP, chain G: LHT-22 V_H)

	Chain G (LHT-22 V _H)	Dist. (Å)	Chain A (LAP)
1	G:THR 124 [OG1]	3.29	A:GLU 213 [OE1]
2	G:TYR 125 [OH]	2.30	A:GLU 215 [OE2]
3	G:ASP 50 [OD1]	2.89	A:LYS 123 [NZ]
4	G:ASP 49 [O]	2.51	A:LYS 123 [NZ]
5	G:ASP 122 [OD2]	3.57	A:ILE 214 [N]

Salt bridges (pairwise interactions, chain A: LAP, chain G: LHT-22 V_H)

Chain G (LHT-22 V _H)	Dist. (Å)	Chain A (LAP)
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1 G:ASP 50[OD1] 2.89 A:LYS 123[NZ]

Full list of interfacing residues (not pairwise, chain A: LAP, chain G: LHT-22 V_H)

Chain G (LHT-22 V _H)			Chain A (LAP)		
1	G:ASP	49	1	A:GLU	119
2	G:ASP	50 (H*/S**)	2	A:ILE	120
3	G:TYR	51	3	A:ASP	122
4	G:ARG	71	4	A:LYS	123 (H/S)
5	G:TRP	72	5	A:PHE	124
6	G:ASN	73	6	A:PHE	134
7	G:VAL	75	7	A:LYS	173
8	G:THR	76	8	A:TYR	174
9	G:GLY	119	9	A:SER	175
10	G:SER	120	10	A:ASN	176
11	G:ILE	121	11	A:ASN	177
12	G:ASP	122 (H)	12	A:ARG	210
13	G:LEU	123	13	A:GLY	211
14	G:THR	124 (H)	14	A:GLY	212
15	G:TYR	125 (H)	15	A:GLU	213 (H)
			16	A:ILE	214 (H)
			17	A:GLU	215 (H)

*H: Hydrogen Bond

**S: Salt Bridge

SUPPLEMENTAL TABLE 2

Interaction between mouse latent TGF- β 1 (LAP) and a Fab derived from LMT-12
Analysis performed with PISA server (<https://www.ebi.ac.uk/pdbe/pisa/>)

LAP – LMT-12 V_L interaction

Hydrogen bonds (pairwise interactions, chain A: LAP, chain I: LMT-12 V_L)

	Chain I (LMT-12 V _L)	Dist. (Å)	Chain A (LAP)
1	I:TYR 52 [OH]	3.11	A:GLN 210 [OE1]
2	I:TYR 53 [N]	3.40	A:ASP 212 [O]
3	I:TYR 112 [OH]	3.31	A:ASN 209 [O]

Full list of interfacing residues (not pairwise, chain A: LAP, chain I: LMT-12 V_L)

	Chain I (LMT-12 V _L)		Chain A (LAP)
1	I:SER 55	1	A:SER 138
2	I:SER 46	2	A:TYR 174
3	I:GLY 50	3	A:SER 175
4	I:GLY 51	4	A:ASN 176
5	I:TYR 52 (H*)	5	A:ASN 209 (H)
6	I:TYR 53 (H)	6	A:GLN 210 (H)
7	I:TYR 112 (H)	7	A:GLY 211
8	I:SER 113	8	A:ASP 212 (H)
9	I:SER 117	9	A:GLY 213
		10	A:ILE 214
		11	A:GLN 215

*H: Hydrogen Bond

LAP – LMT-12 V_H interaction

Hydrogen bonds (pairwise interactions, chain A: LAP, chain H: LMT-12 V_H)

	Chain H (LMT-12 V _H)	Dist. (Å)	Chain A (LAP)
1	H:LYS 125 [NZ]	3.46	A:SER 138 [OG]
2	H:TYR 52 [OH]	2.27	A:PRO 145 [O]
3	H:SER 71 [OG]	2.84	A:GLU 146 [OE1]
4	H:GLY 75 [N]	3.36	A:GLU 146 [OE1]
5	H:TYR 52 [OH]	3.26	A:ARG 141 [NH1]
6	H:ARG 122 [O]	3.86	A:GLU 142 [N]
7	H:TYR 124 [O]	2.68	A:ASP 212 [N]

Full list of interfacing residues (not pairwise, chain A: LAP, chain H: LMT-12 V_H)

	Chain H (LMT-12 V _H)		Chain A (LAP)
1	H:SER 49	1	A:SER 138 (H)
2	H:TYR 52 (H)	2	A:ARG 141 (H)
3	H:SER 71 (H)	3	A:GLU 142 (H)
4	H:ILE 72	4	A:PRO 145 (H)
5	H:GLY 73	5	A:GLU 146 (H)
6	H:GLY 75 (H)	6	A:PRO 147
7	H:SER 76	7	A:PRO 148
8	H:TRP 78	8	A:LEU 149

9	H:ASN 120	9	A:LEU 208
10	H:SER 121	10	A:ASN 209
11	H:ARG 122 (H)	11	A:GLN 210
12	H:SER 123	12	A:GLY 211
13	H:TYR 124 (H)	13	A:ASP 212 (H)
14	H:LYS 125 (H)	14	A:LEU 265
		15	A:GLU 266

*H: Hydrogen Bond

SUPPLEMENTAL TABLE 3

List of primers used for RT-qPCR

Primer Sequence (5' - 3')	Definition
TCAAGTGGCATAGATGTGGAAGAA	Forward primer in <i>mlfng</i> mRNA
TGGCTCTGCAGGATTTTCATG	Reverse primer in <i>mlfng</i> mRNA
TCACCATCCTTTTGCCAGTTCCTCCAG	Taqman probe in <i>mlfng</i> mRNA
AAGAGAGCAAGGACAACACTC	Forward primer in <i>mGzmb</i> mRNA
CATGTCCCCCGATGATCTC	Reverse primer in <i>mGzmb</i> mRNA
ACAAGGTCAGCAGTAGCAGGAGGA	Taqman probe in <i>mGzmb</i> mRNA
CAGTAGAGTGTCGCATGTACAG	Forward primer in <i>mPrf1</i> mRNA
GATGAGCCTGTGGTAAGCAT	Reverse primer in <i>mPrf1</i> mRNA
TCGCCTGGTACAAAAACCTCCACTC	Taqman probe in <i>mPrf1</i> mRNA
CATCTTCTCAAATTGAGTGACAA	Forward primer in <i>mTnfa</i> mRNA
GGGTTGTACCTTGTCTACTCCCA	Reverse primer in <i>mTnfa</i> mRNA
CACGTCGTAGCAAACCACCAAGTGGA	Taqman probe in <i>mTnfa</i> mRNA
CAAGACCACATCCACAAACATC	Forward primer in <i>mTbx21</i> mRNA
TTCAACCAGCACCAGACAG	Reverse primer in <i>mTbx21</i> mRNA
TCACTAAGCAAGGACGGCGAATGT	Taqman probe in <i>mTbx21</i> mRNA
CCTTATCAAGCCCAAGCGAA	Forward primer in <i>mGata3</i> mRNA
GTCCCCATTAGCGTTCCTC	Reverse primer in <i>mGata3</i> mRNA
TGTCCCTGCTCTCCTTGCTGC	Taqman probe in <i>mGata3</i> mRNA
GCTCCAGAAGGCCCTCAG	Forward primer in <i>mI17a</i> mRNA
CTTTCCTCCGCATTGACA	Reverse primer in <i>mI17a</i> mRNA
ACCTCAACCGTTCCACGTCACCCTG	Taqman probe in <i>mI17a</i> mRNA
TCTGCAAGACTCATCGACAAG	Forward primer in <i>mRorc</i> mRNA
GAGGTGCTGGAAGATCTGC	Reverse primer in <i>mRorc</i> mRNA
CTAGCCAAGCTGCCACCCAAAG	Taqman probe in <i>mRorc</i> mRNA
CCCCGCCACCTGGAAGAAT	Forward primer in <i>mFoxp3</i> mRNA
CTCCCTTCTCGCTCTCCAC	Reverse primer in <i>mFoxp3</i> mRNA
CCATCCGCCACAACCTGAGCC	Taqman probe in <i>mFoxp3</i> mRNA
CTGTGTTGCTGTGAATCTTACG	Forward primer in <i>mSmad7</i> mRNA
CATTGGGTATCTGGAGTAAGGAG	Reverse primer in <i>mSmad7</i> mRNA
AGATCAACCCCGAGCTGGTGTG	Taqman probe in <i>mSmad7</i> mRNA
CGCAAAGAGTCTACATGTCTAGG	Forward primer in <i>mCol1a1</i> mRNA

CATTGTGTATGCAGCTGACTTC	Reverse primer in <i>mCol1a1</i> mRNA
CCGGAGGTCCACAAAGCTGAACA	Taqman probe in <i>mCol1a1</i> mRNA
AGTAACTTCGTGCCTAGCAAC	Forward primer in <i>mCol1a2</i> mRNA
CATCAACACCATCTCTGCCT	Reverse primer in <i>mCol1a2</i> mRNA
CCCTTTCGTA CTGATCCCGATTGCA	Taqman probe in <i>mCol1a2</i> mRNA
GAGCTATCCATTTTCACCTTCAGA	Forward primer in <i>mFn1</i> mRNA
TTGTTCTAGACACTGGAGAC	Reverse primer in <i>mFn1</i> mRNA
CAGGAGATTTGTTAGGACCACGGCA	Taqman probe in <i>mFn1</i> mRNA
GAGCTACGAACTGCCTGAC	Forward primer in <i>mActa2</i> mRNA
CTGTTATAGGTGGTTTCGTGGA	Reverse primer in <i>mActa2</i> mRNA
CCGCTGACTCCATCCCAATGAAAGA	Taqman probe in <i>mActa2</i> mRNA
AAGAACCAGGAGCATGTAGAA G	Forward primer in <i>mFap</i> mRNA
TTGTAACCATCCTTGTGCGCTA	Reverse primer in <i>mFap</i> mRNA
ACA AAG AAT CCA CCA GCC CAT CCT	Taqman probe in <i>mFap</i> mRNA
CTATGGTGAAACAGGTGGACT	Forward primer in <i>mSerpine1</i> mRNA
CGTGTCAGCTCGTCTACAG	Reverse primer in <i>mSerpine1</i> mRNA
ACCTTTGGTATGCCTTTCCACCCA	Taqman probe in <i>mSerpine1</i> mRNA

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TGF- β 1 activating antibodies to treat chronic graft-versus-host disease

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KEYWORDS

Graft-versus-host disease, transforming growth factor beta 1, regulatory T cells, monoclonal antibodies, thymic Tregs, thymus, autoimmunity

ABSTRACT

Graft-versus-host disease (GVHD) is a frequent and severe complication of allogeneic hematopoietic stem cell transplantation caused by uncontrolled donor T cell activity against recipient antigens. The acute phase is triggered mainly by mature alloreactive donor T cells present in the graft, whereas chronic GVHD also results from the auto-reactive T cell repertoire that reconstitutes in recipients after transplantation. Current therapeutic options for acute and chronic GVHD are limited. Clinical approaches under development aim at enhancing immune tolerance by promoting regulatory T cell (Treg) immunosuppression. Tregs suppress other immune cells at least in part by producing active TGF- β 1. We recently developed monoclonal antibodies (mAbs) that activate latent TGF- β 1 and showed that they exert therapeutic effects in a Treg-dependent manner in a murine model of acute GVHD. Here, we report that TGF- β 1 activating mAbs could also serve to treat chronic GVHD. A TGF- β 1 activating mAb decreased disease severity and increased survival in irradiated mice grafted with allogeneic bone marrow cells and splenocytes, which induce a chronic form of GVHD characterized by severe cutaneous damage. Treatment with the mAb was associated with reduced destruction of the hypodermis. Expression of TGF- β 1 target genes tended to increase in thymus and spleen after 6 weeks of treatment, indicating that the mAb enhanced TGF- β 1 signaling in lymphoid organs. The therapeutic effect was associated with higher proportions of CD8⁺, CD4⁺FOXP3⁻, and CD4⁺FOXP3⁺ single positive (SP) T cells within thymocytes, arguing for enhanced thymic function and development of SP T cells, including thymic Tregs (tTregs). In spleen and blood, we observed decreased proportions of activated cells within CD8⁺ T lymphocytes. In line with this, treatment with the TGF- β 1 activating mAb reduced expression of genes associated with type 1 adaptive immune responses in several organs (skin, kidney, lung and liver). Treatment also decreased proportions of innate immune cells in the spleen. Altogether, our results suggest that TGF- β 1 activating mAbs may serve as a treatment for chronic GVHD in patients, by improving thymic function and controlling type 1 adaptive T cell responses and inflammation in the periphery.

INTRODUCTION

GVHD is a frequent and life-threatening complication of allogeneic hematopoietic stem cell transplantation (allo-HSCT) caused by donor T lymphocytes reacting against recipient antigens. Acute GVHD (aGVHD) is characterized by massive immune cell infiltration in tissues and major inflammation occurring less than 100 days after transplantation. It is mainly driven by alloreactive mature T cells that recognize major or minor histocompatibility antigens (MHC and miHA, respectively).¹ Chronic GVHD (cGVHD), on the other hand, is mainly characterized by auto-immune manifestations associated with aberrant tissue repair and fibrosis.²⁻⁴ Lack of immune tolerance during cGVHD mainly results from thymic dysfunction in reconstituted graft recipients.^{5,6} In healthy individuals, the thymus supports development of a very diverse mature naïve T cell repertoire able to recognize foreign peptides presented by self MHC molecules (foreign pMHCs) and remain tolerant to self (self pMHCs). Such a diverse but tolerant T cell repertoire establishes as a result of random recombinations of *TCRA* and *TCRB* gene segments in hematopoietic lymphoid progenitors, which become double-positive ($CD4^+CD8^+$; DP) T progenitors ready to undergo sequential positive and negative selections.⁷ DP cells with TCRs that bind self pMHC with sufficient affinity in the thymic cortex are positively selected and survive to become $CD4^+$ or $CD8^+$ single positive (SP) progenitors. In the thymic medulla, negative selection eliminates $CD4^+$ or $CD8^+$ SP progenitors that have high affinity for self pMHC, while $CD4^+$ SP T progenitors with intermediate affinity develop into thymic Tregs (tTregs) in response to strong co-stimulatory signals and cytokines such as IL-2, IL-7 and TGF- β 1.⁸⁻¹¹ Mature naïve $CD8^+$ T cells, $CD4^+FOXP3^-$ T cells and $CD4^+FOXP3^+$ tTregs then egress from the thymus to reach the periphery, where they can exert effector functions. tTregs are mainly immunosuppressive and are indispensable to maintain immune tolerance in the periphery.¹² After allo-HSCT, the thymus supports development of T cells from donor lymphoid progenitors, but thymic injury caused by aGVHD impairs negative selection and tTreg development.^{5,6} Consequently, more autoreactive T cells that are less efficiently controlled by tTregs can be activated in the periphery, leading to chronic inflammation, production of auto-antibodies by B cells and collagen deposition by macrophages in peripheral tissues.

Despite prophylactic treatments designed to limit excessive donor T cell reactivity, 30-50% of patients will develop acute and/or chronic GVHD.^{13, 14} Ongoing clinical trials explore strategies to enhance immune tolerance after allo-HSCT through adoptive transfer of donor Tregs, or *in vivo* expansion of donor Tregs by administration of low dose IL-2. Both strategies have shown promising early results¹⁵⁻¹⁹ but their application in clinical practice remains limited by treatment resistance and significant technical challenges.

