TGF-β1 activating antibodies for the immunotherapy of allo- and auto-immune diseases

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ABBREVIATION LIST

aa	Amino acid
ADMIDAS	Adjacent to Metal Ion-Dependent Adhesion Site
allo-HCT	allogeneic hematopoietic cell transplantation
AML	Acute Myeloid Leukemia
ANA	Anti-Nuclear Antibodies
APC	Antigen-Presenting Cell
BCR	B cell receptor
BrdU	Bromodeoxyuridine
CNS	Central Nervous System
Cryo-EM	Cryo-electron microscopy
CTL	Cytotoxic T lymphocyte
DAMP	Danger-Associated Molecular Pattern
DC	Dendritic cell
DT	Diphteria toxin
DTR	Diphteria toxin receptor
DSS	Dextran Sulfate Sodium
EAE	Experimental Autoimmune Encephalomyelitis
ECM	Extracellular Matrix
GARP	Glycoprotein A Repetition Predominant
GvHD	Graft-versus-Host Disease
GvT	Graft-versus-Tumor
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDM	Host Dust Mite
HIV	Human Immunodeficiency Virus
HSC	Hematopoietic stem cell
IBD	Inflammatory Bowel Disease
INF-α	Interferon-α
INF-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cell
KO	Knock-out
LAP	Latency Associated Peptide
LCMV	Lymphocytic Choriomeningitis Virus
LRR	Leucine Rich Repeat
LRRC32	Leucine Rich Repeats Containing protein 32
LRRC33	Leucine Rich Repeats Containing protein 33
LTBP	Latent TGF-β1 Binding Protein
mAb	Monoclonal antibody

MHC-I	Major Histocompatibility Complex class I
MHC-II	Major Histocompatibility Complex class II
MK	Megakaryocyte
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cell
NK	Natural Killer cell
NMR	Nuclear Magnetic Resonance
NSG	NOD SCID Gamma
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
pDC	Plasmacytoid Dendritic Cells
SLE	Systemic Lupus Erythematosus
Tc1	Cytotoxic CD8+ T cell (Type 1)
Tc2	Cytotoxic CD8+ T cell (Type 2)
Tc17	Cytotoxic CD8+ T cell (Type 17)
TCR	T Cell Receptor
Tfh	T Follicular helper cell
TGF-β	Transforming Growth Factor Beta
Th	Helper T Cell
TNF-α	Tumor Necrosis Factor alpha
Treg	Regulatory T cell
TSP-1	Thrombospondin-1
tTreg	Thymic Regulatory T cell
TβRI	TGF-β Receptor class I
TβRII	TGF-β Receptor class II
pTreg	Peripheral Regulatory T cell
xGvHD	Xenogeneic Graft-versus-Host Disease

ABSTRACT

ABSTRACT

TGF-β1 is a potent immunosuppressive cytokine produced as a latent homodimer, in which mature TGF-β1 is kept inactive by the Latency Associated Peptide (LAP). TGF-β1 requires activation to bind to its receptor, a process tightly regulated and cell-type specific. The most documented mechanisms of activation involve mechanical deformation of LAP anchored to cell membranes or extracellular matrix through covalent bonds with a tethering protein, which presents latent TGF-β1 to integrins. Within the immune system, Tregs activate latent TGF-β1 through the collaboration of transmembrane protein GARP and integrin $\alpha_V\beta_8$. However, the molecular processes governing this activation are not resolved yet. Due to its immunosuppressive functions, TGF-β1 appears as an interesting therapeutic target for a set of diseases associated with immune dysfunction, such as allo- or auto-immune diseases.

To uncover the molecular mechanisms underlying TGF-β1 activation and decipher the roles played by TGF-β1 in these diseases, we derived monoclonal antibodies (mAbs) capable of activating human or mouse TGF-β1 anchored on cells by a transmembrane protein. Using biochemical and Cryo-electron microscopy (cryo-EM) analyses, we unveil that mAb-mediated activation requires bivalent binding close to the LAP dimerization interface and crosslinking of two membrane-bound GARP:TGF-β1 complexes. Administration of mAbs to mice with graft-versus-host-disease (GvHD) reduced disease severity and increased survival. This beneficial outcome is associated with an increase in regulatory T cells (Tregs), which are required for the therapeutic effect. In this work, we show that activation of membrane-bound TGF-β1 *in vivo* is achievable with mAbs, opening avenues for potential immunotherapy of allo- or auto-immune diseases characterized by dysregulated T cell activity insufficiently controlled by Tregs.

INTRODUCTION

Transforming Growth Factor β 1 (TGF-β1) belongs to the TGF-β superfamily, which comprises 33 structurally related members: Bone Morphogenic Proteins (BMP), Growth and Differentiation Factors (GDF), activins, Nodal and TGF-βs. Three TGF-β isoforms (TGF-β1, 2 and 3) are expressed in mammals. They are encoded by three distinct genes and have pleiotropic roles on cell proliferation, differentiation, migration and survival. TGF-β1, 2 and 3 share high levels of amino-acid sequence identities and signal through the same receptor, but they exert very different functions in the organism.

TGF-β1 plays major roles *in utero* during development and in immunity after birth. It is the predominant TGF-β isoform expressed in the immune system. Its major roles are illustrated by the phenotype of *Tgfb1*$^{-/-}$ mice, which either die *in utero* from profound developmental defects, or are born without gross developmental anomalies but die from a fatal multi-organ inflammation within 3 to 4 weeks after birth [1, 2]. This introduction will focus on the roles that TGF-β1 may play in immune-related diseases. Beyond the role of TGF-β1 on immune cells, particular attention will also be paid to its involvement in the process of wound healing and fibrosis, features that are part of many immune-related pathologies.

TGF-β1 activity is regulated mostly at a post-translational level: it is produced as a latent, inactive cytokine that requires an additional step of activation to enable signaling through the TGF-β receptor. The first part of the introduction describes the wonderful travel of this cytokine, from biosynthesis to signaling.

1. TGF-β1: from biosynthesis as a latent cytokine to activation

1.1. Biosynthesis and bioavailability

TGF-β1 is first synthesized as a large precursor called pre-pro-TGF-β1 which contains a signal peptide sequence, a large (25 kDa) N-terminal domain called the latency-associated-peptide or LAP, and a short (12,5 kDa) C-terminal mature TGF-β1 peptide (Fig. 1). After signal peptide removal in the secretory pathway, the pro-TGF-β1 assembles into a homodimer thanks to formation of three interchain disulfide bonds: two disulfide bridges link the monomers in the LAP region, while a third bridge stabilizes the homodimer in the mature TGF-β1 region. The polypeptidic chains of the pro-TGF-β1 homodimer are then processed by the convertase Furin, leading to proteolytic cleavage of the mature cytokine from the LAP. The resulting LAP and mature TGF-β1 homodimers nevertheless remain non-covalently associated, forming a complex called latent TGF-β1. Latent TGF-β1 is inactive because the LAP encircles mature TGF-β1, thereby preventing its binding to the TGF-β receptor [3, 4].

Almost all cell types express the *TGFB1* gene and produce latent TGF-β1, which is either secreted in the extracellular milieu as a soluble molecule called “free latent TGF-β1” or bound to other proteins via disulfide linkage with cysteine residues (Cys33) from each LAP monomer. These TGF-β1-tethering proteins enhance the rate of folding and secretion of TGF-β1 and include Latent TGF-β1 Binding Proteins (LTBPs), Leucine Rich Repeats Containing protein 32 (LRRC32), also called Glycoprotein A Repetition Predominant (GARP), and LRRC33 [5, 6].

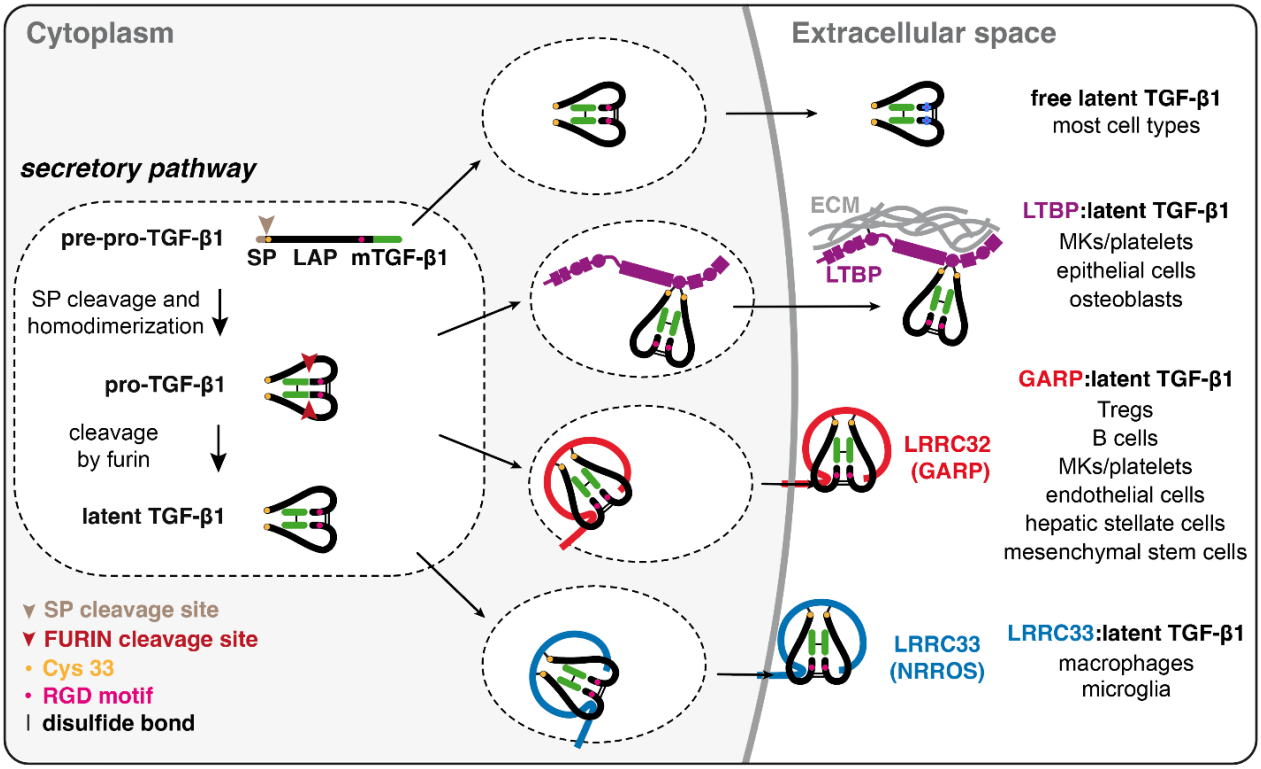

Figure 1: Schematic representation of TGF-β1 processing and trafficking. Figure adapted from [3]. TGF-β1 is first produced as a precursor protein called “pre-pro-TGF-β1”. After signal peptide cleavage and homodimerization via formation of disulfide bonds, the resulting pro-TGF-β1 is cleaved by protease Furin, leading to formation of “latent TGF-β1”. In this inactive complex, the N-terminal dimer or Latency Associated Peptide (LAP) remains non-covalently associated to the C-terminal dimer or mature TGF-β1. Latent TGF-β can be secreted as such by most cells, or covalently associates to tethering proteins such as LTBP, GARP or LRRC33 in specific cell types. To bind to its receptor, mature TGF-β1 must be released from the LAP, a process called “TGF-β1 activation”. SP, signal peptide; LAP, latency associated peptide; mTGF-β, mature TGF-β.