Increasing the activity of transforming growth factor beta 1 (TGF- β 1) to treat GVHD is an interesting alternative to the transfer or *in vivo* expansion of donor Tregs. TGF- β 1 indeed plays critical roles in suppressing autoreactive T cells²⁰, as best illustrated in mice deficient in *Tgfb1* or lacking TGF- β 1 signaling in T cells (*CD4^{Cre}xTgfb1^{2-/-}*), which succumb within 3-4 weeks after birth from severe inflammation and autoimmunity.²¹⁻²³ TGF- β 1 activity is tightly regulated at a post-transcriptional level. Virtually all cell types can produce TGF- β 1 as an inactive, latent complex composed of mature TGF- β 1 non-covalently bound to the latency-associated peptide (LAP). LAP prevents binding of mature TGF- β 1 to the TGF- β receptor.^{24, 25} Only a few cell types can release mature TGF- β 1 from the LAP through cell-type specific mechanisms, in a process called “latent TGF- β 1 activation”. We and others previously showed that integrin α v β 8 activates latent TGF- β 1 presented on the surface of TCR-stimulated Tregs by a transmembrane protein called GARP.²⁶⁻²⁹ In mice and humans, the absence of functional Tregs leads to a fatal inflammatory disease that closely resembles TGF- β 1 deficiency.^{30, 31} In addition to tTregs, which develop in the thymus from CD4⁺FOXP3⁻ SP progenitors, so-called peripherally-induced Tregs or pTregs can also differentiate from mature naïve CD4⁺ T cells in the presence of TGF- β 1 in the periphery.³² Noteworthy, it remains difficult to distinguish tTregs from pTregs due to the lack of unambiguous markers to distinguish these cell subtypes in various tissues, particularly in humans. Human and mouse Tregs (tTregs and pTregs) exert immunosuppression at least in part via production of active TGF- β 1.^{25, 29, 32-34}

We recently derived antibodies that enhance TGF- β 1 activation, with the objective to address diseases characterized by deleterious T cell activity insufficiently controlled by Tregs.³⁵ We showed that the mAbs exert therapeutic effects in a murine model of aGVHD. The therapeutic effect was associated with increased TGF- β 1 activity and Treg numbers in the spleen, and was abrogated in the absence of Tregs. Here, we investigated whether TGF- β 1 activating mAbs could also exert therapeutic activity in cGVHD, and if so, by which mechanisms.

RESULTS

A TGF- β 1 activating mAb decreased disease severity and increased survival in murine chronic GVHD characterized by severe cutaneous damage.

To induce chronic GVHD in mice, we transplanted a mix of T cell-depleted BM cells (7.5×10^6) and splenocytes (25×10^6) isolated from B10.D2 donors into lethally irradiated female BALB/c recipients (**Figures 1A and S1**). In this model, often referred to as “sclerodermatous cGVHD”³⁶⁻³⁸, donor and recipient mice share the same MHC antigens (H-2^d). GVHD is caused by mature donor T cells activated by minor histocompatibility antigens. All recipients and control mice transplanted with BM cells only (BM controls) develop moderate symptoms linked to aGVHD or irradiation during the first two weeks after transplantation (**Figure S2A**). Approximately 5% of the mice reach a predefined humane endpoint and are euthanized before day 14. Recipients surviving this acute phase develop cutaneous symptoms 3 weeks after transplantation. Symptoms include fur loss, ear sclerosis, conjunctivitis, and scaling of the tail, allowing to establish a cGVHD score (**Figure 1A and S2B**). BM controls do not develop detectable signs of cGVHD. To establish whether TGF- β 1 activating mAbs could serve to treat cGVHD, recipient mice received weekly injections of a TGF- β 1 activating mAb (LMT-12) or an isotype control starting one day before transplantation (**Figure 1A**). All recipients receiving the isotype control developed cutaneous symptoms 4 weeks after the graft, with 72% of the mice exhibiting a cGVHD score ≥ 1 at week 7 (mean score $\pm$ sem : 3.1 ± 0.4). At this timepoint, 25% of the mice had reached a cGVHD score of 4.5 and were euthanized, with an overall mortality of 40% at the end of the experiment (**Figure 1B and S2B**). In contrast, only 24% of mice treated with the TGF- β 1 activating mAb developed a cGVHD score ≥ 1 at week 7 (0.7 ± 0.2) and none had to be euthanised before week 9. Notably, 88% of mice treated with the TGF- β 1 activating mAb achieved long-term survival. Our results indicate that a TGF- β 1 activating mAb decreases disease severity and increases survival in cGVHD. When treatment with the TGF- β 1 activating mAb was started 2 weeks after transplantation however, we did not observe significant effects on disease severity or survival by comparison to the isotype control (**Figure S3**).

We analyzed dorsal skin samples collected 6 weeks after transplantation. Increased ratios of [dermis] to [dermis + hypodermis] thickness is used to measure cutaneous damage in many murine cGVHD models.³⁹⁻⁴¹ As illustrated in **Figure 1C**, we observed significantly increased ratios in recipients transplanted with BM cells and splenocytes (mean $\pm$ sem : 0.82 ± 0.04) by

comparison to BM controls (0.41 ± 0.02). This increase was mainly driven by a marked reduction in hypodermal thickness (mean $\pm$ sem : 240 ± 32 vs 46 ± 13 μ m in BM controls or recipients with cGVHD, respectively). As expected from clinical cGVHD scores, treatment with the TGF- β 1 activating mAb significantly reduced the [dermis] to [dermis + hypodermis] ratio by comparison to the isotype control (0.67 ± 0.03), resulting from a 2-fold increase in hypodermal thickness (87 ± 11 μ m). Altogether, results above indicate that a TGF- β 1 activating mAb decreased disease severity, assessed by clinical symptoms and skin histology, and increased survival in mice with cGVHD.

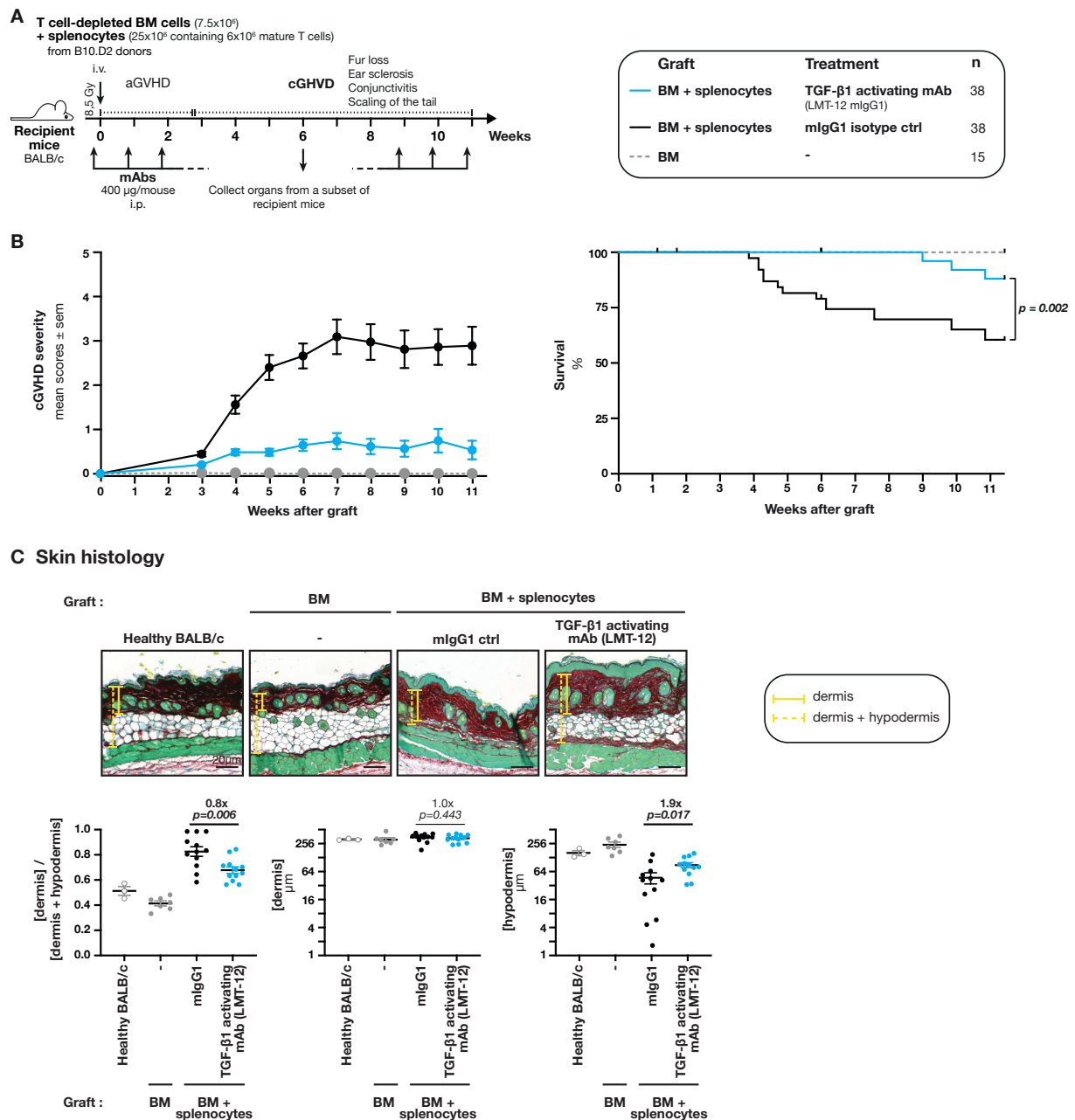


Figure 1. A TGF- β 1 activating mAb reduced disease severity and increased survival in murine cGVHD, characterized by severe cutaneous damage.

(A) Schematic experimental design. T cell-depleted bone marrow cells (BM) and splenocytes isolated from B10.D2 donors were transplanted into lethally irradiated female BALB/c recipients. BM controls were grafted with BM cells only. Recipient mice received weekly injections of a TGF- β 1 activating mAb (LMT-12 mIgG1) or an isotype control (mIgG1 ctrl) starting one day before transplantation. Several organs were collected 6 weeks after transplantation from a predefined subset of mice (n=13 for mice transplanted with BM cells and splenocytes and receiving either TGF- β 1 activating mAb or isotype control, and n=8 for BM controls). **(B) Left graph** : Evolution of the mean of cGVHD scores, established based on fur loss (1 if fur loss < 1 cm²; 2 if fur loss of 1-2,5 cm²; 3 if fur loss $\geq$ 2,5 cm²), ear sclerosis (1 if 1 ear affected; 2 if both ears affected), conjunctivitis (0.25 if 1 eye affected; 0.5 if both eyes affected) and scaling of the tail (0.25 if slight scaling; 0.5 if severe scaling). Mice were euthanized when reaching a score $\geq$ 4.5, or when reaching predefined humane endpoint. The maximal score (death) is 5. For each experimental week, a representative day was selected to allow comparison of two combined experiments in which mice were analyzed on different days. Error bars denote sem. **Right graph** : Kaplan-Meier plots representing the proportion of mice alive at the indicated weeks after transplantation. P values calculated using a Gehan-Breslow Wilcoxon test. Vertical mark : censored data. **(C)** Representative pictures (sirius red 150x) and histological analyses of dorsal skin samples collected 6 weeks after transplantation. Ratios were calculated by dividing thickness of [dermis] (full yellow line) by thickness of [dermis + hypodermis] (from basal epidermis to muscle layer; dashed yellow line). Healthy BALB/c were non irradiated non grafted controls. Fold change (x) values above each graph represent ratios of mean values in the group receiving the TGF-1 activating mAbs to that of the isotype control, with p values indicated below. Fold changes $\geq$ 1.4 and p values < 0.05 are highlighted in bold. Statistical analysis were performed using a Mann-Whitney unpaired t-test. Threshold for statistical significance: 0.05.

The TGF- β 1 activating mAb enhanced TGF- β 1 signaling in lymphoid organs of mice with cGVHD.

We next assessed whether the TGF- β 1 activating mAb enhanced TGF- β 1 signaling, and if so, in which organs. We previously showed that TGF- β 1 activating mAbs increased TGF- β 1 activity in spleen, but not in other organs, during murine aGVHD.³⁵ We thus examined TGF- β 1 activity in primary and secondary lymphoid organs (thymus and spleen), and in non-lymphoid organs, including skin, kidney, lung and liver. In the thymus and spleen of mice treated with the TGF- β 1 activating mAb, the expression of all 5 examined TGF- β 1 target genes increased ≥ 1.4 -fold by comparison to the isotype control, although these increases did not always reach statistical significance (**Figure 2**). In non-lymphoid organs however, expression of at least 3 of the 5 TGF- β 1 target genes did not increase in treated mice.

Overall, these findings suggest that the TGF- β 1 activating mAb enhances TGF- β 1 signaling, at least in lymphoid organs like the spleen and the thymus.

TGF-β1 signaling

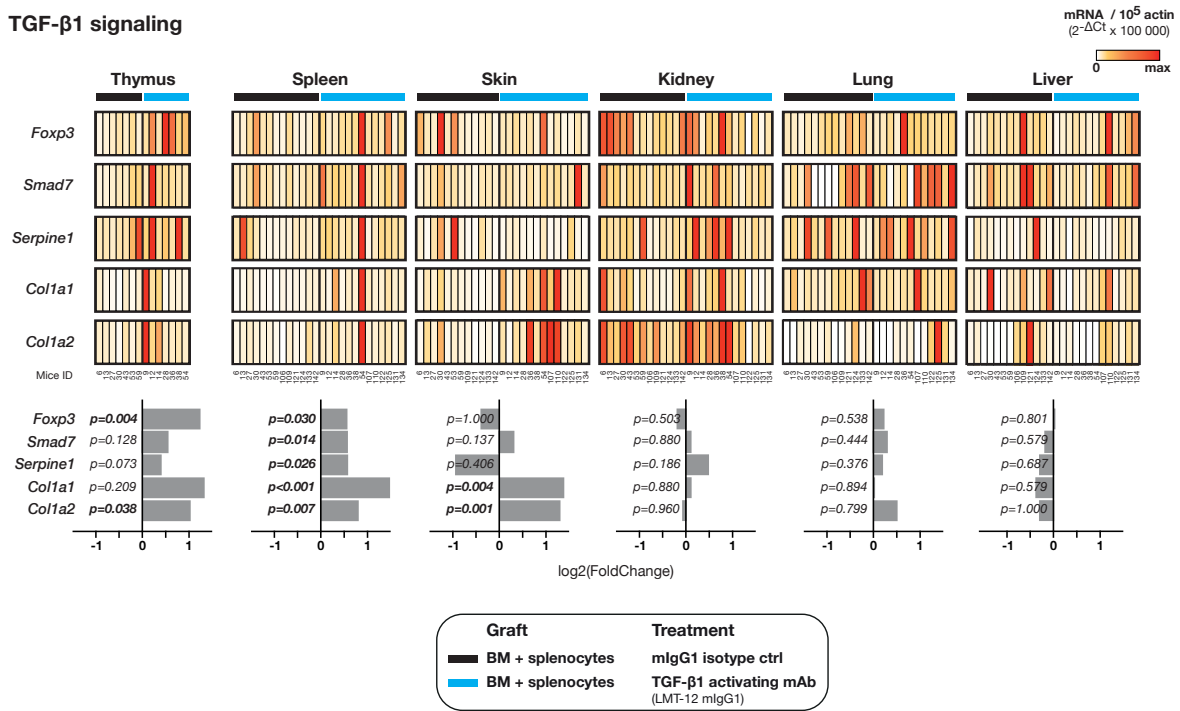


Figure 2. The TGF-β1 activating mAb enhanced TGF-1 signaling in lymphoid organs of mice with cGVHD.

Organs collected in subsets of mice 6 weeks after transplantation were analyzed by RT-qPCR to measure expression of five TGF-β1 target genes (*Foxp3* is taken here as a TGF-β1 target gene). Results from 2 independent experiments (except for thymi, collected in one experiment only) are depicted as heatmaps with a color scale ranging from 0 (white) to maximum (red) gene expression per organ and gene. Fold change (x) values expressed in log2 below each organ represent ratios of mean values in the group receiving the TGF-β1 activating mAbs to that of the isotype control, with p values indicated below. P values < 0.05 are highlighted in bold. Statistical analysis were performed using a Mann-Whitney unpaired t-test. Threshold for statistical significance: 0.05.

The TGF- β 1 activating mAb enhanced thymic function and development of tTregs in mice with cGVHD.

TGF- β 1 activity was suggested to influence important thymic functions, including establishment of immune tolerance via negative selection and differentiation of thymic tTregs.^{10, 11, 42} We thus examined thymic function in mice undergoing cGVHD and treated with the TGF- β 1 activating mAb.

Proportions of CD8⁺ SP T cells, CD4⁺ SP conventional T cells and tTregs within total thymocytes were significantly increased (1.3 to 1.7 fold) in cGVHD mice treated with the activating mAb by comparison to the isotype control (**Figure 3A**). The vast majority of the three SP T cell subsets corresponded to naïve cells (mean $\pm$ sem : $79 \pm 2\%$, $95 \pm 1\%$ and $70 \pm 2\%$ for CD8⁺ SP T cells, CD4⁺ SP conventional T cells and tTregs, respectively), whereas only a minority corresponded to activated, potentially recirculating T cells ($9 \pm 1\%$, $2 \pm 0\%$ and $7 \pm 1\%$, respectively) (**Figure 3A**). As the proportions of activated T cells within the various subsets did not increase in mice treated with the TGF- β 1 activating mAb, the increased proportions of the various SP cells within thymocytes reflect enhanced thymic SP T cell development, including development of tTregs. Conversely, proportions of CD4⁺CD8⁺ double positive (DP) T cells were significantly reduced in cGVHD mice treated with the activating mAb (**Figure 3B**). Observed differences in the various T cell subsets between recipients receiving BM cells and splenocytes and those receiving BM cells only may reflect different kinetics of T cell reconstitution when splenocytes are added to the graft to induce GVHD in this model.

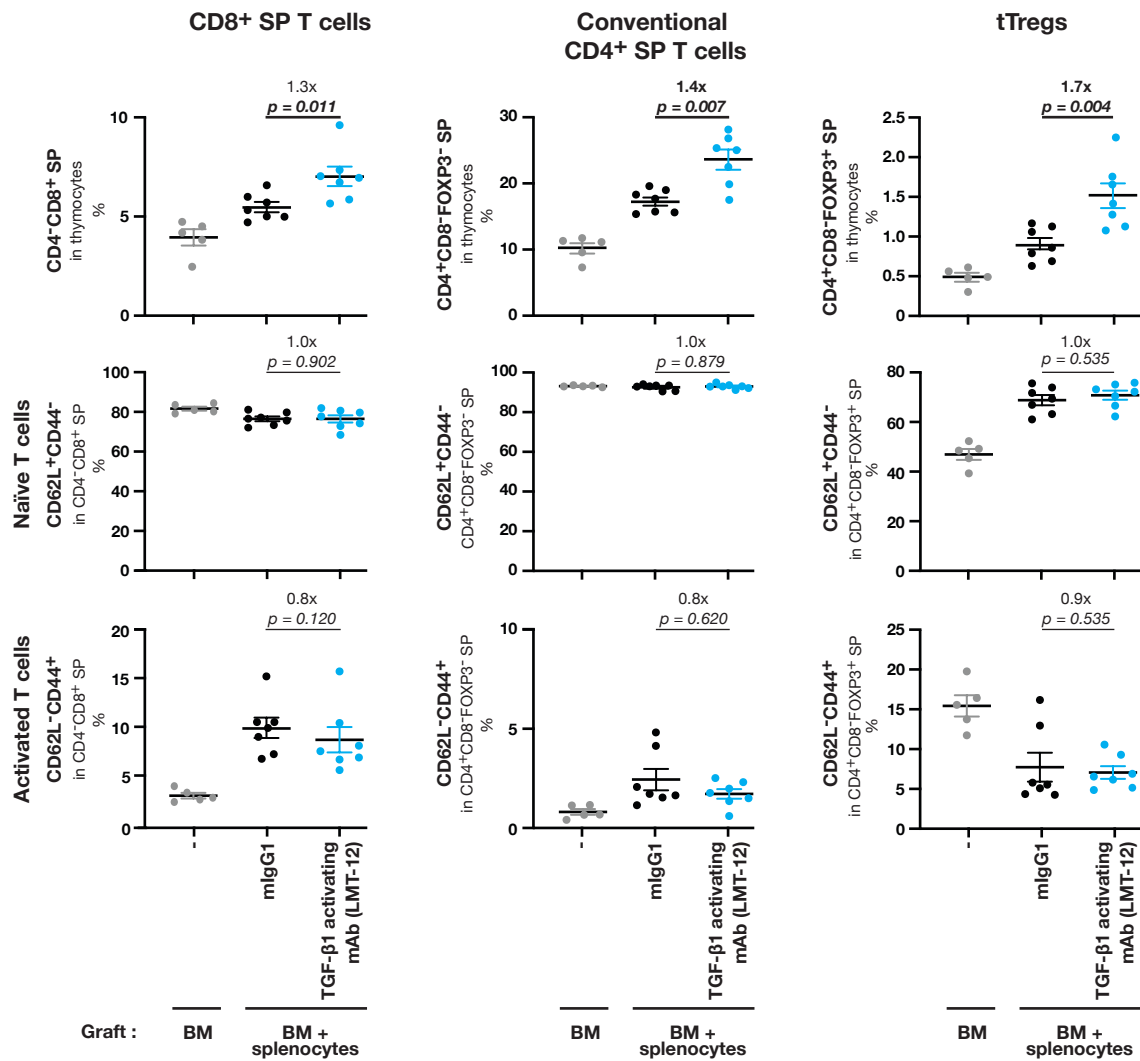
Collectively, these findings suggest that the TGF- β 1 activating mAb may promote thymic function, including tTreg development, following allo-HSCT in mice.

Figure 3. The TGF- β 1 activating mAb enhanced thymic function and development of tTregs in mice with cGVHD.

Thymi were collected from recipient mice 6 weeks after transplantation, and thymocytes were analyzed by flow cytometry. Each data point represents the value in one mouse, with horizontal bars representing mean $\pm$ sem. Fold changes (x) indicate ratios of mean values in the group receiving the TGF- β 1 activating mAbs by comparison to that of the isotype control, with p values indicated below. Fold changes ≥ 1.4 and p values < 0.05 are highlighted in bold. Statistical analysis were performed using a Mann-Whitney unpaired t-test. Threshold for statistical significance: 0.05. Data are representative of 2 independent experiments.

Thymus - 6 weeks after transplantation

A



B

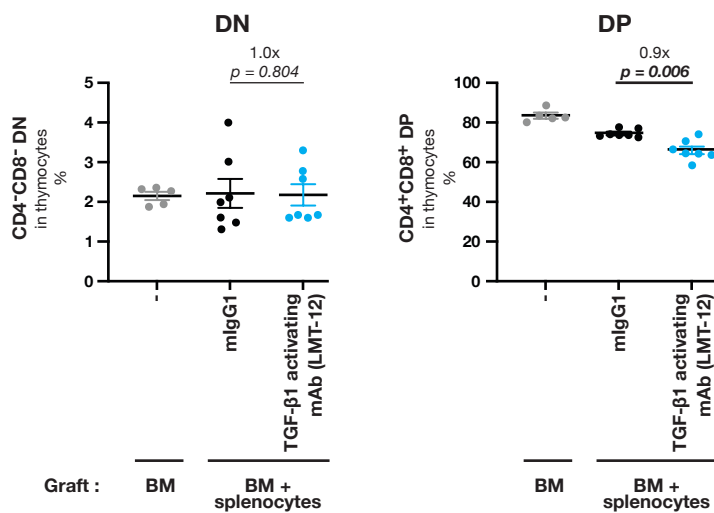


Figure 3. The TGF-β1 activating mAb enhanced thymic function and development of tTregs in mice with cGVHD.

The TGF- β 1 activating mAb decreases type 1 adaptive immune responses during cGVHD.

Chronic GVHD is characterized by sustained T cell-mediated inflammation in lymphoid and non-lymphoid organs. We thus investigated the impact of the TGF- β 1 activating mAb on adaptive immune responses in spleen, blood and various non-lymphoid organs.

We first examined numbers and proportions of major cell subsets in spleen and blood. Platelet counts in the blood were similar across the various groups, but red blood cell and leukocyte counts as well as total numbers of splenocytes were reduced 6 weeks after transplantation in cGVHD mice by comparison to BM controls. This indicates that reconstitution of the lymphoid lineage and most subsets of the myeloid lineage is still ongoing 6 weeks after transplantation in mice with cGVHD (**Figure S4**). No significant difference in proportions or numbers of major cell subsets in the spleen or blood was observed in cGVHD mice treated with the activating mAb by comparison to the isotype control, with the exception of conventional CD4⁺ T cells and Tregs, the proportion of which moderately increased in splenocytes, in line with our observation of increased development of SP CD4⁺ T cells and tTregs in the thymus (**Figures 2 and S4**).

In contrast, treatment with the TGF- β 1 activating mAb significantly reduced by more than 2-fold the proportion of activated cells within CD8⁺ T splenocytes (mean $\pm$ sem: 21 $\pm$ 3% vs 49 $\pm$ 7% in cGVHD mice receiving the activating mAb or the isotype control, respectively). As expected, this reduction was accompanied by a corresponding increase in naïve cells within CD8⁺ T splenocytes (51 $\pm$ 4% vs 35 $\pm$ 5%) (**Figure 4**). These results suggest that the TGF- β 1 activating mAb reduces activation of CD8⁺ T cells in the spleen. A similar 2-fold reduction in activated cells within CD8⁺ T cells was observed in the blood (17 $\pm$ 3% vs 9 $\pm$ 1%), suggesting that reduced CD8⁺ T cell activation in secondary lymphoid organs results in reduced circulation of activated CD8⁺ T cells in the blood (**Figure 4**). No significant difference in activated or naïve cells within conventional CD4⁺ T cells or Tregs was observed in the blood and spleen (**Figure 4**).

We examined whether type 1 cytotoxic CD8⁺ T cell responses could also be decreased in non-lymphoid organs, in line with the reduction in CD8⁺ T cell activation we observed in lymphoid tissues. Expression of *Gzmb* decreased by at least 30% across all organs examined in mice treated with the TGF- β 1 activating mAb, although the decrease did not always reach statistical significance (**Figure 5**). A similar 30% decrease was also observed for at least one of 3 other

genes related to type 1 immunity (*Prfl*, *Infg*, *Cd8b*) in skin and kidney. Expression of genes associated with Th2 and Th17 effector cytokines was very low in all lymphoid or non-lymphoid organs. It also did not change in response to treatment, with the exception of *Il17a* in the skin (**Figure 5**). Overall, our results suggest a global reduction in type 1 cytotoxic CD8⁺ T cell responses in non-lymphoid organs of cGVHD mice treated with the TGF- β 1 activating mAb.

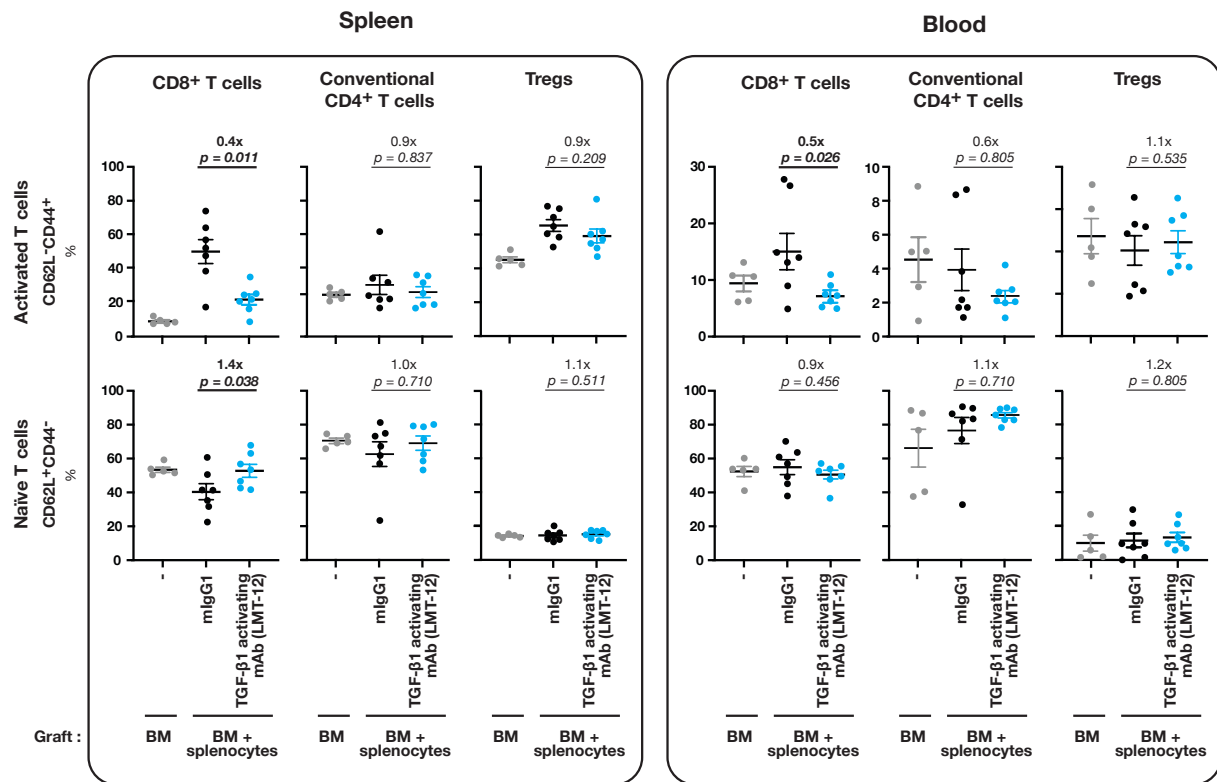


Figure 4. The TGF- β 1 activating mAb decreased proportions of activated cells within CD8⁺ T lymphocytes in spleen and blood of cGVHD mice.

Spleens and blood were collected from recipient mice shown in Fig 3. Splenocytes and leukocytes were analyzed by flow cytometry. Each data point represents the value in one mouse, with horizontal bars representing mean $\pm$ sem. Data are representative of 2 independent experiments. Fold changes (x) indicate ratios of mean values in the group receiving the TGF- β 1 activating mAbs by comparison to that of the isotype control, with p values indicated below. Fold changes ≥ 1.4 and p values < 0.05 are highlighted in bold. Statistical analysis were performed using a Mann-Whitney unpaired t-test. Threshold for statistical significance: 0.05. Data are representative of 2 independent experiments.