1.1.1. LTBPs

Many cell types, such as epithelial cells and osteoblasts, produce latent TGF-β1 in association with the LTBPs. LTBPs are a family of large (120-240 kDa) multidomain glycoproteins composed of numerous calcium-binding EGF-like repeats and several globular domains containing 8 cysteines (8-Cys motifs). TGF-β1 can associate covalently with LTBP-1, -3 and -4 through disulfide bond formation between the Cys33 of the two LAP monomers and two cysteines located in the third 8-Cys motif of a single LTBP molecule [7] . After secretion, LTBPs deposit in the matrix by forming

covalent interactions with extracellular matrix proteins such as fibrillin and fibronectin, thus providing a reservoir of latent TGF-β1 that can be rapidly mobilized [4].

1.1.2. LRRC32/GARP

By contrast to LTBPs, LRRC32 or GARP is a type 1 transmembrane protein anchoring latent TGF-β1 to the cell surface. GARP contains a large extracellular region composed of 20 Leucin Rich Repeats (LRRs) and a short intracellular region [8]. Due to the presence of leucine repeats, the extracytoplasmic region adopts a horseshoe-like tridimensional structure prone to support protein–protein interactions [9]. Like LTBPs, covalent binding of GARP to latent TGF-β1 engages Cys33 of each LAP monomer with two cysteines of a single GARP molecule (Cys211 and Cys350). In addition to binding latent TGF-β1, GARP is also able to bind latent TGF-β2 and 3 [10]. GARP:(latent)TGF-β1 complexes are expressed only on a few cell types, comprising subsets of hematopoietic cells, namely regulatory T cells (Tregs) [11-13], stimulated B cells [14], megakaryocytes and activated platelets [10, 15], and non-hematopoietic cells such as endothelial cells [15, 16], subsets of fibroblasts [17], mesenchymal stromal cells [18, 19] and hepatic stellate cells [3, 20]. Finally, soluble GARP:TGF-β1 complexes have been described in the supernatant of cultured Tregs, resulting from a putative shedding from the cell surface by an unidentified protease [5].

1.1.3. LRRC33/NRROS

The closest relative of GARP, namely LRRC33, has been described recently as a docking molecule that anchors latent TGF-β1 to the surface of myeloid cells such as macrophages and microglial cells. Despite only 34% amino-acids sequence identity between the GARP and LRRC33, the two proteins share a highly similar structure with a short intracytoplasmic tail and a large extracellular domain containing the same number of LRRs. Moreover, the cysteines of GARP implicated in disulfide binding to LAP are also conserved in LRRC33 [21-23]. However, in contrast to GARP, LRRC33 only binds latent TGF-β1, and does not bind latent TGF-β2 or 3.

1.2. TGF-β1 latency: lessons learned from structural data

1.2.1. Mature TGF-β1 and its receptor

Grasping the concept of latency begins with understanding the interaction between the cytokine and its receptor (Fig. 2). TGF-β1 signals through a unique hetero-tetrameric receptor, composed of two homodimers of “type I” (TβRI) and two homodimers of “type II” (TβRII) receptor chains.

The mature TGF-β1 homodimer presents a butterfly-like shape, in which each monomer forms a slightly curved left hand containing three fingers and a rigid palm. The thumb and the wrist are respectively formed by α3 and α1 helix of one monomer, while the two other fingers are formed by

a flexible loop and by anti-parallel β strands (β6 and β7) from the other monomer. The palms are stabilized by a cysteine knot made of three intrachain disulfide bonds. In addition, the two TGF-β1 monomers are linked together at the palms by an interchain disulfide bond [24, 25].

Binding of the ligand to its receptor starts by a first contact between the tips of the fingers of mature TGF-β1 with the TβRII homodimer (zone 1). Supported by salt bridges and hydrogen bonds, this first interaction zone constitutes a high affinity interaction. After binding of mature TGF-β1 to the TβRII homodimer, the latter recruits two monomers of TβRI that are now able to interact with the palm face of the two other fingers (zone 2) and the thumb (zone 3). These interactions with TβRI are mainly sustained by hydrophobic patches and thus constitute a low affinity interface [26]

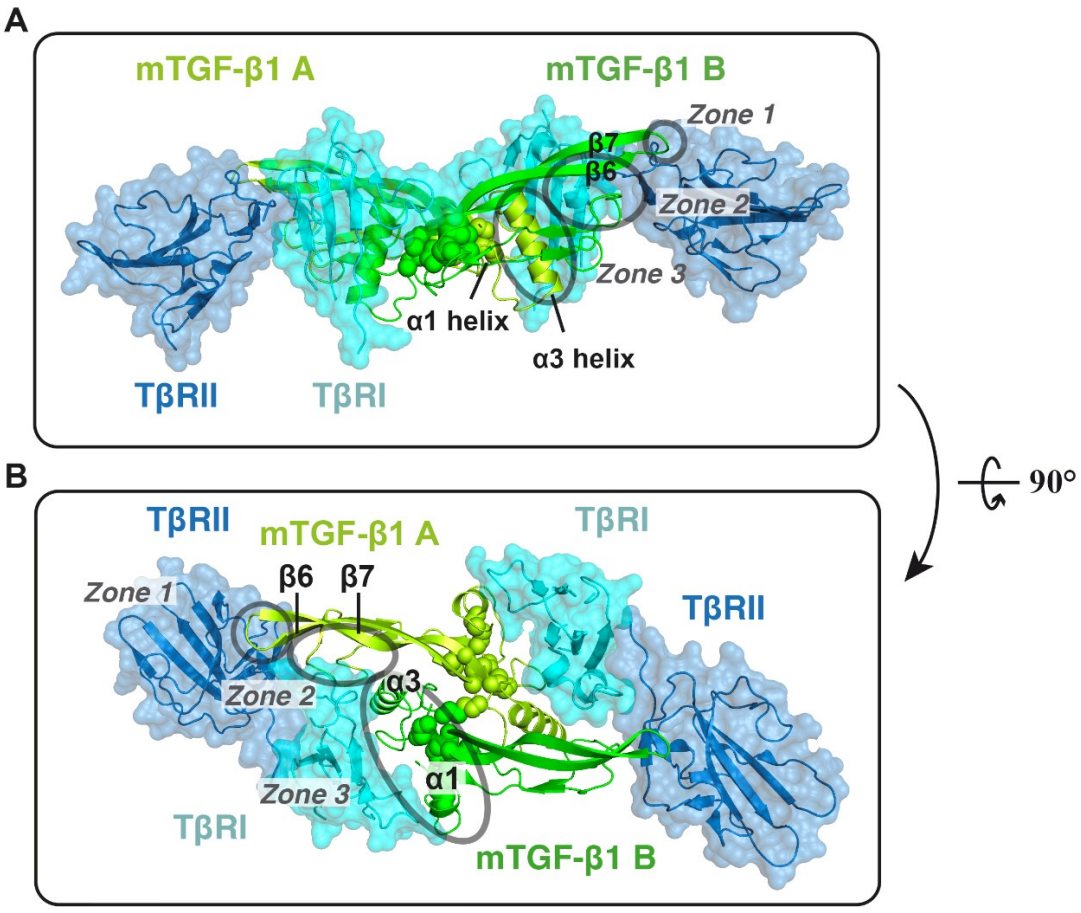

Figure 2: Structure of human active TGF-β1 bound to the extracellular domain of TβRI and TβRII. Cartoon representation, associated with surface transparency for TβRI and TβRII. Zones of interactions are depicted by grey circles. A. Vue from "front" illustrating the butterfly shape of the active TGF-β1. B. Vue from "top" illustrating all the interactions zones. The mature TGF-β1 chains are shown in green (light green monomer A, dark green, monomer B) and the TβRI and TβRII chains are shown in light and dark blue respectively. Crystal structure published by Radaev et al. 2010 (PDB 3KFD) [26].

1.2.2. Latent TGF-β1

Solved in 2011 by the group of Timothy Springer, the crystallographic structure of porcine latent TGF-β1 shed the light on the concept of latency and laid the foundation for reflexions on the structural changes likely induced during activation [27]. Each LAP monomer is composed of a rigid "arm" domain and a so-called straitjacket domain (corresponding to the fastened forearm) that maintains the two hands of mature TGF-β1 confined in the LAP. The two arms are disulfide linked together at the neck, forming a strong dimerization interface (Fig. 3A-B).

- **The forearms**

Each straitjacket domain of LAP is formed by the α1 helix and the latency lasso, a flexible loop that connects α1 to the arm domain. This structure confers latency by masking the three major zones of interaction between the mature TGF-β1 cytokine and its receptor. The tips of the fingers of mature TGF-β1 are encircled by the latency lasso, precluding interaction with TβRII by obstructing the first zone of interaction. The palm face of the fingers (zone 2) is partially covered by the latency lasso and the α1 helix. Finally, the α1 helix is so deeply buried between the two first fingers and the thumb (zone 3) that it moves the thumb away from fingers by comparison to their position in active TGF-β1 bound to the receptor (Fig.3C). Thus, in addition to restrict receptor access by masking key residues, the LAP holds mature TGF-β1 in a sharply different conformation.

- **The arm**

The rigidity of the arm domain come from its structure composed of two anti-parallel, four-stranded β-sheets. The α2 and α5 helices of the arm domain complete the encirclement of the mature TGF-β1. A strong interaction between the C-terminal segment of the arm and the N-terminal part of helix α1 from the straitjacket, called the fastener, ensures the stabilization of the straitjacket, and completes covering the palm face of the two first fingers. At the shoulder region of the arm domain, a flexible loop between two β-strands of the arm domain (β7 and β10) contains a RGDLXX(L/I) motif known to be bound by a subset of integrins.

- **The dimerization interface**

At the neck of the latent TGF-β1 structure, the two monomers are welded together by two interchain disulfide bonds between the arm domains, in a shape recalling a bowtie. This dimerization interface is further reinforced by two salt bridges and a cation-π bond. The importance of this dimerization interface in latency is attested by the Camurati-Engelmann disease, where patients carrying loss of function mutations in one of the LAP amino acids involved in the stabilization of this interface suffer from long bone and skull malformations attributed to an excessive TGF-β1 activity [28].

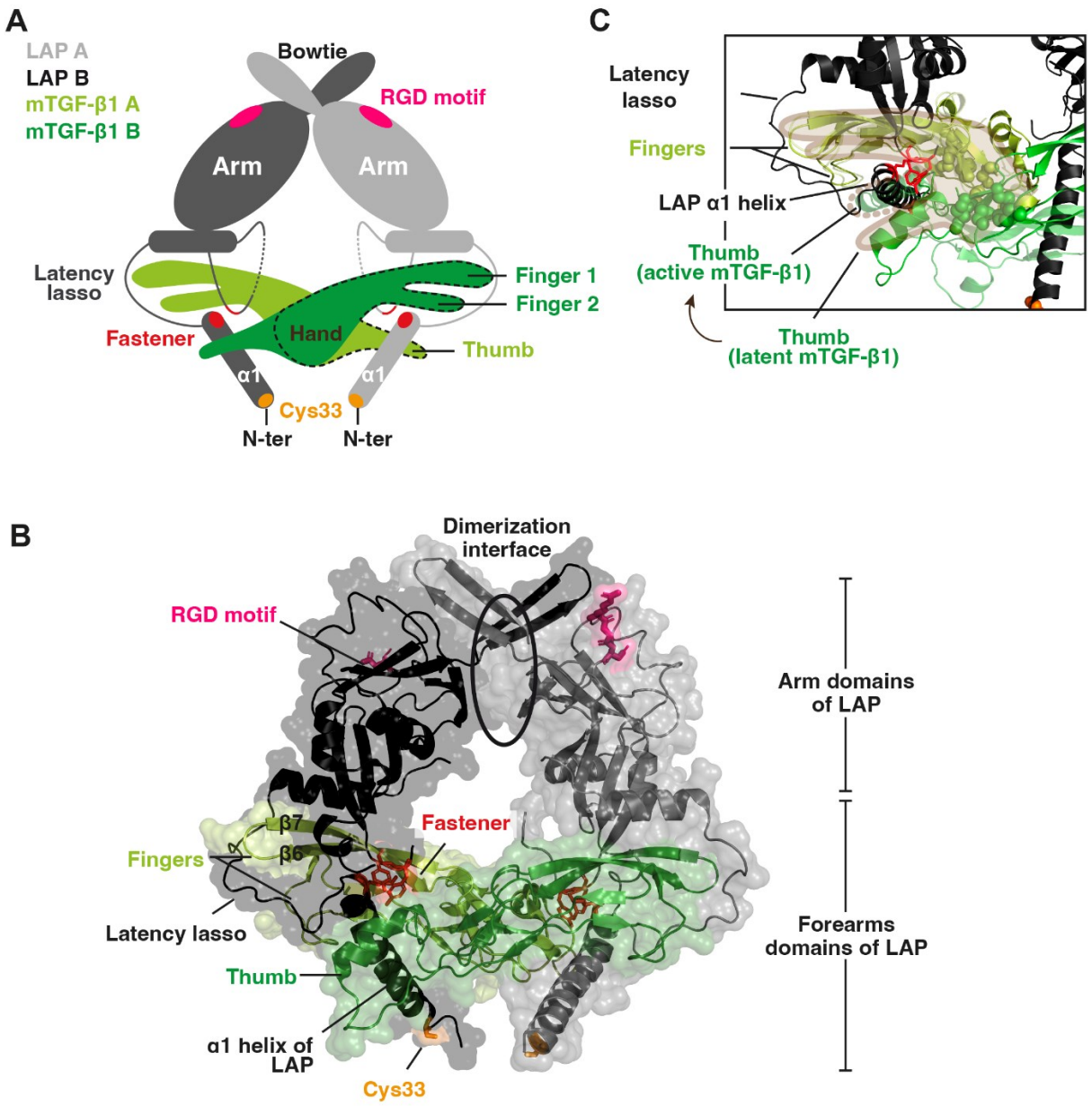

Figure 3: Structure of porcine latent TGF-β1. A. Schematic representation of the latent TGF-β1 structure, from Stephanie Liénart's thesis. B. Cartoon overlaid with surface transparency representations of the crystal structure of porcine latent TGF-β1, as originally published by Shi et al. [27] and re-refined by Zhao et al. (PDB 5VQF) [29]. D. Illustration of the conformational change occurring within mature TGF-β1 upon activation. Representation of the open hand in latent TGF-β1 and of the close hand in active TGF-β1. Overlay of latent TGF-β1 (5VQF - cartoon, no transparency) and of position of helix α3 in active TGF-β1 (3KFD - cartoon, transparency). Amino acids closing the fastener are depicted as sticks.

1.2.3. Tethering of latent TGF-β1 by other proteins

Linkage of the straitjacket C-termini to one of the TGF-β1-tethering proteins further completes the encirclement of mature TGF-β1 and reinforces latency [27].

- **LTBPs**

Due to their size and their multidomain features, no 3D structure of a full-length LTBP or a LTBP:TGF-β1 complex have been solved yet. Nevertheless, the third 8-Cys domain that interacts with latent TGF-β1 was solved by NMR in 2003. This globular domain consists of six β-strands and two α-helices stabilised by four disulphide bridges. The “2-6” disulfide bridge can be disrupted to associate with the LAP homodimer without domain unfolding. The interchain disulfide bond formation is fostered by electrostatic interaction of residues in the close vicinity of the cysteine from each protein [30, 31].

- **GARP and LRRC33**

The tri-dimensional structures of GARP:TGF-β1 and LRRC33:TGF-β1 complexes were solved by X-ray crystallography (Liénart et al) or cryoEM (Duan et al), respectively [32, 33].

The structures of GARP and LRRC33 are illustrated in figure 4. GARP and LRRC33 are two closely related members of the Leucin-Rich-Repeat (LRR) family. The 20 LRR motifs found in the two proteins comprise 20 to 30 residues highly enriched in hydrophobic amino acids leucine and asparagine, spaced in a consensus motif (LxxLxLxxNxL, where "L" correspond to Leu, Iso ou Val and "N" to Asn, Thr, Ser ou Cys). The hydrophobic core of each LRR motif folds in a loop including a short β strand. Consecutive motifs arrange in a solenoid horseshoe shape in which the β strands associate in hydrogen-bounded β sheets at the concave side of the curve. GARP presents a nearly perfect circular curvature. LRRC33 was suggested to present an inward movement in the central region [32], which I failed to illustrate using PyMol (Fig. 4). The hydrophobic core of GARP is protected by two caps formed of two anti-parallel β strand at the N- and C- termini. The N-terminus of LRRC33 is apparent in figure 4, although both the C- and N- termini were not solved according to Duan et al. Secondary structures protruding on the convex side of LRR proteins arise depending on the length of the LRR motifs. This diversity of shape is well illustrated in GARP and LRRC33, which display an irregular mix of such secondary structures on their convex side, with loops of various lengths and short α helices.

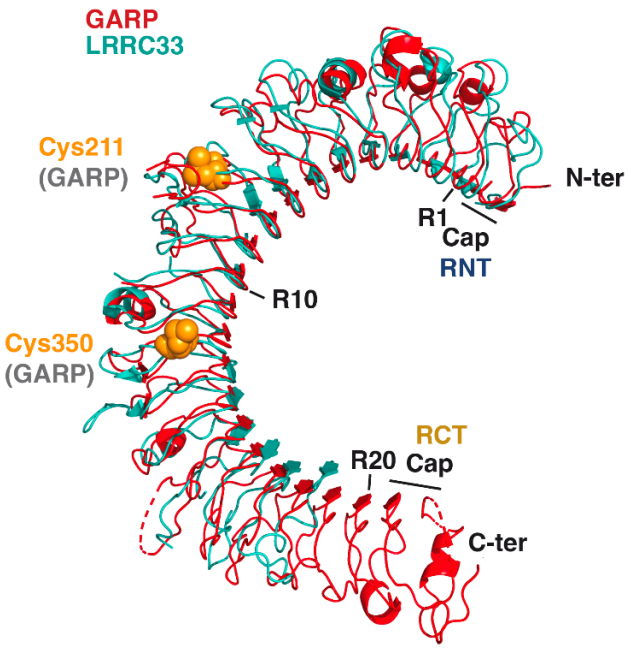

Figure 4: Structural alignment of GARP and LRRC33. Cartoon representation of GARP (red) and LRRC33 (blue) 3D structures, as observed in the structures of GARP:TGF-β1 (PDB 6GFF) and LRRC33:TGF-β1 (PDB 7Y1R) solved by X-ray crystallography or cryoEM, respectively. LRR motifs are numbered from R1 to R20, cysteines forming disulfide bonds with LAP are represented by orange spheres. N-ter, N-terminus ; C-ter, C-terminus; R, leucine-rich repeat motif ; RNT, leucin-rich repeat N-terminal; RCT, leucin-rich repeat C-terminal.

In GARP:TGF-β1 and LRRC33:TGF-β1 complexes, latent TGF-β1 raises in a similar manner on the lateral side of the LRR proteins. Its structure is also globally similar to that of the previously described “free” latent TGF-β1. The intermolecular disulfide bonds linking the two LAP monomers to GARP or LRRC33 are conserved in the two LRR proteins. The N-terminus of one monomer (LAP_A) is burried in a hydrophobic pocket, while the N-terminus of the second (LAP_B) floats on the lateral surface of LRRC where it is stabilized by van der Waals interactions. Finally, the mature TGF-$\beta1_B$ monomer (α3 helix and the preceding loop comprising residues 323 to 337) contacts the LRR proteins in a non-covalent interaction.

Despite a globally conserved shape by comparison to “free” latent TGF-β1, binding of latent TGF-β1 to any one of the LRR proteins induces conformational changes at the interface with GARP or LRRC33 [32, 33]. These variations reflect plasticity that could explain why latent TGF-β1 is able to associate with two structurally distinct classes of proteins, namely LRRs and LTBPs. Flexibility is also observed by cryo-EM in the LRRC33:TGF-β1 complex, in the loop containing the RGD motif, which might provide enough mobility for integrin recognition [32].

The most notable difference between the structures of GARP:TGF-β1 and LRRC33:TGF-β1 is a 35° rotation between the LRR proteins in the two complexes. It is important to note however that the crystals used to diffract X-rays and solve the structure of GARP:TGF-β1 complexes were generated in the presence of an anti-GARP:TGF-β1 antibody (not shown on Fig. 5), whereas the structure of LRRC33:TGF-β1 complexes was modelled by cryo-EM in absence of any other protein.