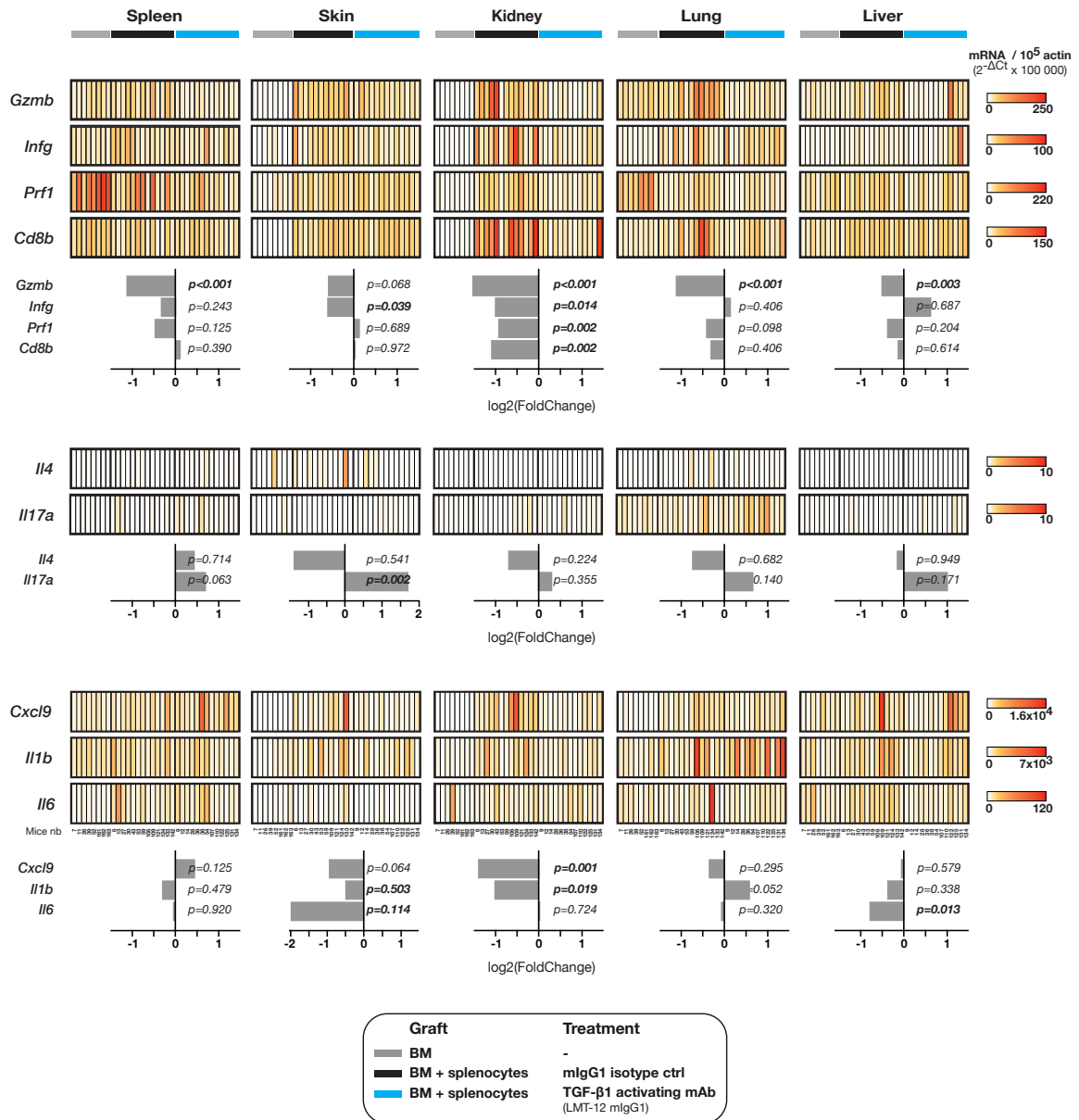


Figure 5. A TGF-β1 activating mAb reduced expression of genes associated with type 1 adaptive immune responses.

Organs collected in subsets of mice 6 weeks after transplantation were analyzed by RT-qPCR to measure gene expression. Results from 2 independent experiments are depicted as heatmaps with a color scale ranging from 0 (white) to maximum (red) gene expression per gene. Fold change (x) values expressed in log2 represent ratios of mean values in the group receiving the TGF-β1 activating mAbs to that of the isotype control, with corresponded p values indicated. P values < 0.05 are highlighted in bold. Statistical analysis were performed using a Mann-Whitney unpaired t-test. Threshold for statistical significance: 0.05.

The TGF- β 1 activating mAb decreases innate immune cells and inflammation during cGVHD.

Innate immune cells also contribute to cGVHD severity. Mice treated with the TGF- β 1 activating mAb displayed a significant, ≥ 2 -fold decrease in proportions of NK cells, neutrophils, eosinophils and monocytes/macrophages among splenocytes (**Figure 6**). Together, these innate immune cells represented only 16% of total splenocytes in cGVHD mice receiving the activating mAb, by comparison to 34% for the isotype control. No such striking difference was observed in the proportions of innate cells circulating in the blood, with the exception of a moderate increase in circulating neutrophils. Thus, treatment with the TGF- β 1 activating mAb appears to reduce innate immune cell recruitment in secondary lymphoid organs, at least in the spleen. We also examined expression levels of proinflammatory cytokine genes in various organs. In skin and kidney, the TGF- β 1 activating mAb reduced the expression of 2 out of 3 pro-inflammatory genes by at least 40%, although this did not always reach statistical significance (**Figure 5**). Altogether, our data suggest that treatment with a TGF- β 1 activating mAb decreases innate immune cell recruitment in the spleen and inflammation in skin and kidney of cGVHD mice.

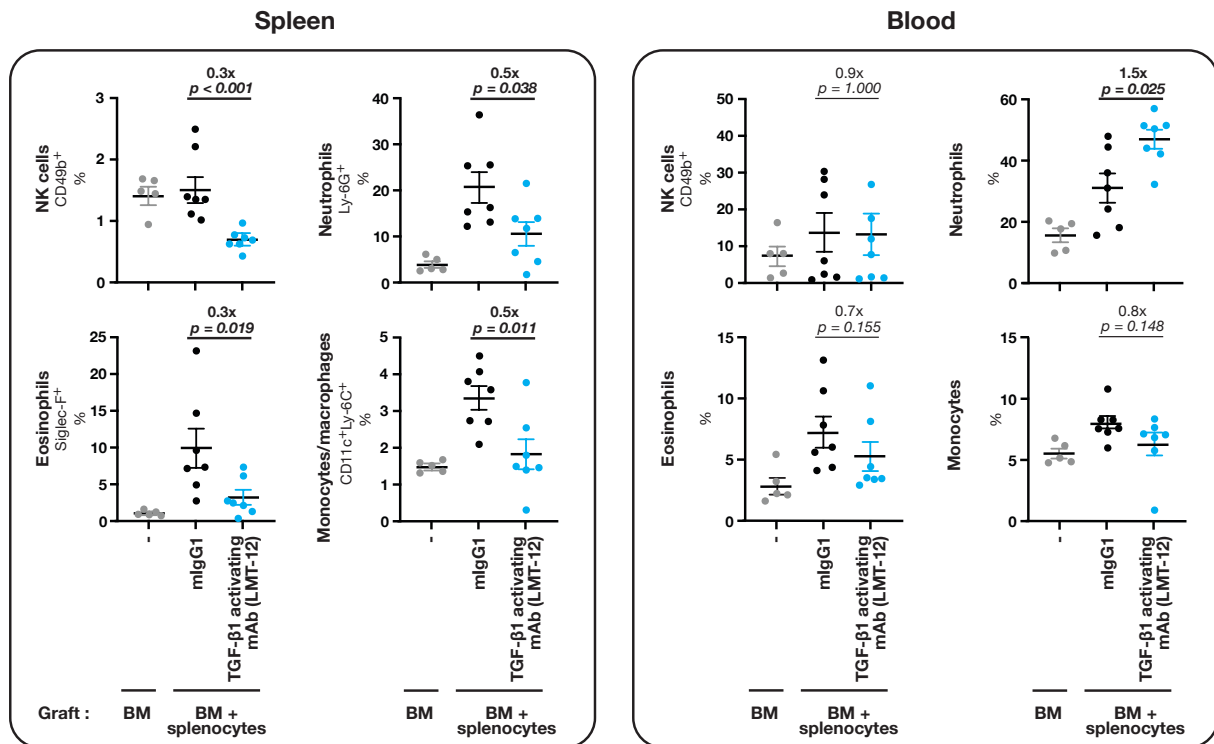


Figure 6. The TGF-β1 activating mAb decreased proportions of innate immune cells in spleen.

Spleens and blood were collected from recipient mice shown in Fig 3. Splenocytes and leukocytes were analyzed by flow cytometry. Among leukocytes, NK cells were analyzed by flow cytometry and neutrophils, eosinophils and monocytes by hematological analyzer. Each point represents the value in one mouse, and horizontal bar the mean $\pm$ sem. Statistical analysis were performed between cGVHD mice receiving either the TGF-β1 activating mAb or the isotype control using Mann-Whitney unpaired t-test. Statistical significance ≥ 0.05 is highlighted in bold. Data are representative of 2 independent experiments.

DISCUSSION

Altogether, we report here that mice treated with TGF- β 1 activating mAbs experience longer survival and reduced severity of a chronic form of GVHD characterized by severe cutaneous damage. Treatment with the activating mAbs appeared to enhance TGF- β 1 signaling in lymphoid organs. The therapeutic effect was associated with enhanced thymic function, including tTreg development, and reduced CD8⁺ T cell activation and recruitment of innate immune cells in the spleen. It also appeared to reduce type 1 T cell responses in peripheral tissues or organs such as skin and kidney. We thus propose that TGF- β 1 activating mAbs may serve as a treatment for chronic GVHD in patients.

We had previously showed that TGF- β 1 activating mAbs exert therapeutic effects in a model of aGVHD caused by transplantation of H-2^b (C57BL/6) splenocytes in H-2^{bd} (C57BL/6 x DBA/2) unirradiated recipients.³⁵ Similar to our observations in aGVHD, we observe here in cGVHD that TGF- β 1 signaling is increased upon mAb treatment in lymphoid organs, but not or only very weakly in non-lymphoid organs. Lymphoid organs display a very high immune-cell density, dominated by T and B cell populations.⁴³ We previously demonstrated that TGF- β 1 activating mAbs can only activate TGF- β 1 *in vitro* by crosslinking two distinct latent TGF- β 1 molecules presented by transmembrane proteins such GARP or LRRC33 on the surface of cells. This led us to suggest that TGF- β 1 activation by mAbs can only occur *in vivo* when latent TGF- β 1 is presented at a high density on the surface of single cells or in tissues that are abundantly infiltrated by spatially connected cells. This could explain why we observe mAb-mediated TGF- β 1 activation in lymphoid, but not non-lymphoid organs.

In contrast to aGVHD, our results here show that the TGF- β 1 activating mAbs enhance thymic function in mice undergoing cGVHD. This includes increased tTreg development. Previous reports showed that TGF- β 1 signalling in thymocytes is required for efficient tTreg development. Numbers of tTregs were reduced 3 to 5 days after birth in mice lacking TGF- β 1 signaling in T cells.^{10, 42} Because deletion of the pro-apoptotic factor Bim restored normal tTreg numbers in the thymus of *CD4^{Cre}xTgfb β 2^{fl/fl}* mice, it was suggested that TGF- β 1 protects tTreg precursors from apoptosis, thereby increasing tTreg development.^{10, 11} McCarron and colleagues further suggested that TGF- β 1 signaling in thymocytes is required for efficient negative selection of CD4⁺FOXP3⁻ SP T cells¹⁰. Indeed, increased numbers of autoreactive

CD4⁺ T cells were found in the thymus of *CD4^{Cre}xTgfb β 2^{fl/fl}* mice. These cells appeared to have escaped negative selection and drive the lethal systemic inflammation observed in these mice. Altogether, their findings indicate that TGF- β 1 may both promote tTreg development and negative selection of CD4⁺FOXP3⁻ SP T cells in the thymus. An alternative hypothesis was proposed by Ouyang and colleagues, who suggested that TGF- β 1 protects all autoreactive thymocytes, including tTregs and CD4⁺FOXP3⁻ SP T cells, from apoptosis during negative selection.¹¹ In mice expressing a transgenic TCR specific for a chicken ovalbumin–derived peptide (OT-II) and the cognate antigen in medullary thymic epithelial cells (TECs), these authors showed increased deletion of OT-II T cells in absence of TGF- β 1 signalling in T cells (*CD4^{Cre}xTgfb β 2^{fl/fl}*). This suggests that TGF- β 1 signaling indeed protects autoreactive CD4⁺ T cells against negative selection. Moreover, they showed that TGF- β 1 regulate expression of IL-7R α during thymic development, which is important to promote SP T cell development.⁴⁴ Our observation that cGVHD mice treated with a TGF- β 1 activating mAb exhibit increased development of SP T cells, including tTregs, in the thymus during cGVHD suggests that TGF- β 1 could improve the ability of the thymus to support the development of all T cell subsets. Such effects could contribute to the generation of a more diverse and self-tolerant T cell repertoire. Because autoimmune-like manifestations, including destruction of the skin hypodermis, are reduced in cGVHD mice treated with activating mAbs, we hypothesise that the dominant effect of TGF- β 1 activity in the thymus is to promote tTreg development.

In addition to these Treg-dependent effect, TGF- β 1 can also directly restrain autoreactive T cells independently of Tregs.^{45, 46} Indeed, murine experiments showed that loss of TGF- β 1 signaling in T cells led to earlier onset and increased severity of autoimmune disease, even in the absence of Treg dysfunction, and is associated with enhanced recruitment and activation of innate immune cells.⁴⁶ In contrast, in their experiment, Foxp3-deficient mice developed autoimmune disease with a later onset and less severe phenotype. These observations highlight that TGF- β 1 maintains peripheral T cell tolerance via direct suppression of T cell activation and cross-talk with innate immune cells.⁴⁶ Consistent with these findings, cGVHD mice treated with the activating mAbs displayed reduced CD8⁺ T cell activation and recruitment of innate inflammatory cells in the spleen, as well as reduced type 1 CD8⁺ T cell responses in peripheral organs. We could not discriminate whether these effects result from direct immunosuppression by TGF- β 1 activated by the mAbs locally, or indirectly result from improved immune tolerance in the periphery due to improved thymic function. We therefore propose that TGF- β 1 activating

mAbs reduce cGVHD severity and increase survival through a combination of direct suppression of pathogenic T cell responses and promoting of immune tolerance via enhanced thymic Treg development.

A potential concern of treatment with TGF- β 1 activating mAbs is that they could exacerbate pathological fibrosis in cGVHD. In our cGVHD model, we observed no increased cutaneous fibrosis, as assessed by dermal thickness. Macrophages activated by proinflammatory cytokines and auto-antibodies are implicated in the development of fibrosis during cGVHD because they stimulate fibroblasts to secrete collagen. Given the immunosuppressive effects observed on innate immune cells upon treatment with TGF- β 1 activating mAbs, we propose that the mAbs may rather decrease susceptibility to develop fibrosis as a consequence of reduced macrophage activation.

Collectively, our findings support the clinical evaluation of TGF- β 1 activating mAbs in patients with cGVHD. Moreover, prophylactic or early post-transplantation administration may help to preserve or even enhance immune T cell reconstitution, including tTregs, and thereby potentially reduce the incidence of GVHD by promoting a more tolerant immune reconstitution.

MATERIAL AND METHODS

Mice

BALB/c (BALB/cJ) and B10.D2 (B10.D2-H2D/N2SNJ) mice were bred at the SPF animal facility of the UCLouvain. The facility is controlled to maintain the temperature between 20 and 24°C; humidity rate between 40 and 65% and day–night cycles of 12 h–12 h. All animal studies were performed in accordance with national and institutional guidelines for animal care, under permit numbers 2023/UCL/MD/37 at the UCLouvain. Female mice of 7–13 weeks of age were used as donors and recipients to induce GVHD.

Induction and scoring of murine GVHD

Recipient BALB/c mice were exposed to 850 cGy irradiation with the use of a ¹³⁷Cs source the day before transplantation. Splenocytes and BM cells were collected from a pool of B10.D2 donor mice. BM were flushed from femurs and tibias, while spleen were mechanically dissociated, then both passed through 70 μ m filters. Red blood cells (RBC) were lysed using a standard RBC lysis buffer. T cells in BM were depleted with the use of biotin-conjugate anti-

CD3 and streptavidin-conjugated micro-magnetic beads (Miltenyi 130-094-973), followed by passage through a MACS cell sorter. Mixture of 25×10^6 splenocytes and 7.5×10^6 T cell-depleted BM cells were grafted by tail vein injections in BALB/c mice to induce GVHD. Starting one day before, or 14 days after transplantation, recipients received weekly intraperitoneal injections of the TGF- β 1 activating mAb (LMT-12 mIgG ; 400 μ g/mouse) or an isotype control (Motavizumab mIgG1 ; 400 μ g/mouse). Mice were evaluated for the development of acute GVHD symptoms every two days until the development of a first symptom in one of the mice (usually around day 4), then daily thereafter (from days 4 to 14). Acute GVHD scores were established based on weight loss (1 if $\geq 10\%$; 2 if $\geq 20\%$), posture (1 if humped posture; 2 if humped posture + tiptoe), reduced mobility (1 if limited activity; 2 if no activity), ruffled fur (1 if visible but slight; 2 if visible and severe), anemia (0.5 if white ears; 1 if white ears and tail), and conjunctivitis (0.5 if one eye affected; 1 if both eye). Mice were euthanized when reaching a global score ≥ 8 , if weight loss $> 20\%$ without weight gain within 3 days, or in absence of activity. Death corresponds to a maximum score of 10. Recipient mice surviving this acute phase are scored three times a week from day 21 to 81 after transplantation. A global cGVHD was established based on the presence of the following symptoms : fur loss (1 if fur loss < 1 cm²; 2 if fur loss of 1-2.5 cm²; 3 if fur loss ≥ 2.5 cm²), ear sclerosis (1 if 1 ear affected; 2 if both ears affected); conjunctivitis (0.25 if 1 eye affected; 0.5 if both eyes affected), and scaling of the tail (0.25 if slight scaling; 0.5 if severe scaling). The mice were euthanized when they reached a score ≥ 4.5 , or if weight loss $> 20\%$ without weight gain within 3 days, or in absence of activity. The maximal score (death) is 5.

A subset of mice (n=13 for mice transplanted with BM cells and splenocytes treated with the TGF- β 1 activating mAb or the isotype control, and n=8 for BM controls) were predefined before the administration of mAbs and euthanized 6 weeks after transplantation to collect blood, spleen, thymus, skin, kidney, liver and lung for analyses.

Flow cytometry

Mouse organs were harvested immediately after euthanasia. Spleens and thymi were mechanically dissociated, while 200 μ L of blood were collected by retro-orbital bleeding with Minivette® (Sarstedt #17.2113.050) into EDTA-coated Microvette® (Sarstedt #20.1341). Blood cells were counted on a Melet Schloesing MS9-5 hematological analyzer. Homogenates of blood, thymus and blood were clarified through 70 μ m filters and red blood cells (RBC) were lysed using a standard RBC lysis buffer. All single cell suspensions were counted on a Luna®

cell counter with a live-dead cell marker (Acridine Orange/Propidium Iodide Stain, Logos Biosystem), centrifuged, resuspended in FACS Buffer (2 mM EDTA and 1% FCS in PBS, filter-sterilized) and clarified through 40µm filters prior to incubation with antibodies coupled to fluorochromes. All antibodies used in flow cytometry experiments are listed in the Table S1. Cells were sequentially incubated with primary antibodies coupled to fluorochromes during 20 minutes at 4°Cs. After staining for surface markers, splenocytes were washed, fixed and permeabilized with the Foxp3/Transcription Factor staining buffer set (eBioscience), then incubated with primary antibodies targeting intracellular proteins. Cells were analyzed on a FACS LSR Fortessa and CYTEK Aurora flow cytometer and data were computed using the FlowJo software (Tree Star).

Real-time quantitative polymerase chain reaction

Murine organs were collected, weighted and immersed in RNA later (Invitrogen) for 24 hours before being stored at -80°C until processing. Tissue disruption was performed using the TissueLyser (Qiagen) for 5 minutes at 50Hz. Total RNA was extracted using NucleoSpin Mini Columns (Macherey Nagel) and RNA concentration was determined using the NanoDrop spectrophotometer (Thermo Scientific). Reverse transcription was carried out with the Maxima First Strand cDNA Synthesis Kit (Thermofisher). For real-time qPCR, a master mix was prepared containing Takyon Master Mix (0,025 U/µl, Eurogentec), primers (300nM of each), Takyon probe (100nM) and 1,25 µl of cDNA. Reactions were performed on a QuantStudio3 device (Thermofisher) under either standard conditions (95°C for 3 minutes; 45 cycles of 95°C for 10 seconds and 60°C for 60 seconds) or fast conditions (95°C for 3 minutes; 95°C for 10 seconds and 60°C for 30 seconds) depending on amplicon size. Primers and probes sequences are listed in Supplementary Table S2. Data analysis was conducted using the Thermofisher Design and analysis software. Expression of genes of interest are expressed as a ratio of mRNA/ 10^5 *b-actin* (housekeeping gene), calculated as $10^5 \times 2^{-\Delta Ct}$ (where $\Delta Ct = Ct$ for gene of interest – Ct for *b-actin*).

Histopathology

Dorsal skin samples were collected, fixed in 10% formalin, and routinely processed for paraffin embedding. Four-micrometer sections were stained with Sirius Red for histologic examination. Measures were blindly assessed.

Quantification and statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 10). Groups were compared with Mann-Whitney unpaired t-test. Survival curves were generated using the Kaplan-Meier method and compared using a log-rank test (Mantel-Cox). Threshold for statistical significance: 0.05.

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AUTHOR CONTRIBUTIONS

S.W and S.L. conceived and designed the overall project. S.W., E.D., M.J., F.B., N.C. and J.S. designed and performed the murine GvHD experiments *in vivo*. S.W. and S.L. wrote the manuscript with input from all authors.

DECLARATION OF INTERESTS

Lore Marien, Bas van der Woning and Manon Lesage are full-time employees of argenx. Patents pertaining to activating antibodies against TGF- β 1 have been filed under the Patent Cooperation Treaty (International application Number PCT/EP2023/056082), with Sophie Lucas, Fanny Lambert, Lore Marien, Bas van der Woning as inventors, and UCLouvain as applicant. All other authors declare no competing interests.

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SUPPLEMENTAL FIGURES

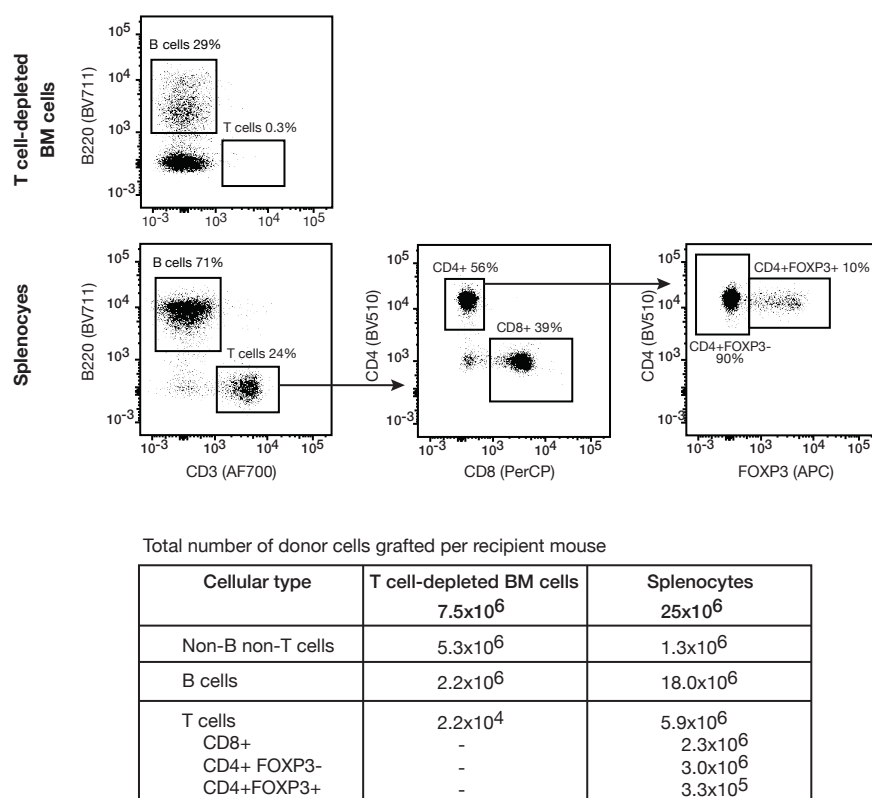


Figure S1. Cellular composition of the graft

Dot plots of flow cytometry analysis performed on pool of donor T cell-depleted BM cells and splenocytes. The absolute numbers presented in the table were obtained by multiplying the proportion of each cell type by the total number of grafted cells per recipient mouse (7.5×10^6 for T cell-depleted BM cells and 25×10^6 for splenocytes).

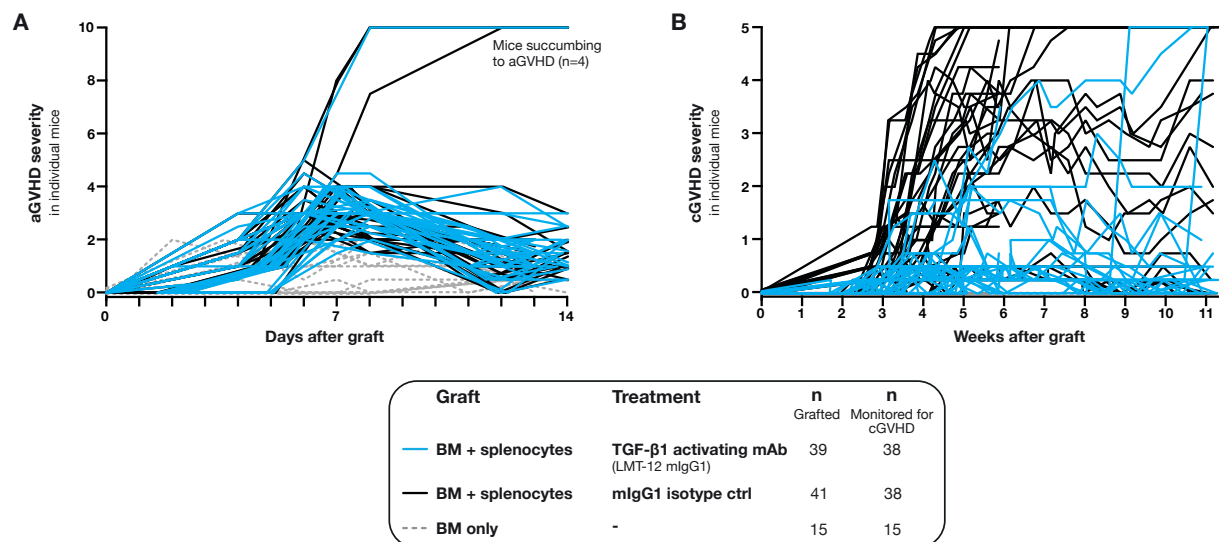


Figure S2. Individual curves of aGVHD and cGVHD severity

Evolution of aGVHD (**A**) and cGVHD (**B**) scores in individual mice. Each line represents one mouse. Mice were scored for symptoms of aGVHD (maximum score: 10; mice were euthanized when reaching a score of 8) or cGVHD (maximum score: 5; mice were euthanized when reaching a score of 4.5). Detailed scores are indicated in the Material and methods.

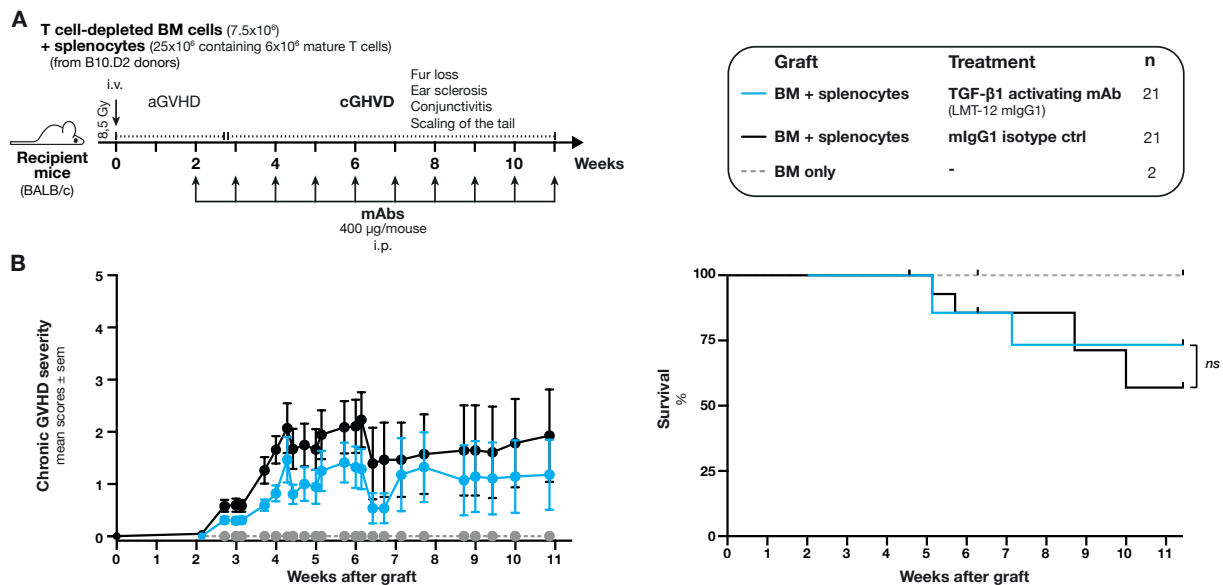


Figure S3. A TGF-β1 activating mAb treatment started 2 weeks after allo-HSCT did not decrease disease severity or increase survival in cGVHD mice.

(A) Schematic experimental design. T cell-depleted bone marrow cells (BM; 7.5×10^6 per mouse) and splenocytes (25×10^6 per mouse) isolated from B10.D2 donors were transplanted into lethally irradiated female BALB/c recipients. BM controls were grafted with BM cells only. Recipient mice received weekly injections of a TGF-β1 activating mAb (LMT-12 mIgG1) or an isotype control (mIgG1 ctrl) starting 14 days after transplantation. **(B) Left graph** : Evolution of the mean of cGVHD scores, established based on fur loss (1 if fur loss $< 1 \text{ cm}^2$; 2 if fur loss of $1\text{--}2.5 \text{ cm}^2$; 3 if fur loss $\geq 2.5 \text{ cm}^2$), ear sclerosis (1 if 1 ear affected; 2 if both ears affected), conjunctivitis (0.25 if 1 eye affected; 0.5 if both eyes affected) and scaling of the tail (0.25 if slight scaling; 0.5 if severe scaling). Mice were euthanized when reaching a score ≥ 4.5 , or when reaching predefined humane endpoint. The maximal score (death) is 5. Error bars denote sem. **Right graph** : Kaplan-Meier plots representing the proportion of mice alive at the indicated weeks after transplantation. P values calculated using a Gehan-Breslow Wilcoxon test. Vertical mark : censored data. Data from a single experiment.

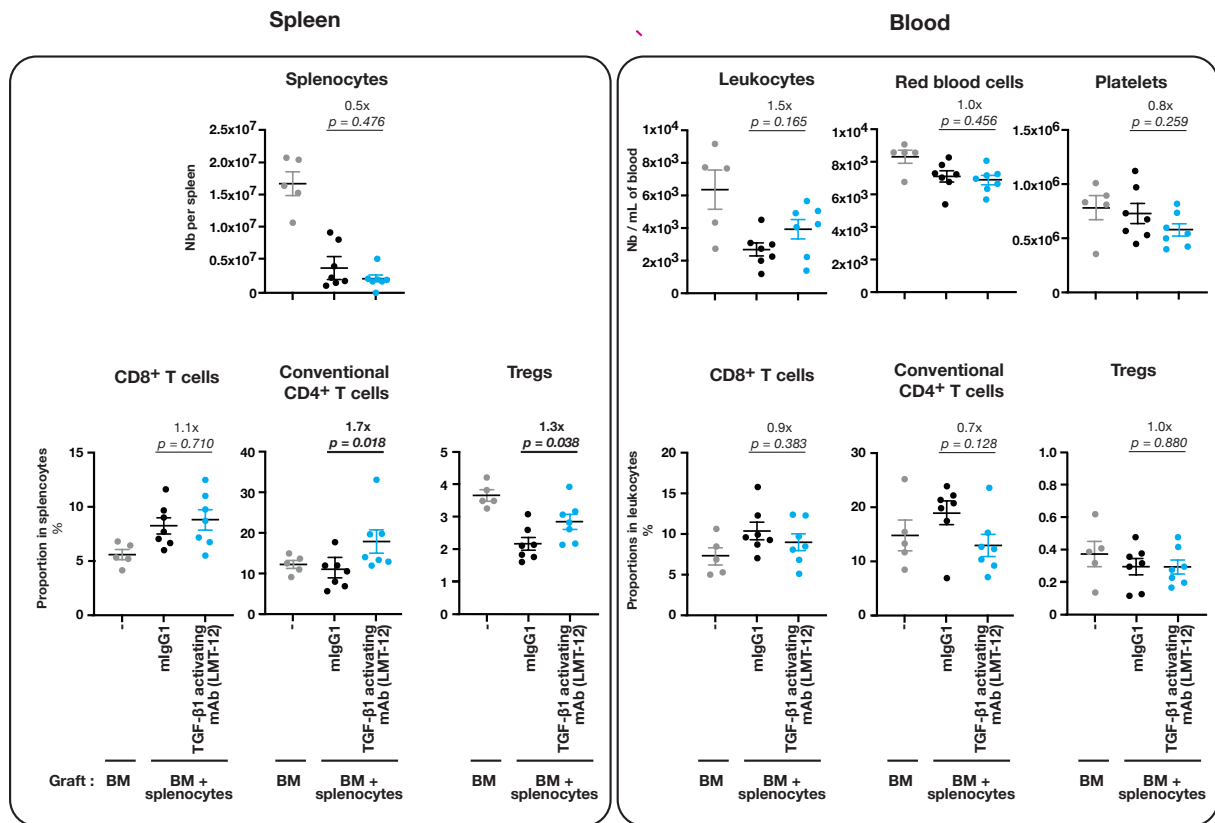


Figure S4. The TGF- β 1 activating mAb did not modify proportions of CD8⁺ T cells, conventional CD4⁺ T cells and Tregs in splenocytes of mice with cGVHD.