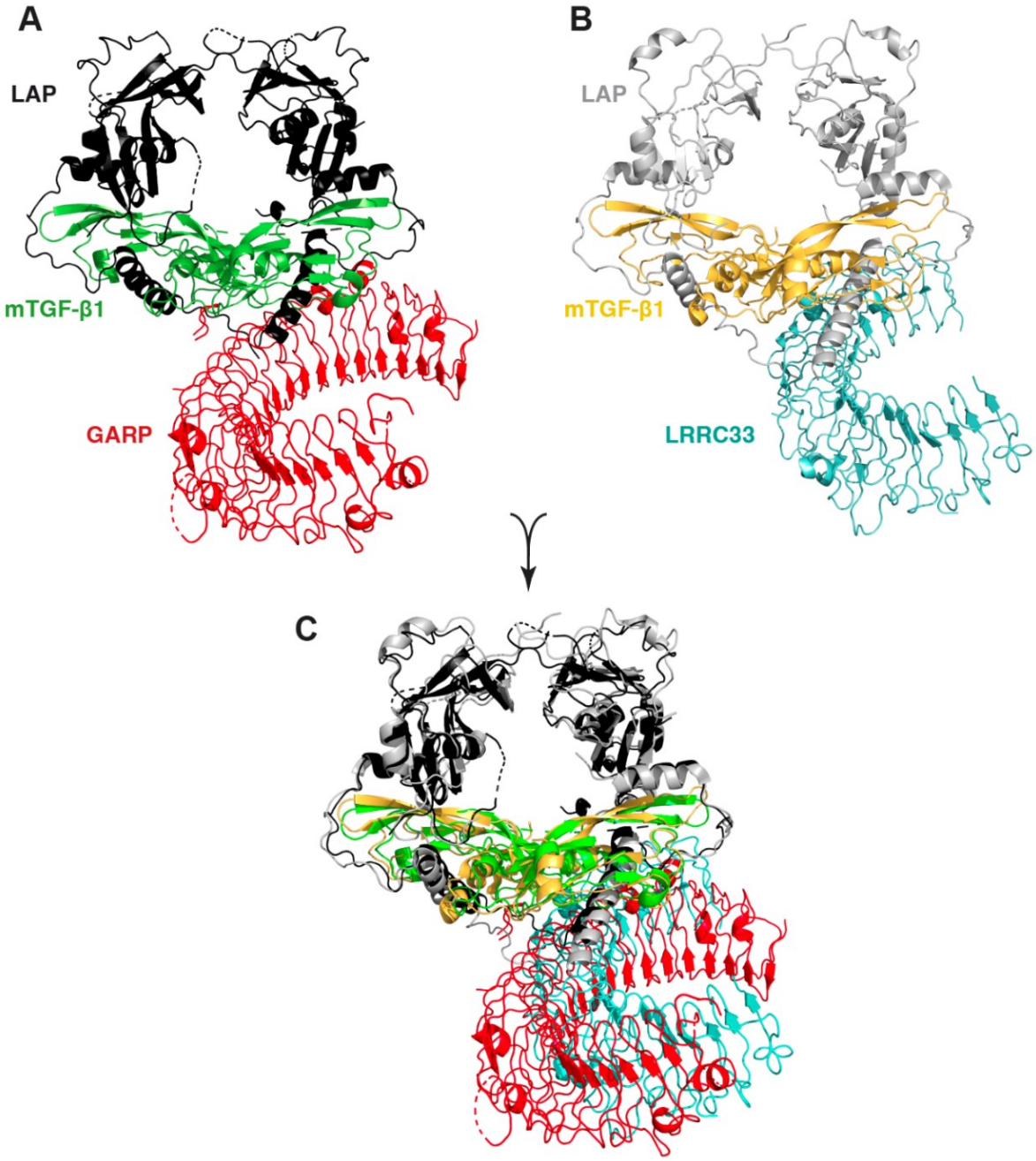

Figure 5: Structures of GARP:TGF-β1 (A) and LRRC33:TGF-β1 complexes (B), either represented individually or aligned. Superposition of L-TGF-β1:GARP and L-TGF-β1:LRRC33 (PDB codes: 6GFF and 7Y1R, respectively) complexes according to L-TGF-β1. Compared with GARP, LRRC33 rotates about 35 degrees relative to the axis of L-TGF-β1 dimer. Representation and alignment were performed using PyMol.

1.3. Activation: how and by whom?

To bind its receptor and trigger a signaling cascade in target cells, mature TGF-β1 needs to be released from LAP, a process called TGF-β1 activation. Given the pleiotropic effects of TGF-β1 and its production in a latent form by most cells, this process is tightly regulated and appears to occur in only a few cell types. The molecular mechanisms of TGF-β1 activation that have been identified thus far are cell-type specific and involve either mechanical deformation or proteolytic cleavage of LAP. The most described model is the mechanical deformation of LAP. It implies cooperation between one of the TGF-β1-tethering proteins and an RGD-binding integrin. Thrombospondin-1 (TSP-1) is implicated in the second most studied mechanism of activation, where a peptide of TSP-1 would compete with a portion of LAP to liberate key amino acids from mature TGF-β1. Finally, several proteases such as the serine protease plasmin, and MMP2 or MMP9 metalloproteases were shown to activate TGF-β1 *in vitro* by proteolytic degradation of LAP. However, the relevance of these mechanisms *in vivo* is unclear [4, 34].

1.3.1. Roles of TGF-β1-tethering proteins

Even though their association with TGF-β1 contributes to latency, tethering proteins also appear to play a role in TGF-β1 activation. This is well illustrated by the phenotype of *Tgfb1*$^{C33S/C33S}$ mice, where covalent binding of LAP to TGF-β1-tethering proteins cannot occur. These mice display multi-organ inflammation and a shorter lifespan (with half of the mice dying within 30 days, while the others can survive up to 5 months), which is associated with reduced TGF-β signalling. The phenotype of *Tgfb1*$^{C33S/C33S}$ mice suggests an overall reduction in TGF-β1 activity, rather than impaired TGF-β1 latency. Nevertheless, the phenotype is milder than that observed for *Tgfb1*$^{-/-}$ mice, suggesting that TGF-β1 activation can also occur independently of LTBPs or LRRC proteins [35].

- **LTBPs**

Ltbp1-, *Ltbp3*- and *Ltbp4*-deficient mice display miscellaneous tissue abnormalities or early fatalities that can be attributed to a lack of appropriate TGF-β secretion and/or activation. Dominant features of these severe phenotypes comprise a lethal defect in heart development (*Ltbp1* and *Ltbp1-L*), skeletal malformation (*Ltbp3*) and abnormal lung development, cardiomyopathy, emphysema, inflammation of colonic wall, splenomegaly and colorectal cancer (*Ltbp4*) [36-39]. Of note, *Ltbp2*- and *Ltbp4*-deficient mice also display TGF-β1 independent phenotypes that are related to their function in fibrillin stabilization.

- **GARP**

The phenotype of mice with a germline mutation of *Garp* is almost identical to that of *Tgfb3*$^{-/-}$ mice. Both strains display lethal defects in palatogenesis, leading to death in the immediate perinatal period [40] and our own, unpublished observations). Together with the observed co-localization of GARP and TGF-β3 proteins in epithelial cells of the developing palate, these studies show a non-redundant role of GARP in TGF-β3 activity during development. Construction of mouse strains harbouring cell type-specific deletions of *Garp* circumvented the lethal phenotype of the *Garp* germline deletion, allowing to study the roles of GARP in TGF-β1 activation after birth (cf 1.3.3.).

- **LRRC33**

Nrros$^{-/-}$ mice show abnormal microglial development and neurological disorders that lead to death by 5 months of age. These neurological features are also found in mice with *Tgfb1* deficiency limited to the central nervous system [21, 41]. Moreover, transcriptional profiling of *Nrros*$^{-/-}$ microglia demonstrates defective TGF-β1 signaling in this compartment [22].

1.3.2. Proteins triggering TGF-β1 activation

- **RGD-binding integrins**

Integrins are heterodimeric surface proteins involved in various functions, among which cell adhesion. A subset of integrins is able to bind the integrin-binding RGDLXX(L/I) motif in the LAP of TGF-β1. The motif is located in the shoulder region of the arm domain, at the opposite side of the cysteine bound by the TGF-β1-tethering proteins [42, 43] (Fig.3B). Integrins are composed of one α and one β chain that are non-covalently associated. Both α and β chains contain a short intracytoplasmic tail that can associate with cytoskeleton proteins, and several extracellular domains organized in “legs” and “head”. α and β subunits associate at the head level by their β-propeller and βI domain, respectively (Fig.6A).

The relevance of TGF-β1 activation by RGD-binding integrins *in vivo* is demonstrated by the phenotype of *Tgfb1*$^{RGE/RGE}$ mice, in which the substitution of the aspartate by a glutamate in the RGD motif of LAP precludes integrin binding. Similar to their *Tgfb1*$^{-/-}$ mice counterparts, *Tgfb1*$^{RGE/RGE}$ mice die from vascular defects during embryonic development or from multi-organ inflammation 3 weeks after birth while producing normal levels of latent TGF-β1 [44]. The general mechanism by which integrins are activated and fully exert traction forces on their ligands implies two steps: integrin activation and headpiece opening (Fig.6B). Resting integrins are generally found in a “bent-closed” and inactive conformation stabilized by a salt bridge between the α and β subunits. Activation occurs as a result of the association between the β chain intracytoplasmic tail with a cytoskeleton protein called tailin, which disrupts the salt bridge [45]. The resulting “extended-closed” integrin conformation binds the ligand with low affinity. The interaction between the ligand and a metal ion in the βI domain of the β subunit, called ADMIDAS, launch conformational changes in the

βI and hybrid domains leading to the headpiece opening, separating the legs of the α and β subunits and placing them in an optimal position to transmit the contractile force generated by the movement of the cytoskeleton to the ligand [46]. To date, integrins $\alpha_V\beta_1$, $\alpha_V\beta_6$ and $\alpha_V\beta_8$ were shown to bind and activate latent TGF-β1 *in vivo*.

Integrin $\alpha_V\beta_1$

Individually, α_V and β_1 subunits contribute to various integrin heterodimers, and are expressed in nearly every cell type. In spite of this wide pattern of expression, only a few cell types present the $\alpha_V\beta_1$ heterodimer on their surface. Sharing of α_V or β_1 subunits between multiple integrin heterodimers renders the study of the specific role(s) of $\alpha_V\beta_1$ by genetic inactivation mostly impossible. To circumvent this difficulty, Dean Sheppard's team synthetised a highly specific small-molecule inhibitor of $\alpha_V\beta_1$. Activated fibroblasts are known to express four α_V-containing integrins, namely $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, and $\alpha_V\beta_8$. Using this new inhibitor and genetic deletions of the 3 other β chains, either globally or conditionally, they found a non-redundant role of $\alpha_V\beta_1$ in TGF-β1 activation by activated fibroblasts [47].

Integrin $\alpha_V\beta_6$

Integrin $\alpha_V\beta_6$ is exclusively expressed in epithelial cells, where it is upregulated upon tissue injury or inflammation. In connection with this restricted expression pattern, *Itgb6*$^{-/-}$ mice present hair loss, accumulation of activated lymphocytes in the lungs and hyperresponsive airways, suggesting an alteration of immune homeostasis at epithelial barriers [48]. In addition to this mild inflammatory phenotype observed in skin and lungs, mice are protected against fibrosis, a condition in which TGF-β1 plays a crucial role [49].