Spleens and blood were collected from recipient mice shown in Fig 4. Splenocytes were counted with automated cell counter, and leukocytes were counted by hematological analyzer. Phenotypes were analyzed by flow cytometry. Each point represents the value in one mouse, and horizontal bar the mean $\pm$ sem. Statistical analysis were performed between cGVHD mice receiving either the TGF- β 1 activating mAb or the isotype control using Mann-Whitney unpaired t-test. Statistical significance ≥ 0.05 is highlighted in bold. Data are representative of 2 independent experiments.

SUPPLEMENTAL TABLE 1

Flow cytometry antibodies		
Antibodies	Clone	Supplier
CD11b-eF450	M1/70	Thermo
CD11c-eF450	N418	Thermo
Ly-6G/Ly-6C-eF450	RB6-8C5	Thermo
TER-119-eF450	TER-119	Thermo
F4/80-eF450	BM8	Thermo
FceRa-eF450	MAR-1	Thermo
TCRgd-BV480	GL3	BD Biosciences
CD49b-BV605	HMa2	BD Biosciences
NKp46-BV785	29A1.4	Biolegend
CD44-NovaFluorBlue610-70S	IM7	Thermo
CD45-NovaFluor Blue 660-120S	30-F11	Thermo
CD8a-RB705	53-6.7	BD Biosciences
IgA-PE	mA-6E1	Invitrogen
CD127-PE-CF594	SB/199	BD Biosciences
CD62L-NovaFluor Yellow 660	MEL-14	Thermo
CD3-PE-Cy7	145-2C11	Thermo
CD4-R718	RM4-5	BD Biosciences
B220-APC-eF780	RA3-6B2	Thermo
CD3e-BV480	145-2C11	BD Biosciences
CD5-BV480	53-7.3	BD Biosciences
CD19-BV480	1D3	BD Biosciences
NK-1.1-BV480	PK136	BD Biosciences
Ly-6G-BV605	1A8	BD Biosciences
CD64-BV650	X54-5/7.1	BD Biosciences
I-A/I-E-BV711	M5/114.15.2	BD Biosciences
CD11b-BV786	M1/70	BD Biosciences
Siglec-F-BB515	E50-2440	BD Biosciences
F4-80-RB545	T45-2342	BD Biosciences
CD11c-RB613	N418	BD Biosciences
CD45-NovaFluor Blue 660-120S	30-F11	Thermo
FceR1-RY610	M290	BD Biosciences
Ly-6C-RY703	AL-21	BD Biosciences
XCR1-PE-Cy7	ZET	BD Biosciences
CD317-APC	927	Biolegend
CD172a-R718	P84	BD Biosciences
B220-APC-eF780	RA3-6B2	Thermo

SUPPLEMENTAL TABLE 2

Primers sequences

Sequence (5' - 3')

Definition

CCCCGCCACCTGGAAGAAT	Forward primer in <i>mFoxp3</i> mRNA
CTCCCTTCTCGCTCTCCAC	Reverse primer in <i>mFoxp3</i> mRNA
CCATCCGCCACAACCTGAGCC	Taqman probe in <i>mFoxp3</i> mRNA
CTGTGTTGCTGTGAATCTTACG	Forward primer in <i>mSmad7</i> mRNA
CATTGGGTATCTGGAGTAAGGAG	Reverse primer in <i>mSmad7</i> mRNA
AGATCAACCCCGAGCTGGTGTG	Taqman probe in <i>mSmad7</i> mRNA
CTATGGTGAAACAGGTGGACT	Forward primer in <i>mSerpine1</i> mRNA
CGTGTCAGCTCGTCTACAG	Reverse primer in <i>mSerpine1</i> mRNA
ACCTTTGGTATGCCTTTCCACCCA	Taqman probe in <i>mSerpine1</i> mRNA
CGCAAAGAGTCTACATGTCTAGG	Forward primer in <i>mColla1</i> mRNA
CATTGTGTATGCAGCTGACTTC	Reverse primer in <i>mColla1</i> mRNA
CCGGAGGTCCACAAAGCTGAACA	Taqman probe in <i>mColla1</i> mRNA
AGTAACTTCGTGCCTAGCAAC	Forward primer in <i>mColla2</i> mRNA
CATCAACACCATCTCTGCCT	Reverse primer in <i>mColla2</i> mRNA
CCCTTTCGTACTIONGATCCCGATTGCA	Taqman probe in <i>mColla2</i> mRNA
GTGGTTGATGTCCCTTCTACAA	Forward primer in <i>mCd8b</i> mRNA
TCCGCACACAGTAAAAGTAGAC	Reverse primer in <i>mCd8b</i> mRNA
AATGCCAGCAGAAGCAGGATGCAGACTA	Taqman probe in <i>mCd8b</i> mRNA
TCAAGTGGCATAGATGTGGAAGAA	Forward primer in <i>mIfng</i> mRNA
TGGCTCTGCAGGATTTTCATG	Reverse primer in <i>mIfng</i> mRNA
TCACCATCCTTTTGCCAGTTCCTCCAG	Taqman probe in <i>mIfng</i> mRNA
AAGAGAGCAAGGACAACACTC	Forward primer in <i>mGzmb</i> mRNA
CATGTCCCCCGATGATCTC	Reverse primer in <i>mGzmb</i> mRNA
ACAAGGTCAGCAGTAGCAGGAGGA	Taqman probe in <i>mGzmb</i> mRNA
CAGTAGAGTGTGCGCATGTACAG	Forward primer in <i>mPrfl</i> mRNA
GATGAGCCTGTGGTAAGCAT	Reverse primer in <i>mPrfl</i> mRNA
TCGCCTGGTACAAAAACCTCCACTC	Taqman probe in <i>mPrfl</i> mRNA
GAACGAGGTCACAGGAGAAGG	Forward primer in <i>mIl4</i> mRNA
GGACTCATTCATGGTGCAGCTTA	Reverse primer in <i>mIl4</i> mRNA
CCTCACAGCAACGAAGAACACCACAG	Taqman probe in <i>mIl4</i> mRNA
GCTCCAGAAGGCCCTCAG	Forward primer in <i>mIl17a</i> mRNA
CTTTCCCTCCGCATTGACA	Reverse primer in <i>mIl17a</i> mRNA
ACCTCAACCGTTCCACGTCACCCTG	Taqman probe in <i>mIl17a</i> mRNA
GATCTCCGTTCTTCAGTGTAGC	Forward primer in <i>mCxl9</i> mRNA
CAAATCCCTCAAAGACCTCAAAC	Reverse primer in <i>mCxl9</i> mRNA
CCCCAAGCCCCAATTGCAACAAA	Taqman probe in <i>mCxl9</i> mRNA
TCCAAACCTTTGACCTGGGCTGT	Forward primer in <i>mIl1b</i> mRNA
GACCTGTTCTTTGAAGTTGACG	Reverse primer in <i>mIl1b</i> mRNA
CTCTTGTTGATGTGCTGCTG	Taqman probe in <i>mIl1b</i> mRNA
AGCCAGAGTCCTTCAGAGA	Forward primer in <i>mIl6</i> mRNA
TCCTTAGCCACTCCTTCTGT	Reverse primer in <i>mIl6</i> mRNA
CCTACCCCAATTTCCAATGCTCTCCT	Taqman probe in <i>mIl6</i> mRNA

DISCUSSION AND PERSPECTIVES

1 How and where does mAb-mediated TGF- β 1 activation occur *in vitro* and *in vivo*?

TGF- β 1 and its receptors are expressed in almost all cell types in the organism. Identifying which cells provide the pool of latent TGF- β 1 that becomes activated upon mAb treatment is therefore essential for understanding both the therapeutic potential and the possible adverse effects of TGF- β 1 activating mAbs. Biodistribution studies using radiolabeled or fluorescently labeled mAbs can visualize where mAbs accumulate *in vivo*, but this approach cannot identify the cellular source of activated TGF- β 1, since mAb binding alone is not sufficient for TGF- β 1 activation. With the collaboration of Mathieu Jomez, another PhD student in our laboratory, we studied in detail the mechanism of TGF- β 1 activation by mAbs.

1.1 How is TGF- β 1 activated by mAbs *in vitro*?

Our data indicate that TGF- β 1 activating mAbs require latent TGF- β 1 to be tightly anchored at a cell surface through a transmembrane protein. When the transmembrane domains of GARP or LRRC33 are truncated, latent TGF- β 1 is secreted as a soluble complex and cannot be activated by mAbs (*Cell Reports*, Fig.2, and data not shown). Along the same line, latent TGF- β 1 associated with LTBP, which lack an intracellular domain, cannot be activated by mAbs due to the lack of sufficient anchorage on the solid surface of tissue culture wells. In contrast to the transmembrane domain, the intra-cytoplasmic domain of latent TGF- β 1 presenting molecules is not required for activation : truncation of the intracellular domains of GARP or LRRC33 does not impair mAb-mediated TGF- β 1 activation (data not shown). Thus, it appears that mAbs can only activate latent TGF- β 1 that is retained on the cell surface, but that a link between the protein that anchors latent TGF- β 1 on the surface and the intracellular actin cytoskeleton is not required. Together, these findings imply that TGF- β 1 activating mAbs cannot activate all latent TGF- β 1 reservoirs *in vivo*, but only those bound to cells expressing transmembrane presenting proteins such as GARP or LRRC33. These cells include immune cells, endothelial cells and fibroblasts.

We also found that bivalent binding is required for mAb-mediated TGF- β 1 activation, as demonstrated using Fab fragments and monovalent chimeric antibodies, which were inactive *in vitro* and *in vivo* (*Cell Reports*, Fig.2 and Fig.5). Structural studies likewise indicated that TGF- β 1 activation requires binding of one bivalent mAb to two membrane-anchored latent TGF- β 1 complexes. To probe this requirement more directly, we developed a bispecific anti-GARP x anti-latent TGF- β 1 antibody using the same Fab-arm exchange approach described for the chimeric antibodies in our article published in *Cell Reports*. The GARPxTGF- β 1 bispecific antibody is distinct from the blocking anti-GARP:TGF- β 1 antibody developed by the laboratory previously. Whereas the blocking anti-GARP:TGF- β 1 binds a mixed conformation epitope comprising amino-acids from GARP, LAP and mature TGF- β 1 and prevents release of mature TGF- β 1 from LAP, the GARPxTGF- β 1 bispecific comprises one arm directed against GARP only, and one arm corresponding to a TGF- β 1 activating mAb. This GARPxTGF- β 1 bispecific induced TGF- β 1 activation only when the antibody could bind GARP expressed on one cell and an LRRC33:TGF- β 1 complex on another (**Figure 12 A**). When the bispecific could bind GARP:TGF- β 1 complexes expressed on the same cell, it was not able to activate latent TGF- β 1. Based on structural insights from Jan Felix (UGent), we hypothesize that the GARPxTGF- β 1 bispecific is capable of engaging a single GARP:TGF- β 1 complex via its two arms (GARP on one hand, LAP on the other), and that

bivalent binding to a single complex does not allow TGF- β 1 activation by mAbs. Consistent with this mechanistic limitation, we did not detect any therapeutic effect of this GARPxTGF- β 1 bispecific in the aGVHD mouse model, supporting the conclusion that GARPxTGF- β 1 bispecifics fail to induce TGF- β 1 activation (**Figure 12 B**). These findings confirmed the requirement for cross-linking of two anchored complexes to support mAb-mediated TGF- β 1 activation. Whether two complexes present on the same cell can also support activation by mAbs remains unresolved. To address this, single-cell microdroplet assays incorporating a TGF- β 1 reporter cell line (e.g., CAGA-GFP or CAGA-mCherry) could determine whether a strictly cis configuration (same cell) supports activation, or whether interaction between two distinct cells are required.

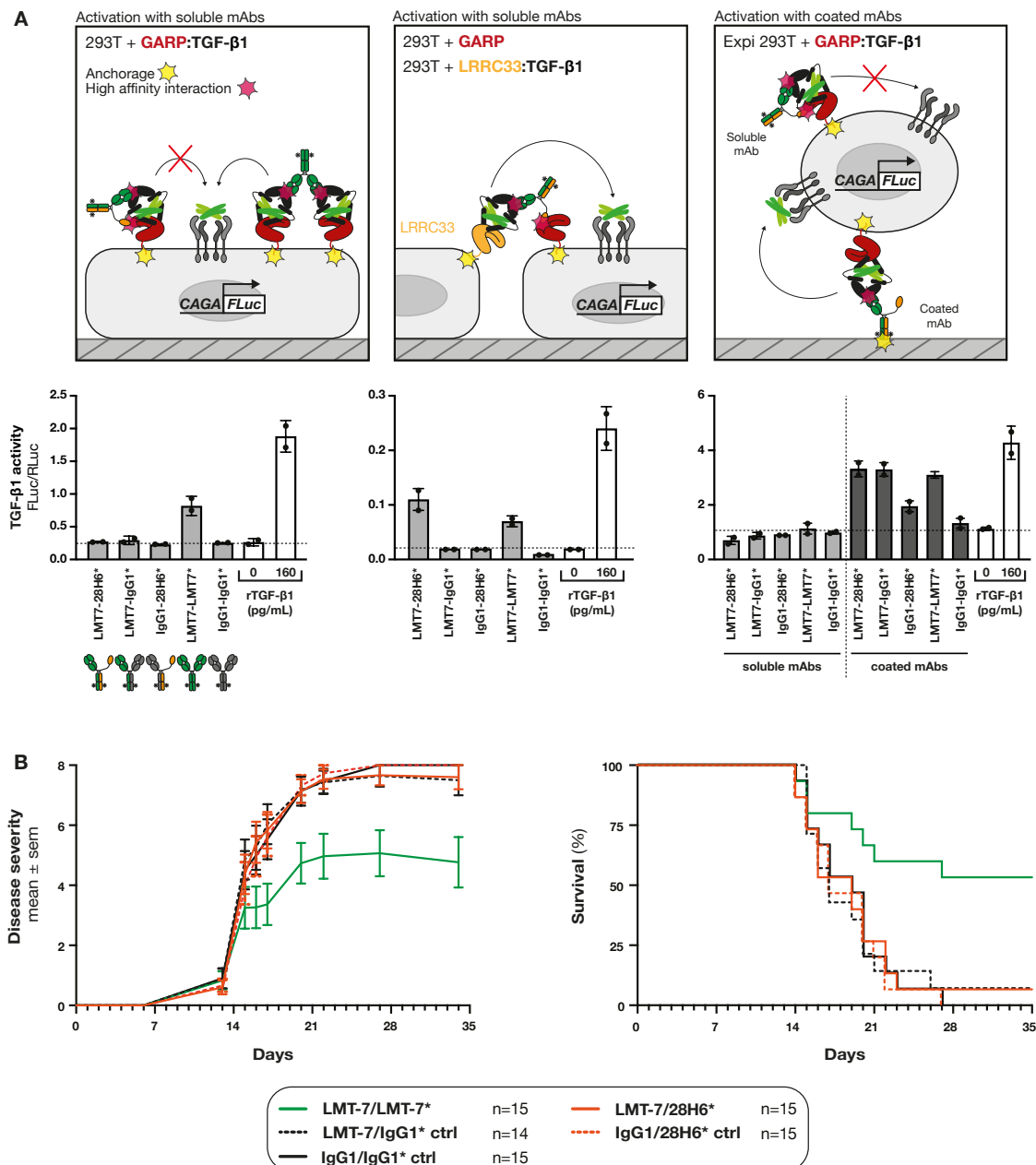


Figure 12. Cross-linking of distinct 2 membrane-anchorage proteins is required for TGF- β 1 activation by mAbs.

(A) Schematic representation of reporter assay measuring TGF- β 1 activation by soluble bispecific chimeric Abs generated by Fab-arm exchange : LMT-7/28H6* (green/orange): chimera with one arm from activating LMT-7 and one arm from anti-GARP 28H6 ; LMT-7/IgG1* (green/grey): chimera with one arm from LMT-7

and one arm from the IgG1 control ; IgG1/28H6* (grey/orange): chimera with one arm from the IgG1 control and one arm from the anti-GARP 28H6. LMT-7/LMT-7* (green): control chimera with two arms from LMT-7 ; IgG1/IgG1* (grey): control chimera with two arms from the irrelevant mouse IgG1. Bar graphs represent ratios of Fluc to Rluc activity (mean of two duplicates + SD). A dotted horizontal line shows FLuc/RLuc in the absence of rhTGF- β 1. Left panel: Reporter 293T cells are co-transfected to express GARP and TGF- β 1. Middle panel: same as (A) with co-culture between reporter cells expressing GARP only with cells expressing LRRC33:TGF- β 1 complexes. Right panel: same as (A) but using non-adherent Expi 293T cells and testing soluble or coated chimeric antibodies. **(B)** Acute GVHD mouse model (as described in *Cell Reports*, Fig 5). Mice were injected with bispecific chimeras once a week for 4 weeks, starting on day -1. Left graph : Evolution of the mean $\pm$ sem of aGVHD. Right graph : Kaplan-Meier plots representing the proportion of mice alive at the indicated weeks after transplantation.

An additional mechanistic observation is that bivalent binding alone is not sufficient. Tensile forces must be transmitted through mAbs to activate latent TGF- β 1. When using non-adherent Expi 293T cells in our reporter assays, we observed that soluble activating mAbs were completely inactive. Activation of TGF- β 1 was restored only when the activating mAbs (including chimeras) were immobilized on culture plates or in presence of adherent cells such as 293T cells (**Figure 12 C**). Moreover, inhibitors of actin polymerization prevented TGF- β 1 activation by mAbs in our reporter assay with adherent 293 T cells. All these findings suggest that mAb-mediated TGF- β 1 activation occurs only when mAbs can provide mechanical resistance by linking two fixed points, allowing the tensile forces generated by cell movement or membrane dynamics to activate TGF- β 1.

Altogether, our data support a model in which mAb-mediated TGF- β 1 activation requires cross-linking of two membrane-anchored proteins, with one bound to latent TGF- β 1, and membrane or cellular movement generating tensile forces to unfasten the LAP and release mature TGF- β 1.

1.2 Which cell types can mediate TGF- β 1 activation by mAbs?

Most mechanistic experiments were initially performed in transfected 293T cells using murine TGF- β 1 activating mAbs. We also evaluated whether other human cells, primary immune or non-immune cells, expressing GARP or LRRC33, could serve as physiological sources for mAb-mediated TGF- β 1 activation.

Human Tregs were challenging to study because only very limited cell numbers can be isolated from healthy donors. Therefore, we used previously expanded Tregs that we thawed from our laboratory collection. Upon TCR stimulation, human Tregs activated TGF- β 1 as expected. TGF- β 1 activation was further enhanced upon treatment with TGF- β 1 activating mAbs. In contrast, human Tregs that had not been stimulated via their TCR did not respond to activating mAbs, consistent with their lack of expression of surface GARP:TGF- β 1 complexes (**Figure 13**).

Our laboratory previously demonstrated that BCR-stimulated B cells can activate TGF- β 1 presented by GARP on their surface (18). Using freshly isolated human B cells (CD19+) from hemochromatosis patients, we confirmed that BCR-stimulated B cells activated TGF- β 1. This activation was further reinforced by TGF- β 1 activating mAbs (**Figure 13**). Unexpectedly, unstimulated B cells also displayed some measurable TGF- β 1 activation upon mAb treatment, although they do not express GARP. These resting B cells nevertheless expressed *LRRC33* mRNA, at levels comparable to THP-1 monocytes (a cell line known to present LRRC33:TGF- β 1

on their surface). This suggests that LRRC33 may provide an alternative membrane anchorage on resting B cells for latent TGF- β 1, which can then be activated by mAbs. However, the presence and functional relevance of the LRRC33 protein on the surface of resting human B cells remain to be demonstrated.

GARP:TGF- β 1 complexes are also expressed on the surface of endothelial cells and fibroblasts. Numerous experiments in our laboratory attempted to determine whether these adherent cell types can activate TGF- β 1 in the presence of activating mAbs. However, we were not able to detect clear mAb-mediated TGF- β 1 activation in fibroblasts. We obtained inconsistent results with endothelial cells, in which we find high basal pSMAD2 signals but no clear increase with mAbs (**Figure 13**). A key technical consideration is that fibroblasts and endothelial cells are adherent and largely immobile. Because mAb-mediated TGF- β 1 activation requires cell-generated tensile force, the limited mobility of these cell types may prevent efficient TGF- β 1 activation by mAbs. Experimental approaches designed to induce cell movement, such as scratch-wound assays, may help determine whether cellular mobility is indeed the limiting factor preventing TGF- β 1 activation in these cell types.

Based on the above, we propose that immune cells, particularly Tregs and B lymphocytes, represent predominant cellular sources of latent TGF- β 1 that can be activated by mAbs. We did not assess mAb-mediated TGF- β 1 activation on myeloid cells, including monocytes or macrophages, which are known to express LRRC33:TGF- β 1 complexes and are implicated in fibrotic lesions in cGVHD (222, 224). Evaluating TGF- β 1 activating mAbs on these populations (e.g., CD14⁺ monocytes cultured with activating mAbs and assessing pSMAD2 or reporter readouts) would be of interest for defining the full range of cells that can be targeted by activating mAbs.

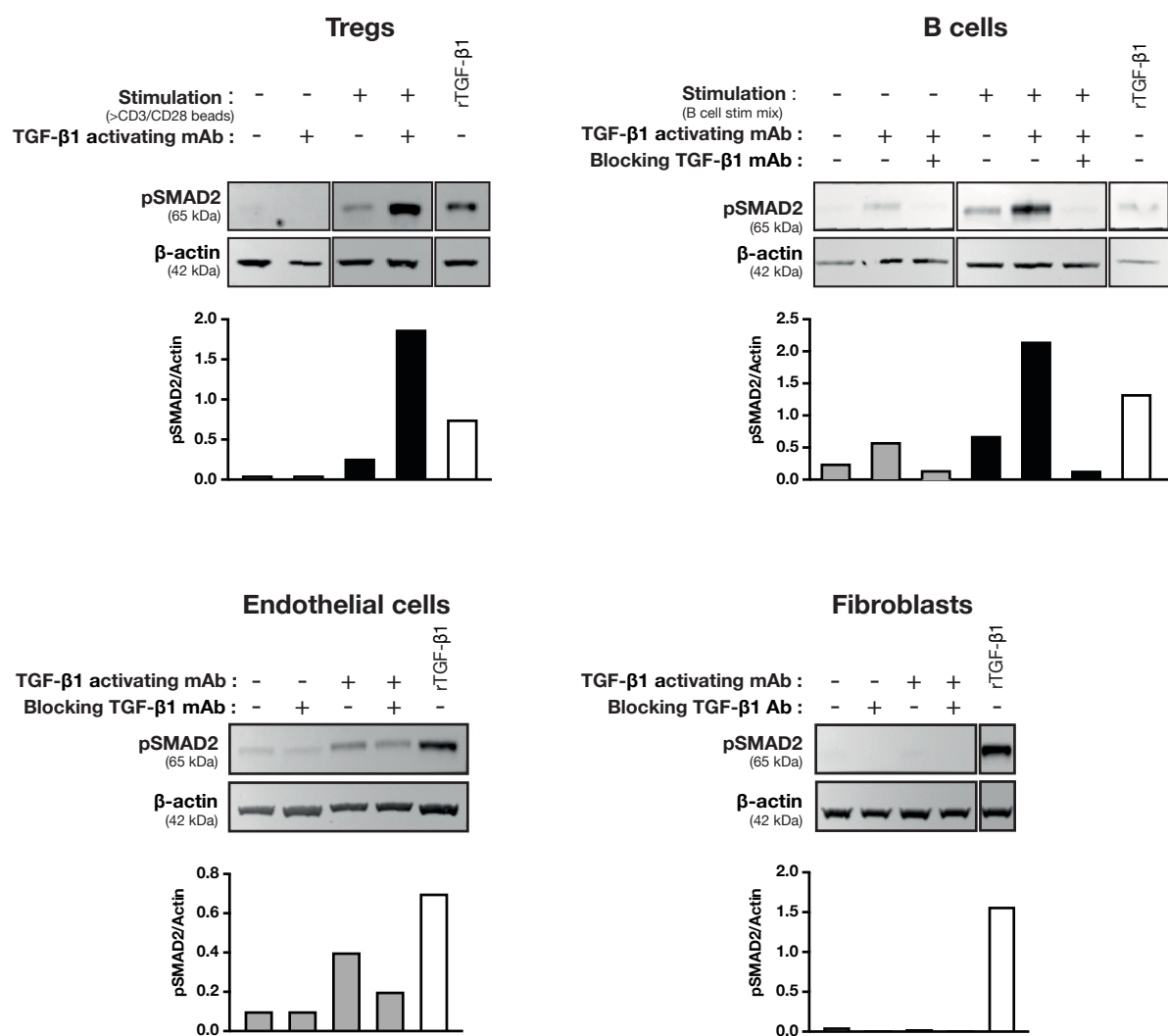


Figure 13. TGF-β1 activating mAbs increased TGF-β1 activity in stimulated B cells and Tregs.

Cells were incubated for 20h with the indicated antibodies (20 μ g/mL), in presence of anti-CD3/CD28 beads for stimulated Tregs and a B cell stim mix (F(ab')² anti-human IgM, IL-21, IL-2 and CPG) for stimulated B cells (FACS-sorted CD19⁺ cells containing ~ 97% CD3⁺CD19⁺). Cell lysates were analyzed by western blot with antibodies against pSMAD2 and β-actin. Bar graphs show quantification of ECL signals. Cells incubated with rhTGF-β1 (1 ng/mL) served as positive controls for pSMAD2 signals. Results are representative of a total of 2 independent experiments.

1.3 In which tissues does mAb-mediated TGF-β1 activation occur during GVHD?

In murine GVHD models, increased TGF-β1 signaling was consistently observed in the spleen of mice treated with the TGF-β1 activating mAbs. In addition, we also observed increased TGF-β1 signaling in the thymus of cGVHD mice treated with the mAbs. The thymus was not assessed in the aGVHD model. Our observations in aGVHD and cGVHD suggest that lymphoid organs may represent the major sites where TGF-β1 activating mAbs can encounter appropriate cellular sources of TGF-β1 (Tregs, B cells) and mediate its activation. TGF-β1 signalling may consequently attenuate acquisition of the effector functions that they will exert in non-lymphoid organs to which they recirculate.

2 Could TGF- β 1 activating mAbs represent a novel therapeutic agent for GVHD in patients?

2.1 Could TGF- β 1 activating mAbs serve to treat aGVHD in patients ?

We showed that TGF- β 1 activating mAbs reduce disease severity and improve survival in an haplo-mismatched model of aGVHD (also called parent-to-F1), in which donor B6 splenocytes are transferred into B6D2 F1 recipients.

This therapeutic efficacy was associated with increased Treg numbers in the spleen, detected as soon as 9 days after transplantation. This increase likely results from the differentiation of naïve CD4⁺ T cells into pTregs. Indeed we do not observe such increase in tTregs, from donor or recipient origins (data not shown). TGF- β 1 is well known to drive the differentiation of pTregs. Whether this increase in newly differentiated pTreg numbers contributes to the therapeutic effect of the mAbs is unclear.

On another line, using FOXP3-DTR mice, we demonstrated that the therapeutic effect of TGF- β 1 activating mAbs was completely abolished when all pre-existing Tregs (i.e. donor Tregs in the graft and Tregs in the recipient), or pre-existing Tregs from recipients only, were depleted with diphtheria toxin prior to transplantation. In contrast, depletion of only the pre-existing donor Tregs in the graft had no impact on the therapeutic outcome. Because recipient Tregs at the time of transplantation vastly outnumber donor Tregs in the graft, we assumed that the abundance of pre-existing Tregs, rather than their origin, determined the therapeutic efficacy of TGF- β 1 activating mAbs.

Whereas the data showing increased (p)Treg numbers after transplantation suggest that the TGF- β 1 activated by the mAbs acts on naïve CD4⁺ T cells to drive their differentiation into new pTregs, the data obtained by depleting Tregs before transplantation rather suggest that pre-existing Tregs are the main cellular sources of the TGF- β 1 activated by the mAbs.

The parent-to-F1 model is often considered as an analog of nonmyeloablative or reduced-intensity allo-HSCT, and therefore may not be generalized to myeloablative conditioning. To address this limitation, we attempted to establish a second aGVHD model by transferring BM cells mixed with purified CD4⁺ and CD8⁺ T cells from B6 donors into lethally irradiated BALB/c recipients. In contrast to the parent-to-F1 model, TGF- β 1 activating mAbs showed no therapeutic efficacy, and all mice rapidly succumbed to severe aGVHD (**Figure 14**). Analysis of immune cell populations on day 6 revealed an almost complete loss of both donor- and recipient-derived Tregs in the spleen. Recipient Tregs had likely been eliminated by irradiation and the allogeneic reaction, while development of new tTregs from donor HSCs had not yet started. We suggest that, in the absence of a minimal number of Tregs in the graft or in the recipient, TGF- β 1 activating mAbs are unable to mediate their protective effects in this severe hyper-acute GVHD. One potential strategy to rescue Treg numbers would be to co-transfer donor Tregs at the time of transplantation. Although we attempted this approach, isolating sufficient donor Tregs to reach the commonly recommended Treg:Teff ratio of 1:2 was technically difficult in our hands. We also explored the possibility to expand Tregs *ex vivo*. However, insufficient initial purity resulted in undesirable expansion of conventional CD4⁺ and CD8⁺ T cells. Further optimization, either through improved culture conditions or more stringent, high-purity FACS sorting, may eventually enable reliable donor Treg supplementation and allow evaluation of TGF- β 1 activating mAbs in a myeloablative aGVHD model.

Finally, it is important to mention that the efficacy of TGF- β 1 activating mAbs was only demonstrated in one murine model of aGVHD, which does not well recapitulate the human disease. More specifically, treatment of the mice was prophylactic, whereas patients are more likely to receive therapy after aGVHD onset. Nevertheless, it is not unreasonable to hypothesize that TGF- β 1 activating mAbs may retain therapeutic potential even if they are administered after the onset of aGVHD clinical manifestations.