In vitro experiments with forced expression of integrin $\alpha_V\beta_6$ showed that binding of the integrin *per se* is not sufficient to activate latent TGF-β1. Efficient activation of latent TGF-β1 by $\alpha_V\beta_6$ requires on the one hand the interaction of the cytoplasmic domain of β_6 with the actin cytoskeleton, and on the other hand the covalent anchorage of latent TGF-β1 to a tethering protein such as LTBP-1 or GARP [49-51]. These requirements suggest a model of mechanical activation of latent TGF-β1, that is also supported by the crystallographic structure of latent TGF-β1 bound to the head of integrin $\alpha_V\beta_6$ (Fig.6C). The RGD motif in LAP displays a strategical position, in a flexible and accessible loop directly connected to the force resistant arm, at the opposite side of the TGF-β1-tethering protein binding site. This RGD motif inserts into a deep pocket at the interface between the β-propeller domain of the α_V subunit and the βI domain of the β_6 subunit. The RGD motif is followed by a LXXL/I motif (LATI) that folds in an amphipathic α-helix upon binding to a hydrophobic pocket in the βI domain of the β_6 subunit. This interface is further reinforced by 3 "specificity determining loops" of the βI domain that directly interacts with LAP. This highly interdigitated interface imposes a well-defined orientation between $\alpha_V\beta_6$ and latent TGF-β1 [43]. The specific orientation and the high affinity of the LAP-integrin interaction paved the way for modelling of the mechanical activation of latent TGF-β1 by integrin $\alpha_V\beta_6$ (Fig.6D). Based on this structure, Timothy

Springer's team simulated the effects of a traction force applied by α_V or β_6 to the RGD at the LAP shoulder, in the presence of a resisting force at the N-terminal α1 helix of LAP. The traction force applied by β_6 to the RGD is transmitted through the force-resistant arms to the fastener residues that stabilize the straitjacket surrounding mature TGF-β1. Unsnapping of the strong interaction between the fastener residues of LAP leads to elongation of the latency lasso, allowing the release of mature TGF-β1 [43, 52]. Simulation of traction forces applied by α_V to RGD did not induce significant changes in LAP.

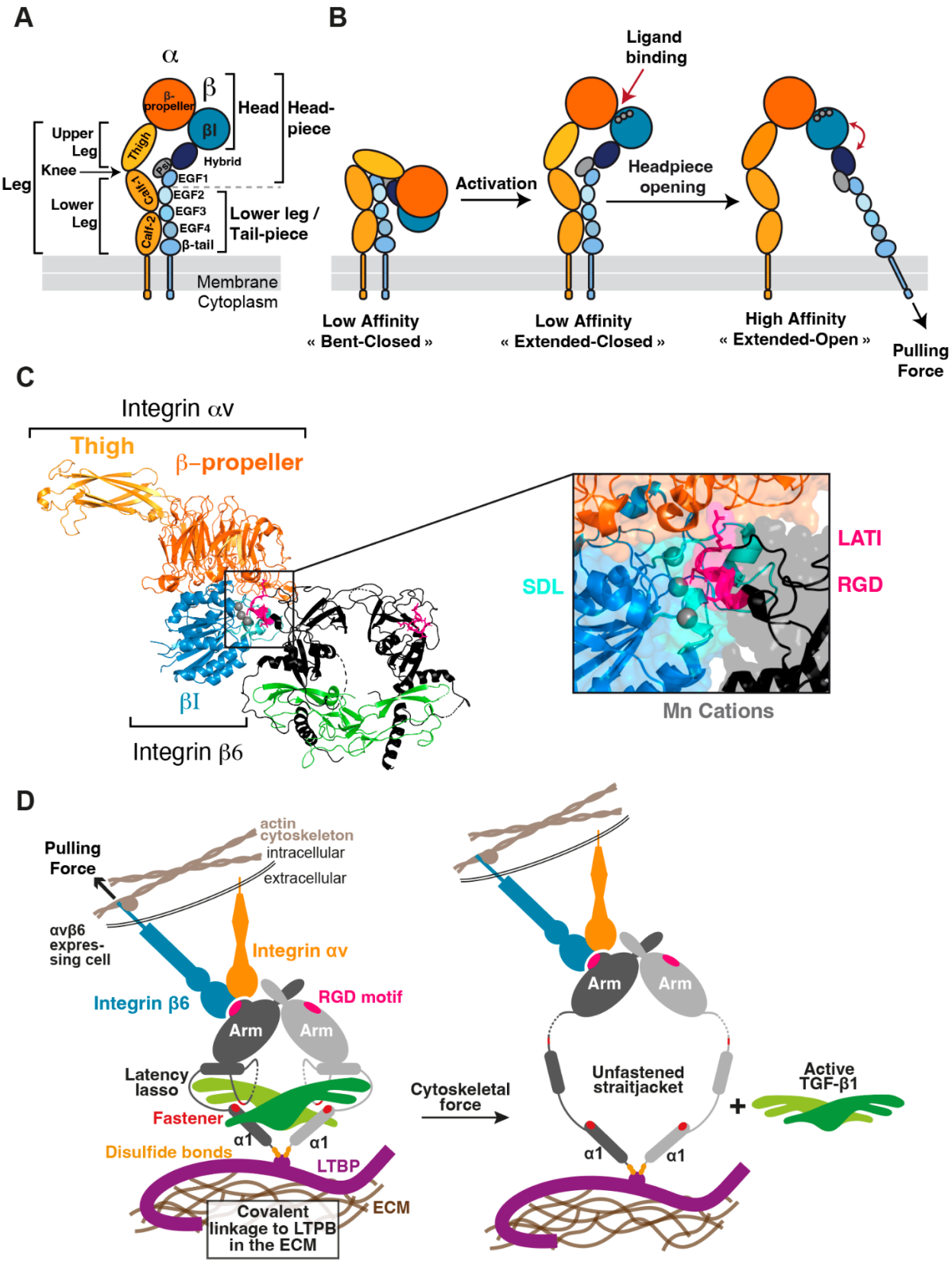

Figure 6: Model of force-driven activation of TGF-β1 mediated by integrin $\alpha_V\beta_6$.
A. Schematic representation of the subunits and domains of integrin $\alpha_V\beta_6$, describing the common nomenclature. B. "Global rearrangement" model of activation mechanisms for integrin $\alpha_V\beta_6$ illustrating the

three main stages and conformational changes. Affinity changes corresponding to movements in the headpiece are indicated by pink arrow. A and B were adapted from [53]. C. Cartoon representation of the crystal structure of *the* $\alpha_V\beta_6$ headpiece in complex with a latent TGF-β1 dimer (1:2 ; PDB codes: 5FFO) as published in [52] with emphasis on interaction interface. Specificity determining loops (SDL) from *βI domain* are represented in light blue, RGDLATI motif from LAP in magenta with sticks and metal ions as grey spheres. D. Schematic representation of mechanical activation of latent TGF-β1 by integrin $\alpha_v\beta_6$. Integrin $\alpha_v\beta_6$ binds the RGD motif in the arm domain of latent TGF-β1. Upon cytoskeleton contraction, the pulling force generated is transmitted through the arm to the straitjacket. The covalent linkage of Cys33 in LAP to LTPB, retained in the ECM, opposes a resisting force to the pulling force. These opposing forces disrupt the fastener and unfold the latency lasso, releasing active TGF-β1. Adapted from Stéphanie Liénart's thesis, based on [27, 52].

Integrin $\alpha_V\beta_8$

Integrin $\alpha_V\beta_8$ is expressed by various cell types such as epithelial cells [54], fibroblasts [55], dendritic cells (DCs) [56, 57], monocytes [58] and Tregs [59, 60]. β_8-deficient mice (*Itgb8*$^{-/-}$) die during embryonic development or at birth due to vascular defects [61], a phenotype shared with mice harboring a specific deletion of β_8 in the neuroepithelium and in glial cells [62]. However, when bred in a specific background (ICR mice), *Itgb8*$^{-/-}$ animals can reach adulthood with a median survival of 2 months. They develop neurological symptoms reminiscent of mice lacking *Itgav* in the central nervous system (CNS) [63]. Conditional deletion of the *Itgb8* gene in fibroblasts leads to pulmonary fibrosis and asthma [55, 64] while deletion in dendritic cells leads to autoimmune colitis and encephalomyelitis [56, 65, 66]. The deletion of *Itgb8* in Tregs does not result in a spontaneous phenotype, but there is evidence suggesting its involvement in the regulation of established inflammation (detailed in 1.3.3.). Altogether, these studies suggest that TGF- β1 activation by $\alpha_V\beta_8$ on the surface of various cell types contributes to immune homeostasis.

The mechanisms by which integrin $\alpha_V\beta_8$ activates latent TGF-β1 are not yet completely elucidated and remain at the core of debates. TGF-β1 activation by epithelial cell lines and monocytes requires the recruitment of the transmembrane metalloprotease MMP14 (also called MT1-MMP) to degrade the LAP [58, 67]. This proteolytic mechanism of activation is not shared by Tregs, in which integrin $\alpha_V\beta_8$-mediated activation of TGF-β1 is independent from proteases, including MMP14 [59, 68]. In Tregs, $\alpha_V\beta_8$-mediated TGF-β1 activation requires anchorage of latent TGF-β1 to the membrane through GARP [11, 50], giving further impetus to mechanical model of activation by integrin $\alpha_V\beta_8$ [33]. However, in contrast to other β subunits, β_8 does not contain talin- or kindling-binding sites required for activation of other integrin chains. Instead, it is described to bind to Band 4.1 and spinophilin that may provide link to the cytoskeleton [69], but their involvement in integrin and latent TGFβ activation has not been studied. Negative stain electron microscopy showed that $\alpha_V\beta_8$ does not adopt a "bent" conformation but rather adopts a constitutively extended conformation, thereby freeing itself from the requirement for an activation step to reach the "extended closed" state (Fig.7A) [70]. In addition, β_8 also lacks the ADMIDAS cation that is crucial for the headpiece opening. This would preclude integrin $\alpha_V\beta_8$ to adopt the « extended-open » conformation [70-72]. However, even in absence of this high affinity conformation, integrin $\alpha_V\beta_8$ has high affinity for latent TGF-β1,

which may be sufficient to transmit pulling forces. The team of Stephen Nishimura suggested a new activation model based on a cryo-EM structure of the ectodomain of one $\alpha_V\beta_8$ integrin bound to a latent TGF-β1 dimer. Major flexibility was observed in latent TGF-β1 outside the interaction site, causing a tilting motion around the $\alpha_V\beta_8$ headpiece. Similar flexibility was also observed in TGF-β1 itself and in the lower legs of integrin $\alpha_V\beta_8$. They postulate that such flexibility is unlikely to generate sufficient force to liberate active TGF-β1 from the complex. Instead, they propose that this flexibility allows the active domain of TGF-β1 to be exposed to a “minimal” TGF-β1 receptor (*i.e.* a single pair of the TβRI:TβRII heterodimer, instead of the “conventional” TβRI:TβRII heterotetramer) without complete dissociation of active TGF-β1 from LAP. This would result in a minimal TGF-β1 signaling cascade (Fig.7B) [72].