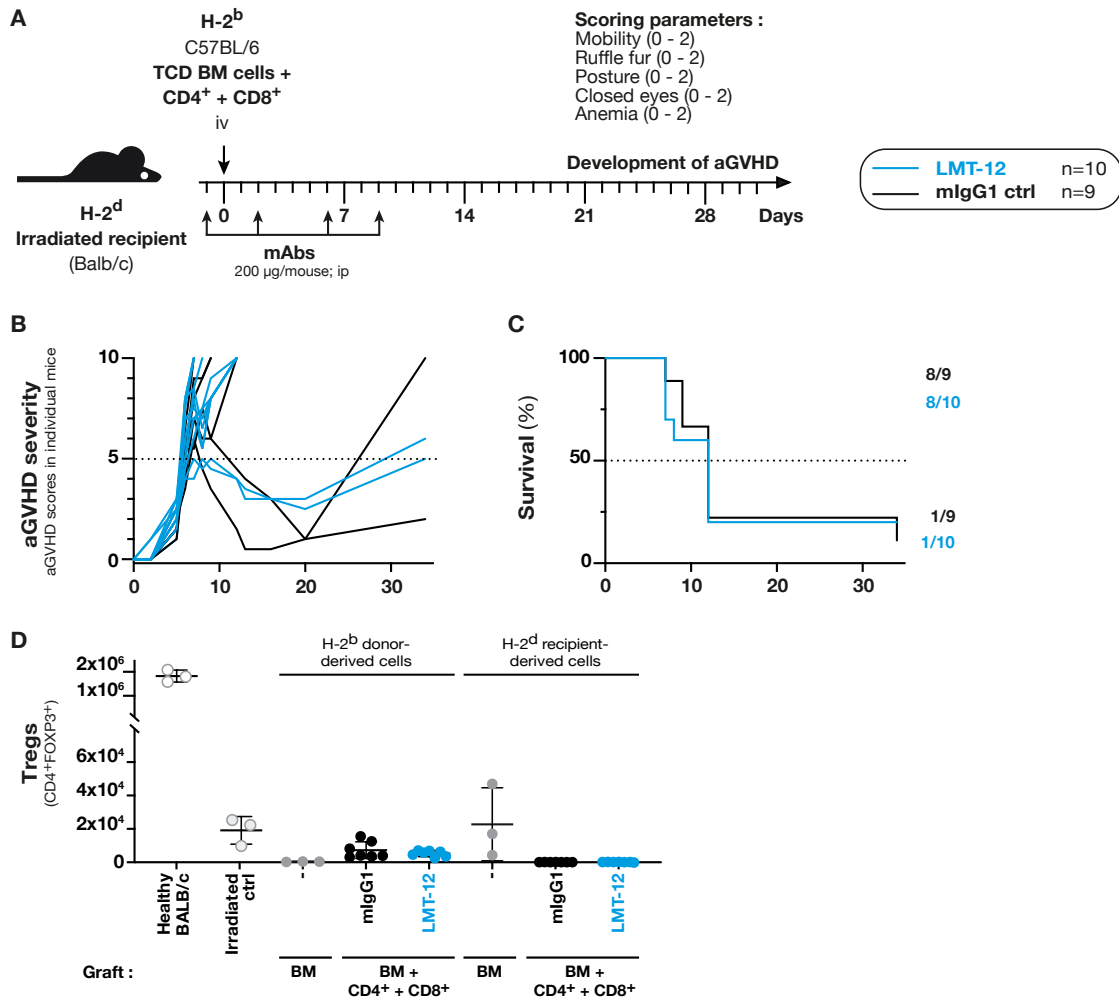


Figure 14. TGF- β 1 activating mAbs do not protect from aGVHD in myeloablative conditioning.

(A) Schematic experimental design. H-2^d mice transplanted with 10^7 T cell-depleted bone marrow (TCD BM) cells, 8×10^5 CD4⁺ and 6×10^5 CD8⁺ T cells isolated from H-2^b mice on day 0. Recipient mice were injected with TGF- β 1 activating mAbs (LMT-12) or isotype controls (mIgG1) twice a week, starting on day -1. Mice were scored for symptoms of aGVHD as indicated (maximum score: 10; mice were sacrificed when reaching a score of 8). (B) Evolution of aGVHD scores in individual mice. (C) Kaplan-Meier plots representing the proportion of mice alive at the indicated days after transplantation. (D) In a repeat experiment, mice were sacrificed 6 days after transplantation, and splenocytes were counted and analyzed by flow cytometry to evaluate numbers of Tregs of donor (H-2^b) and recipient (H-2^d). Healthy mice were non irradiated non grafted. Each point represents the value of one mouse, and horizontal bar the mean $\pm$ sem.

2.2 Could TGF- β 1 activating mAbs serve to treat cGVHD in patients ?

Allo-HSCT is complicated not only by severe aGVHD but also by cGVHD, which develops in nearly half of patients despite immunosuppressive prophylaxis, and remains a major cause of long-term morbidity and mortality. In contrast to aGVHD, driven by a rapid and intense allogeneic response, cGVHD is characterized by chronic immune dysregulation and tissue fibrosis. Given the efficacy of TGF- β 1 activation by mAbs in reducing disease severity in aGVHD, we sought to determine whether controlled TGF- β 1 activation could also ameliorate cGVHD by restoring immune tolerance, without exacerbating fibrotic manifestations. To address this, we decided first to evaluate the effects of TGF- β 1 activating mAbs and a blocking anti-GARP:TGF- β 1 mAb in parallel, in mouse cGVHD induced by transplantation of B10.D2 splenocytes into irradiated BALB/c recipients. We found that administration of blocking anti-GARP:TGF- β 1 mAbs significantly exacerbated the severity of the disease and increased mortality, suggesting that endogenous TGF- β 1 already exerts a protective effect in this model. Accordingly, treatment with TGF- β 1 activating mAbs reduced disease severity and increased survival in cGVHD mice (our second article of the results section). Quite unexpectedly, we observed that TGF- β 1 activating mAbs enhanced thymic function, promoting the development of single positive thymocytes, including tTregs. We thus suggest that increased TGF- β 1 activity promotes reconstitution of a tolerant immune system in the recipient, perhaps by enhancing tTreg development. These tTregs may suppress pathogenic immune responses in affected organs, but they may also contribute to the increased TGF- β 1 activity that we observed in the spleen after treatment with the mAbs, by augmenting the cellular source of latent TGF- β 1 available for mAb-mediated activation. Reduced activation of CD8⁺ T cells in lymphoid organs appears to lead to decreased type 1 adaptive immune responses in affected organs, thereby contributing to the reduced GVHD severity observed in mice treated with the TGF- β 1 activating mAbs. We observed that TGF- β 1 activating mAbs exerted no therapeutic effect when administered 2 weeks after transplantation. This suggests that TGF- β 1 activating mAbs need to be administered in the early post-transplant period to maximize its efficacy. We suggest that increased TGF- β 1 activity could protect thymic T cell development, particularly tTreg differentiation, during the acute phase after transplantation, even in absence of clinical improvement in aGVHD symptoms in this model. This interpretation could be consistent with previous preclinical and clinical studies showing that adoptive Treg transfer is more effective when performed at the time of allo-HSCT, than for established cGVHD lesions (315). This argues that TGF- β 1 activating mAbs may be most beneficial as a prophylactic intervention to decrease the incidence and severity of cGVHD, rather than as a treatment for established disease.

2.3 What could be adverse effects of TGF- β 1 activating mAbs used to treat GVHD after allo-HSCT?

2.3.1 Fibrosis

Because TGF- β 1 is a well-recognized contributor to fibrotic processes, and because blockade of TGF- β 1,2,3 has been shown to attenuate multiorgan fibrosis in murine models of cGVHD (191), one of our concerns was that TGF- β 1 activating mAbs could exacerbate fibrosis in cGVHD. However, in our cGVHD model (characterized by severe cutaneous damage), treatment with TGF- β 1 activating mAbs did not result in increased cutaneous fibrosis, as evidenced by the absence of dermal thickening. Macrophages activated by pro-inflammatory cytokines and autoantibodies are important drivers of fibrosis in cGVHD through their capacity to stimulate collagen production by fibroblasts. In light of the immunosuppressive effects of TGF- β 1 activating mAbs on innate immune cells, we propose that these mAbs may instead limit fibrotic remodeling by reducing macrophage activation. Nevertheless, fibrosis in other organs, particularly the spleen, should be further evaluated using histological staining such as Sirius Red or silver staining, given the increased TGF- β 1 activation observed in lymphoid organs in GVHD mice treated with TGF- β 1 activating mAbs.

2.3.2 Relapse of hematological malignancy

A central concern in treating GVHD is the preservation of the GVL effect, especially because allo-HSCT is mostly performed for malignant hematologic diseases. Enhancing TGF- β 1 activity raises the fear of dampening anti-tumor immunity, given the well-established immunosuppressive properties of TGF- β 1. TGF- β 1 suppresses T cell proliferation and effector function, whereas T cells are the principal mediators of GVL. Therefore, increased TGF- β 1 activity could reduce the ability of the adaptive immune system to eliminate malignant clones. However, reducing excessive inflammation characteristic of aGVHD may also have indirect beneficial effects on anti-tumor immunity by decreasing bystander T cell apoptosis and exhaustion. Moreover, by promoting thymic T cell development, increased TGF- β 1 activation could also improve adaptive immune reconstitution, thereby maintaining GVL activity rather than suppressing it. In addition, cytostatic effects on several hematopoietic malignant cell types have been described for TGF- β 1 (332, 333). It is thus conceivable that a transient increase of TGF- β 1 activity early after allo-HSCT performed for AML or other acute leukemias could restrain blast proliferation, providing time for thymic T cell reconstitution and establishment of a robust donor-derived immune system capable of mediating GVL. However, this cytostatic effect may also be detrimental in other myeloid diseases such as myelodysplasia, where excessive TGF- β 1 signaling contributes to ineffective hematopoiesis, and clinical improvement has been observed with agents that reduce TGF- β activity, such as luspatercept (334, 335). Nevertheless, this raises the possibility that TGF- β 1 activating mAbs might simultaneously mitigate GVHD and preserve GVL effect, although this remains to be demonstrated.

To explore whether TGF- β 1 activating mAbs maintain GVL, at least in mice, we performed a preliminary proof-of-concept experiment using a solid tumor model well-established in our laboratory. CT26 colon carcinoma cells were injected subcutaneously in our cGVHD mouse model, as the rapid mortality associated with parent-to-F1 aGVHD model precludes evaluation of tumor responses. Our initial results indicated that mice with cGVHD exhibited a modest graft-versus-tumor (GVT) effect compared to mice grafted with BM cells only. Importantly, that treatment with TGF- β 1 activating mAbs did not abolish this anti-tumoral response (**Figure 15**). These findings are

encouraging and suggest that enhancing TGF- β 1 activity, possibly through improved thymic function and reduced systemic inflammation, may preserve GVT in this model. Nevertheless, the incomplete tumor rejection was unexpected. The number of injected CT26 cells may overwhelm the immune system after transplantation, and reducing the tumor inoculum could improve the GVT effect with clinical relevance, as allo-HSCT is usually performed in patients with minimal residual disease. Alternatively, solid tumors may not fully recapitulate the biology of hematologic malignancies. To more precisely evaluate the GVL effect, we plan to establish a leukemia model using A20 cells engineered to express firefly luciferase or fluorescent reporter (GFP or mCherry), enabling assessment of tumor burden by noninvasive and quantitative *in vivo* bioluminescence imaging, or longitudinal flow-cytometric analysis on blood samples. Such a system should provide a more relevant setting to determine whether TGF- β 1 activating mAbs preserve or impair the GVL effect across leukemic contexts.

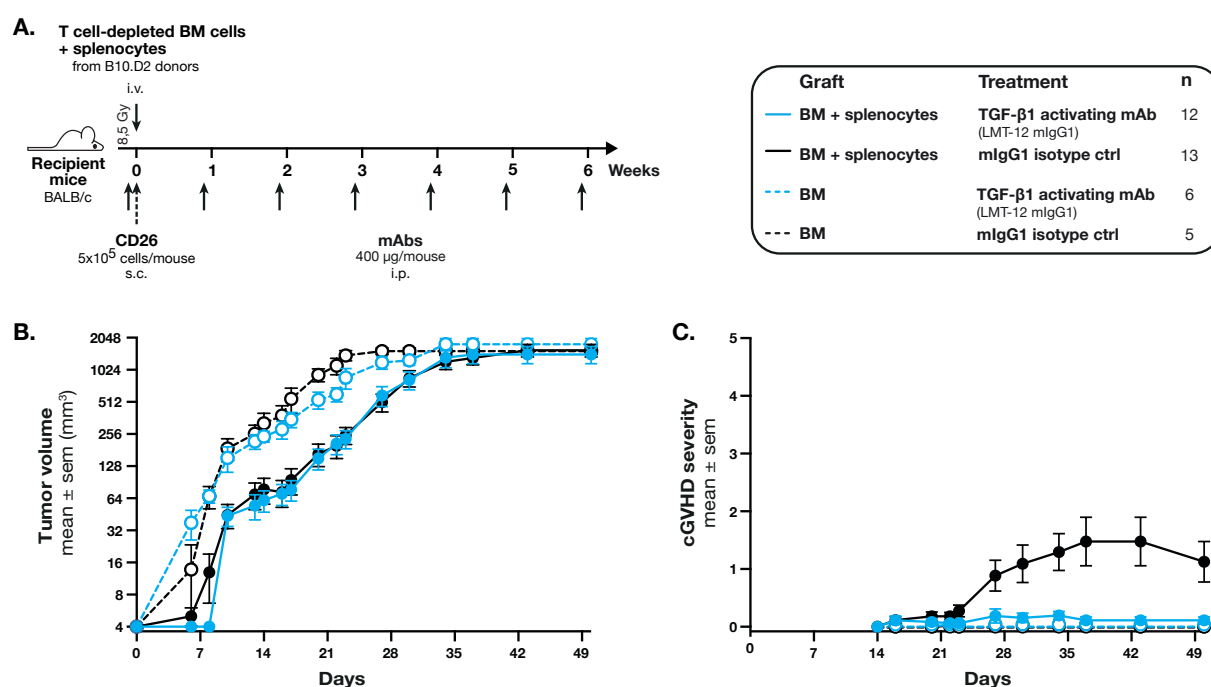


Figure 15. TGF- β 1 activating mAbs could preserve anti-tumoral responses after allo-HSCT.

(A) Schematic experimental design. T cell-depleted bone marrow cells (BM) and splenocytes isolated from B10.D2 donors were transplanted into lethally irradiated BALB/c recipients, while BM were grafted with BM cells only. Recipients were injected s.c. with CT26 cells on day 0. Recipient mice also received weekly injections of a TGF- β 1 activating mAb (LMT-12 mlgG1) or an isotype control (mlgG1 ctrl) starting one day before transplantation. (B) Evolution of the mean of tumor volume $\pm$ sem. Tumor diameters were measured three times a week. Mice were euthanized when the tumor surface was ≥ 200 mm². (C) Evolution of the mean of cGVHD score established as described in the second manuscript (maximum score: 5; mice were sacrificed when reaching a score of 4.5).

2.3.3 Opportunistic infections

Finally, the immunosuppressive functions of TGF- β 1 could potentially impair immune responses to pathogens and favor the development of opportunistic infections. The effects of TGF- β 1 in infectious settings are complex and pathogen-dependent. For example, TGF- β 1 has been shown to inhibit T cell dependent viral clearance in the lung after allo-HSCT, thereby exacerbating pneumonitis (336). Conversely, TGF- β 1 contributes to mucosal immune homeostasis by promoting IgA class switching in B cells and by driving the differentiation of naïve CD4⁺ T cells into Th17 effectors, both being important for protection against extracellular bacteria and maintenance of barrier immunity. In our laboratory, we previously demonstrated that inhibition of TGF- β 1 produced by GARP-expressing cells did not alter either innate or adaptive immune responses in a murine model of intestinal infection with *Citrobacter rodentium* (337). This suggests that GARP-mediated TGF- β 1 activation is not essential for host defense against this pathogen, although it does not exclude the possibility that TGF- β 1 produced or activated by other cell types may still contribute to antimicrobial immunity. In the specific context of post-transplantation, no large or conclusive studies demonstrate that reducing TGF- β 1 activity impacts infection control. However, aGVHD significantly increases the risk of developing life-threatening infections (338). By reducing aGVHD severity, TGF- β 1 activating mAbs might counterbalance its potential immunosuppressive effects, thereby avoiding an overall increase in infection risk. To test this, infections with influenza virus or *Citrobacter rodentium* could be caused in mice treated with TGF- β 1 activating mAbs that survived aGVHD or developed cGVHD, to assess their ability to control pathogens under conditions of increased TGF- β 1 activation.

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