However, our colleagues from the University of Ghent (J. Felix and S. Savviddes) made an interesting observation in the published cryo-EM structure of $\alpha_V\beta_8$ integrin bound to latent TGF-β1: the helix that forms the thumb of mature TGF-β1 adopts a position similar to that observed in latent TGF-β1, rather than the position observed in active TGF-β1. As a reminder, the α1 helix of LAP causes the thumb to move away from the fingers in latent TGF-β1, the thumb adopting therefore a different position from that it occupies when the cytokine is its active state. In this latent configuration, comprising the α1 helix of LAP and this specific thumb position, it is difficult to conceive that an interaction could occur between the thumb and the TβRI receptor subunit, due to severe steric hindrance and clash. The model proposed by Nishimura and colleagues is therefore difficult to reconcile with the observed structure. Furthermore, it is important to consider that the significant structural flexibility observed by Nishimura and colleagues might result from the absence of a tethering protein such as GARP, and may not have physiological relevance.

Recent work from Duan and colleagues suggested a putatively more efficient activation mechanism, induced by binding of two $\alpha_V\beta_8$ heterodimers to one latent TGF-β1 homodimer [32]. They solved a cryo-EM structure of 2 $\alpha_V\beta_8$ headpieces in complex with one latent TGF-β1 dimer (2:2). The most drastic conformational change they observed compared to the previously reported structure was a rotation of each $\alpha_V\beta_8$ heterodimer by ~60° along its vertical axis, that would allow binding of two integrins in this restricted area. With a cell-based assay and surface plasmon resonance experiments, they highlighted a more efficient activation induced by binding of two $\alpha_V\beta_8$ integrins to one latent TGF-β1, due to higher avidity, by comparison to binding of a single $\alpha_V\beta_8$ integrin. They thus propose a model in which latent TGF-β1, presented by LRRC33 (or GARP) on one cell, is bound and activated by two $\alpha_V\beta_8$ integrins on another cell. The traction force between these two adjacent cells would be efficiently transmitted by the high avidity binding of two $\alpha_V\beta_8$ integrins to the single latent TGF-β1 strongly anchored to the other cell surface through LRRC33 (or GARP) (Fig.7C).

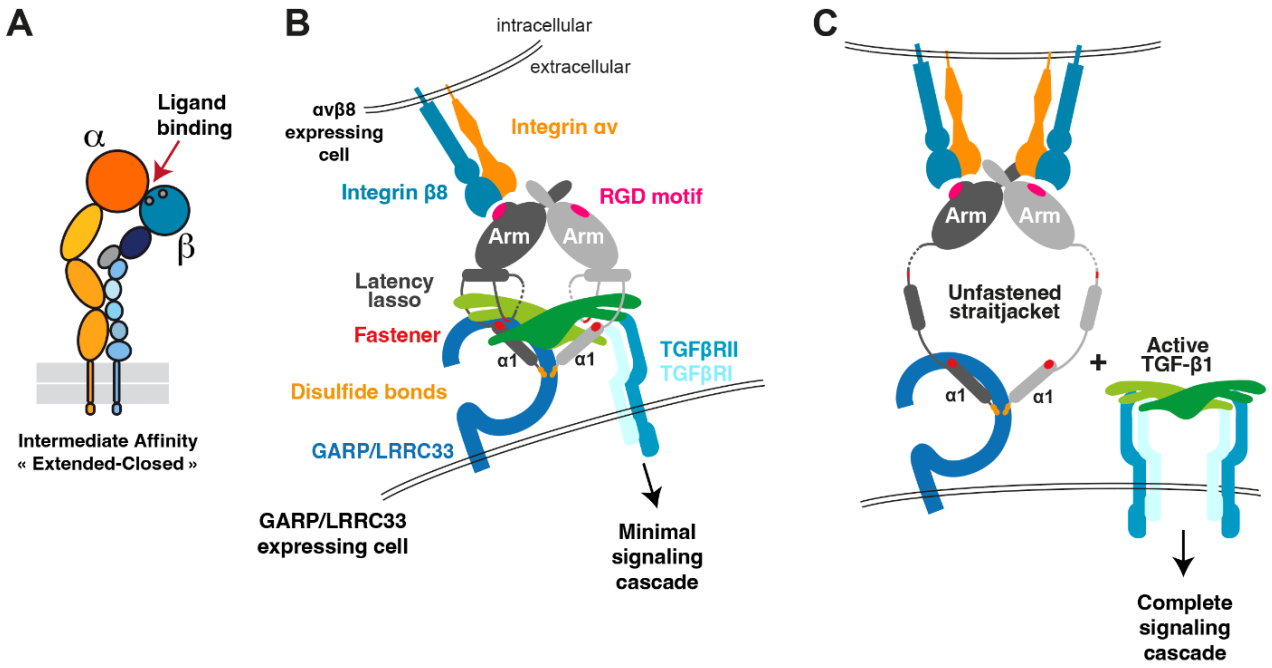

Figure 7: Models of activation of TGF-β1 mediated by integrin $\alpha_V\beta_8$ in absence of MMP-14. A. Schematic representation of the unique "extended-closed" conformation of integrin $\alpha_V\beta_8$. B. Model of a TGF-β1 activation mechanism leading to a "miminal signaling cascade", in which one single integrin $\alpha_V\beta_8$ heterodimer binds to a single RGD motif in the LAP homodimer. In this model, there is no actual release of active TGF-β1, but only minimal changes in the straightjacket allowing interaction of one side of mature TGF-β1 with one TβRI:TβRII heterodimer. C. Model of a TGF-β1 activation mechanism induced by binding of two $\alpha_V\beta_8$ heterodimers to two RGD motifs in the LAP homodimer, leading to unfastening of the straitjacket, release of active TGF-β1, and interaction with the TβRI:TβRII heterotetramer.

- **Thrombospondin-1 (TSP-1)**

Thrombospondin-1 (TSP-1) is an extracellular-matrix protein secreted by multiple cell types such as platelets and endothelial cells. Besides many TGF-β-independent functions, TSP-1 has been described to activate latent TGF-β1 as a result of non-covalent binding of its KRFK motif to the LSKL motif in helix α1 of LAP. The LSKL motif contains the key lysine residue involved in fastening of the arm region with the α1 helix [27, 73-76]. Thereby, the TSP-1_{KRFK}/LAP_{LSKL} interaction would favor release of mature TGF-β1 [34, 77]. Interaction between the TSP-1 or a KRFK-containing peptide with LAP, and subsequent TGF-β1 release were shown in biochemical *in vitro* assays. In cell-based assays, incubation of recombinant latent TGF-β1 with either TSP-1 or KRFK peptides induced release of active TGF-β1 which could be competitively inhibited in the presence of a LSKL peptide [73]. However, this activation mechanism remains somewhat controversial, as other groups failed to observe significant interactions between TSP-1 and latent TGF-β1 by Surface Plasmon Resonance, or to reproduce TGF-β1 activation *in vitro* [78, 79].

Tsp1$^{-/-}$ mice harbor epithelial hyperproliferation in several organs, such as lungs and pancreas, and anomalies of lung capillaries causing hemorrhages, *i.e.* phenotypical features shared with *Tgfb1*$^{-/-}$ mice. However, they display only low-level inflammation by comparison to the lethal autoimmune syndrome developed by *Tgfb1*$^{-/-}$ mice. Treating *Tsp1*$^{-/-}$ pups with a KRFK peptide could prevent some histological anomalies, whereas wild-type (WT) mice treated with a LSKL peptide developed lung and pancreas pathologies similar to *Tsp1*$^{-/-}$ mice. These observations support a role for TSP-1 in TGF-β1 activation, at least during development, but suggest that additional, non-redundant mechanisms of TGF-β1 activation are important to maintain immune homeostasis [80].

1.3.3. Examples of cell-type specific activation

As stated above, the mechanisms of TGF-β1 activation are cell-type specific and require cooperation between several proteins. The few exemples below illuminate this diversity.

- **Regulatory T cells, a collaboration between GARP and ITGB8**

Regulatory T cells (Tregs) are a subset of CD4$^+$ T lymphocytes playing pivotal roles in the maintenance of immunological homeostasis and the prevention of autoimmunity. They are characterized by constitutive expression of transcription factor FOXP3 and high levels of the interleukin-2 (IL-2) receptor α-chain (CD25). Foxp3 serves as a lineage specification factor of Treg cells, as loss-of-function mutations of the *FOXP3* gene is responsible for fatal autoimmune disorder in mice and men [81]. Two main subsets of Tregs can be distinguished based on the anatomical site and the cells from which they differentiate: thymic Tregs (tTregs) differentiate from hematopoietic T cell progenitors in the thymus, whereas peripheral Tregs (pTregs) differentiate from naïve CD4$^+$ T cells in secondary lymphoid organs in the periphery. Tregs exert immunosuppression via multiple mechanisms, which are still under study and are not all completely resolved. The most documented mechanisms, identified via research on rodent Tregs, comprise high CTLA-4 expression, adenosine production, cytolysis by perforin and granzyme, IL-2 consumption and production of immunosuppressive cytokines such as IL-10, IL-35 and TGF-β1 [82].

To investigate the immunosuppressive mechanisms used by human Tregs, our group derived clones of human Tregs, identifying them as Tregs based on the presence of a stable epigenetic mark, corresponding to the demethylation of a regulatory intronic region of gene *FOXP3*. In human cells, this mark is strictly specific of fully differentiated Tregs: it is present in Tregs, but not in activated CD4$^+$ or CD8$^+$ effector T cells, in contrast to *FOXP3* expression itself [83-85]. The human Treg clones stably expressed high *FOXP3* levels, displayed suppressive properties *in vitro*, and provided a unique and pure cell population to study human Treg function. By comparing the transcriptome of human Tregs to that of other human T cells, we discovered that human Tregs produced active TGF-β1 in response to TCR stimulation, a feature which distinguishes them from all other human CD8$^+$ and CD4$^+$ T cells T cells [86, 87]. Active TGF-β1 produced by TCR-stimulated

Tregs acts in autocrine and paracrine fashion on neighbouring cells, but only if Treg-to-target cell contacts were allowed. This suggests that TGF-β1 is activated very close to the Treg surface upon TCR stimulation. Furthermore, our group and others demonstrated that in response to TCR stimulation, human Tregs present latent TGF-β1 on their surface through disulfide-linkage of LAP to GARP [10-13, 88, 89]. To investigate if GARP is required for TGF-β1 activation and the immunosuppressive functions of human Tregs, our laboratory developed anti-h(uman) GARP monoclonal antibodies (mAbs).

Two approaches were employed to generate mAbs against hGARP. First, hybridoma clones were derived from mice immunized with murine cells expressing hGARP. Second, in collaboration with a biotech company called argenx, complementary DNA (cDNA) libraries encoding immunoglobulin light and heavy chain variable regions were prepared from llamas immunized with murine cells expressing hGARP and hTGFB1 or with plasmids encoding these molecules. cDNA libraries were selected for their ability to bind to recombinant hGARP:hTGF-β1 complexes by phage display, and Fab-coding regions obtained from the selected clones were subcloned into a human immunoglobulin G1 (IgG1) backbone. Among the 31 mAbs that we generated, two turned out to block production of active TGF-β1 by human Tregs *in vitro* [17]. By solving the crystal structure of human GARP:TGF-β1 bound to a Fab derived from one of the blocking anti-GARP:TGF-β1 mAbs, we discovered that the antibody recognizes a mixed conformational epitope comprising amino acids from LAP, mature TGF-β1 and GARP [33]. Based on these findings, we hypothesized that the blocking anti-GARP:TGF-β1 mAbs act as a “glue”, preventing the elongation of the latency lasso and thereby impeding the activation process. The blocking mAbs did not recognize mouse GARP. We thus evaluated their ability to block immunosuppression by human Tregs *in vivo* in a model of xenogeneic graft-versus-host disease (xGvHD) induced by transfer of human peripheral blood mononuclear cells (PBMCs) into immunodeficient NSG mice. In this model, co-transfer of autologous Tregs attenuates the severity of xGvHD by suppressing xenogeneic reactive donor T cells. This protection was abrogated by administration of a blocking anti-GARP:TGF-β1 antibody, demonstrating that the production of active TGF-β1 by GARP-expressing human Tregs is essential for their immunosuppressive function in the context of GvHD [17].

In parallel, antibodies were developed to specifically block the production of active TGF-β1 from mouse GARP:TGF-β1 complexes *in vitro*. These antibodies were demonstrated to effectively inhibit the immunosuppressive function of mouse Tregs *in vivo*, in several murine tumor models. First, our group showed that the combined blockade of GARP:TGF-β1 and PD-1 leads to regression of solid transplanted tumors that are resistant to anti-PD-1 immunotherapy alone [16, 90]. Second, reduction in tumor burden was also observed in a murine model of blood cancers, this time with anti-GARP:TGF-β1 alone [91]. The model used here was a model of primary myelofibrosis (PMF), a myeloproliferative neoplasm characterized by abnormal cytokine production and bone marrow fibrosis. The therapeutic benefits observed with anti-GARP:TGF-β1 mAbs in studies with solid and liquid murine cancers were attributed to targeting GARP on Tregs, rather than on B cells, platelets

or megakaryocytes. In these studies, targeting of mouse Tregs enhanced $CD8^+$ T-cell-mediated anti-tumor immune responses, indicating that the production of active TGF-β1 by GARP-expressing Tregs also contributes to Treg immunosuppression in tumor-bearing individuals.

Not all immunosuppressive functions of Tregs are dependent on GARP and active TGF-β production. *In vitro*, Garp-deficient Tregs are still able to suppress the proliferation or cytokine production of effector T cells as efficiently as WT Tregs [92, 93] even though they produce reduced amounts of active TGF-β1 (unpublished data from our group). Mice harboring a deletion of *Garp* specifically in Tregs (*Foxp3*^{cre} x *Lrrc32*$^{fl/fl}$ mice) do not show an overt auto-immune phenotype early after birth (4-6 weeks of age), but older mice develop spontaneous intestinal inflammation and show increased susceptibility to pristan-induced lupus. In addition, Garp-deficient Tregs lose the ability to control DSS-induced colitis, or colitis induced by the transfer of WT naïve $CD4^+$ T cells in immunodeficient mice (*Rag2* $^{-/-}$) [92]. Similar results were obtained with mice bearing a deletion of an enhancer region required for GARP expression in Tregs and containing high risk variants for autoimmune and allergic diseases in humans [94]. Altogether, these studies highlight that GARP-mediated activation of TGF-β1 by Tregs may be dispensable to maintain immune homeostasis under basal conditions early in life, but could play a role in controlling various immune-related pathologies including inflammatory and/or auto-immune pathologies and cancer.

Similar studies were conducted to decipher the role of integrin $\alpha_V\beta_8$ in TGF-β1 activation by Tregs. *In vitro,* Tregs with a deletion of the gene encoding the β_8 chain (*Itgb8*) do not produce active TGF-β1 but exhibit normal suppressive activity on co-cultured cells [68]. *Foxp3*$^{YFP\text{-}cre}$x*Itgb8*$^{fl/fl}$ mice do not develop signs of spontaneous autoimmunity/inflammation, and adoptively-transferred *Itgb8*$^{-/-}$ Tregs could control colitis induced by transfer of naïve T cells when transferred at the same time as the naïve T cells. In contrast, this control was lost when *Itgb8*$^{-/-}$ Tregs were transferred two weeks after the naïve T cells, *i.e.* when inflammation was already installed, whereas WT Tregs prevented colitis efficiently [60]. Our group demonstrated that $\alpha_V\beta_8$ dimers are expressed on human Tregs upon stimulation of their TCR. By using blocking antibodies targeting either α_V or β_8, we confirmed that $\alpha_V\beta_8$ is required for TGF-β1 activation by human Tregs *in vitro*. Using co-immunoprecipitation techniques, we were able to establish a physical interaction between the integrin $\alpha_V\beta_8$ and GARP:TGF-β1 complexes, highlighting the collaborative relationship required for TGF-β1 activation at the surface of human Tregs. Finally, in the xenogeneic GvHD model, treatment with a blocking anti-β8 antibody completely abrogated the protective effect of human Tregs in NSG mice [59]. In line with the results observed for GARP, $\alpha_V\beta_8$ is thus required for TGF-β1 activation and immunosuppression by human and mouse Tregs, at least in conditions where inflammation is ongoing.

- **Platelets**

Platelets are anuclear cellular fragments released into the bloodstream after being shed from megakaryocytes in the bone marrow vasculature. They play a crucial role in various physiological

processes such as hemostasis, wound healing, angiogenesis, inflammation and immunity. Upon activation, platelets perform numerous functions by releasing the content of their α-granules, which contain various molecules including latent TGF-β1. Despite being a major source of latent TGF-β1 in the bloodstream [95, 96], the mechanisms of TGF-β1 activation by platelets and megakaryocytes are still under investigation and not fully elucidated.

TGF-β1 activation by platelets requires the activation of platelets themselves. Platelet activation can be triggered *in vitro* by incubation with recombinant thrombin or by blood coagulation, leading to rapid degranulation and the release of large amounts of latent TGF-β1. Since the purification of TGF-β1 from human platelets in 1988 [95], it has been understood that latent TGF-β1 stored in α-granules is covalently bound to LTBP-1. More recently, GARP:TGF-β1 complexes were also identified at the surface of activated platelets [10, 91, 97]. Platelets can also release active TGF-β1. GARP is required for TGF-β1 activation by platelets during blood coagulation *in vitro*, as platelets isolated from mice lacking GARP in the megakaryocytic lineage (*Pf4*^{Cre} x *Garp*$^{fl/fl}$) were unable to activate TGF-β1 during blood coagulation [91, 97]. In addition, when platelets from WT animals were incubated with a blocking anti-GARP:TGF-β1 mAb, they also failed to activate TGF-β1 during blood coagulation [91]. Analyses of *Pf4*^{Cre} x *Garp*$^{fl/fl}$ mice revealed that GARP expression by platelets was not necessary for hemostasis or thrombosis [15]. The group of Zihai Li reported that similar mice (*Pf4*^{Cre} x *Garp*$^{fl/fl}$ mice) experienced reduced tumor growth compared to WT controls, accompanied by enhanced T cell-mediated anti-tumor immune responses. This suggests that TGF-β1 activation via GARP by platelets may represent a significant immunosuppressive mechanism in the tumor microenvironment [97]. Nevertheless, our group did not observe tumor growth reduction in *Pf4*^{Cre} x *Garp*$^{fl/fl}$ mice [16, 91].

In addition to the involvement of tethering proteins, potential other physical (“mechanical”) and chemical mechanisms have been suggested to mediate TGF-β1 activation. Since platelet-derived latent TGF-β1 is present in the circulation, intravascular shear force was initially proposed as a traction force contributing to TGF-β1 activation in the circulation [98]. Shear-induced activation of latent TGF-β1 nevertheless necessitates covalent binding to a membrane- or ECM- tethering protein, as evidenced by the lack of active TGF-β1 release in stirring experiments conducted *in vitro* with platelets isolated from *Tgfb1*^{C33S} mutant mice. This requirement for molecular anchorage of latent TGF-β1 further supports the mechanical mode of activation. Due to the presence of TSP-1 in platelet releasates, TSP-1 was suggested to activate latent TGF-β1 after both proteins are secreted upon degranulation. However, platelets from *Tsp1*$^{-/-}$ mice exposed to thrombin *in vitro* produced as much active TGF-β1 as WT platelets [99]. In addition, the use of a TSP-1 inhibitor did not affect TGF-β1 activation by human or mouse platelets [100], further confirming that TSP-1 does not play a role in TGF-β1 activation by platelets. Finally, thrombin, a protease produced during blood clotting and known to induce platelet activation, was recently proposed as a partner in GARP-mediated TGF-β1 activation [101]. The authors found that exposure of platelets to recombinant thrombin *in vitro* resulted in GARP cleavage and release of active TGF-β1. GARP contains two

thrombin cleavage sites, and mutation of these sites or use of a GARP peptide containing these sites reduced GARP cleavage and active TGF-β1 production. The authors propose that thrombin-mediated GARP cleavage destabilizes the GARP:TGF-β1 complex, leading to TGF-β1 activation. However, it should be noted that the very high concentrations of thrombin used *in vitro* in this study may not be relevant *in vivo*. Therefore, further investigations are needed to confirm the relevance of this thrombin-dependent mechanism of activation *in vivo*. Additionally, while blocking anti-GARP:TGF-β1 mAbs inhibit TGF-β1 activation during blood coagulation, they fail to impede TGF-β1 activation following exposure of platelets to thrombin (Lecomte S., unublished results). A possible explanation could be that the predicted thrombin cleavage sites in GARP are located on the opposite side of the GARP horseshoe structure, relatively to the amino-acids targeted by the blocking anti-human GARP:TGF-β1 mAb.

- **Other GARP expressing cells**

Similarly to Tregs, our group has demonstrated that B cell receptor (BCR)-stimulated B cells express GARP:TGF-β1 complexes on their surface and can activate TGF-β1, which plays a role in IgA class switch *in vitro*. However, when examining mice with a specific deletion of *Garp* in B cells (*Mb1*^{Cre} x *Garp*$^{fl/fl}$), we did not observe reduced antigen-specific IgA production in response to intestinal bacterial infection by *Citrobacter rodentium,* or after immunization with proteins in adjuvant [102]. This suggests that the active TGF-β1 required for IgA switching does not originate from B cells themselves under these conditions. Additionally, another group suggested that GARP-dependent TGF-β1 production by B cells may also be necessary for the maintenance of immune tolerance and the induction of oral tolerance to T cell-dependent antigens [103]. BCR-stimulated B cells have not been found to express any RGD-binding integrins so far. Therefore, activation is more likely to occur *in trans*, by interaction with integrin-expressing cells, such as subepithelial dendritic cells in Peyer's patches, which express integrin $\alpha_V\beta_8$ [14].

Finally, hepatic stellate cells, mesenchymal stem cells, and liver sinusoid endothelial cells were also found to express GARP. Hepatic stellate cells are liver cells from mesenchymal origin that suppress T cells in a TGF-β1 dependent manner. Genetic inhibition of *GARP* expression in these cells also impairs their ability to suppress T cells *in vitro*, indicating that GARP is required for their activation of TGF-β1 [20]. Similarly, mesenchymal stem cells (MSCs) are suggested to possess immunomodulatory functions through GARP-mediated TGF-β1 activity, as silencing of GARP reduces the ability of mouse MSCs to inhibit T cell proliferation *in vitro [18, 104]*. Additionally, GARP-expressing mouse liver sinusoid endothelial cells have been shown to induce the differentiation of naïve T cells into Treg cells in co-culture assays, suggesting that GARP may contribute to TGF-β1 activation by endothelial cells [105]. However, we failed to detect active TGF-β1 production in GARP-expressing mouse or human endothelial cell lines. The physiological significance of GARP-mediated TGF-β1 activation by these three cell types remains an ongoing area of investigation.

- **Macrophages and LRRC33**

LRRC33, the closest relative of GARP, has been more recently studied for its involvement in TGF-β1 activation, particularly in the monocyte-macrophage lineage [22, 23, 106]. *Lrrc33*$^{-/-}$ mice initially appear normal up to 2 months of age but then develop ascending paraparesis, associated with demyelination and neuron loss in motor-associated brain regions, leading to death by 5 months [22]. LRRC33 is expressed by microglia, a macrophagic subpopulation of the CNS, and its absence in *Lrrc33*$^{-/-}$ mice results in a hyperreactive phenotype. Whole bone marrow transplantation and subsequent repopulation of *Lrrc33*$^{-/-}$ brains by WT microglia halts disease progression. Notably, latent TGF-β1 was absent from the surface of macrophages in *Lrrc33*$^{-/-}$ mice, while still present intracellularly, and Gene Set Enrichment Analysis performed on *Lrrc33*$^{-/-}$ and wt microglia revealed that the TGF-β signaling pathway was the most affected. Altogether, these findings strongly suggest a non-redundant role of LRRC33 in TGF-β1 activation by microglial cells. Additionally, the authors proposed astrocytes as a cellular source providing integrin $\alpha_V\beta_8$ as a partner for TGF-β1 activation.

But the role of LRRC33 extends beyond the microglia and macrophages. The same group observed slowed tumor growth and metastasis in *Lrrc33*$^{-/-}$ mice [106]. In the tumor microenvironment, LRRC33 deficiency led to the absence of surface expression of TGF-β1 on tumor-associated myeloid cells, including macrophages, DCs, and MDSCs. This deficiency resulted in a distinct immune phenotype, characterized by increased infiltration of $CD8^+$ T cells and NK cells, a shift in tumor-associated macrophages toward a tumor-suppressive "M1" phenotype, and decreased infiltration of MDSCs. However, Lrrc33$^{-/-}$ mice treated with a blocking antibody targeting TGF-β1 and TGF-β3 experienced even stronger inhibition of tumor growth, suggesting that LRRC33:TGF-β1 is not the sole source of TGF-β contributing to tumor growth. Additionally, two human acute myeloid leukemia (AML) cell lines were also shown to express high levels of LRRC33 and activate TGF-β1 in an integrin α_V–dependent manner in an *in vitro* co-culture assay [23]. Altogether, these data point out LRRC33 as a new therapeutic target in immune-oncology.

1.4. Signaling

Once activated, TGF-β1 bind to a ubiquitously expressed hetero-tetrameric receptor composed of dimers of the transmembrane serine/threonine kinases TβRI (also known as ALK-5) and TβRII (Fig. 8). As mentioned above, mature TGF-β1 binds first with high affinity to a pre-formed TβRII dimer, which then recruits TβRI. Upon formation of the tetrameric complex, the constitutively active TβRII phosphorylates and activates TβRI, which in turn phosphorylates cytosolic SMAD2 and SMAD3 transcription factors. Phosphorylated SMAD2 and SMAD3 (pSMAD2 and pSMAD3) then form heterotrimeric complexes with SMAD4, which translocate to the nucleus to induce or repress gene expression. Genes induced by TGF-β1 signaling include the inhibitory SMAD7, that modulates the intensity and duration of TGF-β signals in a negative feedback loop. The SMAD2/3 pathway is often qualified as the “canonical” TGF-β signaling pathway. An alternative pathway implying SMADs 1, 5

and 8 has also been described in cells expressing ALK1, such as endothelial cells. Finally, “non-canonical” TGF-β signaling include PI3K-Akt, MAPK or Rho GTPase pathways [107]. It is important to note that the two TβRI:TβRII heterodimers have been suggested to be able to bind and signal autonomously, as shown by a form of TGF-β3 engineered to bind only one heterodimer, which retained one-quarter to one-half of the signalling activity. As explained above, Nishimura and colleagues have suggested that this “minimal signaling cascade” could be induced by asymetric binding of one side of a mature TGF-β1 dimer to a single TβRI:TβRII heterodimer, which could occur when a single integrin $\alpha_V\beta_8$ binds to a latent TGF-β1 homodimer presented by GARP or by LRRC33 ([53] and Fig. 7B). SMAD complexes bind DNA with low affinity and need to associate with various cofactors to regulate gene expression. The identity of the SMAD co-factors expressed in a given cell type or context defines the identity of the genes that will be regulated in response to TGF-β signals. All this diversity in signal transduction results in a vast diversity of TGF-β1 target genes that contributes to the variety of cell type- and context-specific effects of TGF-β. Describing all TGF-β1 effects is beyond the scope of this thesis, which will rather focus on TGF-β1 effects in immunity and fibrosis.

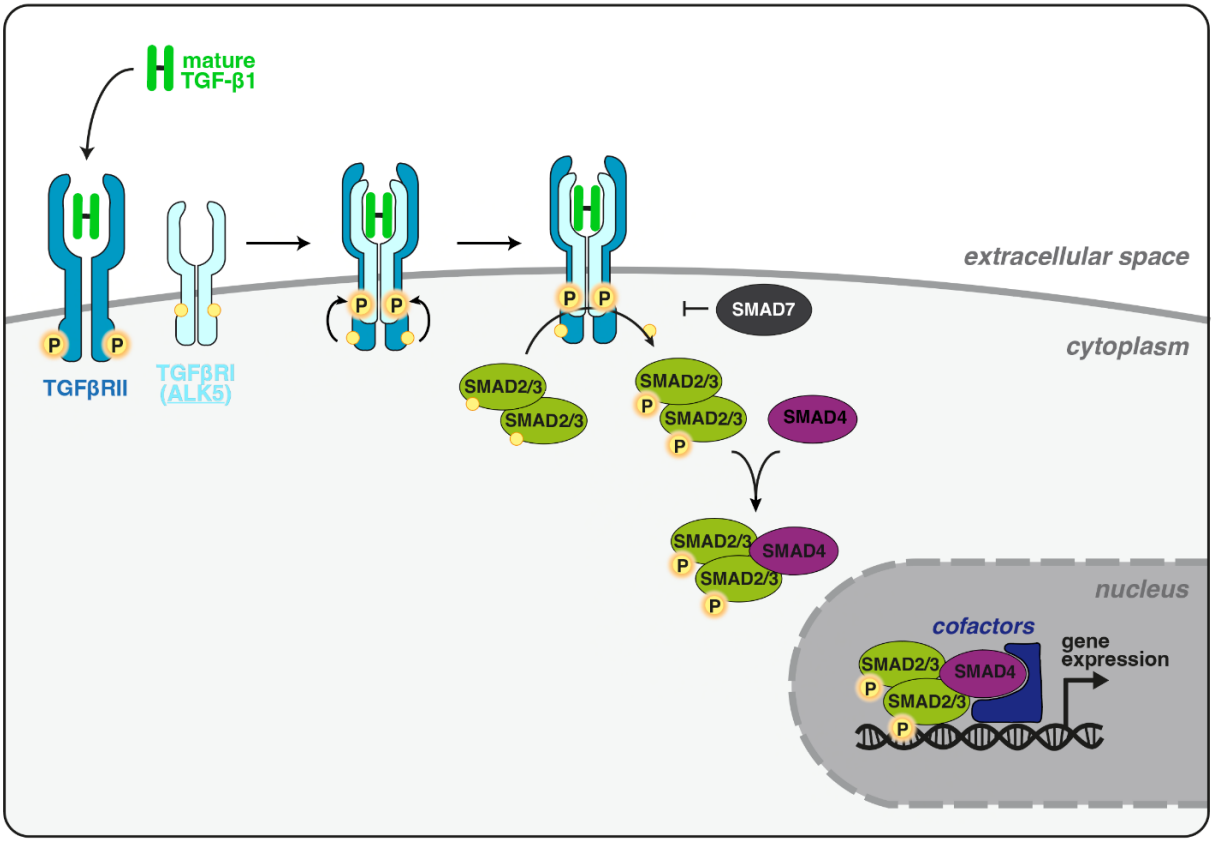

Figure 8: Canonic signaling cascade of TGF-β1. Binding of active TGF-β1 to TβRII triggers the recruitment and phosphorylation of TβRI which in turn phosphorylates SMAD2/3 cytosolic transcription factors. pSmad2/3 associates with SMAD4 to form a trimeric complex that translocates to the nucleus and associates with co-factors to induce or repress the transcription of a variety of genes.

2. TGF-β1 in immunity and diseases associated with immune dysfunctions

2.1. Effects of TGF-β1 on immune cells

Immunity is the ability of an organism to resist a disease caused by an infectious pathogen or by transformed cells while maintaining integrity of self. The immune system is composed of specialized circulating cells working in a highly coordinated fashion, notably through the secretion of cytokines. Immune responses are organized in two main stages that are closely intertwined. A first line of defence, already functional at birth, is triggered immediately upon any infection and is called “innate” immunity. A second line of defence builds up in response to exposure to specific antigens during life, and is called “adaptative” immunity. TGF-β1 controls most aspects of innate and adaptative immunity, by regulating the proliferation, differentiation, activation and functions of virtually all immune cells (Fig.9). The multiorgan, self-destructive inflammation observed early in life in *Tgfb1*$^{-/-}$ mice highlights the major contribution of this cytokine to immune tolerance.

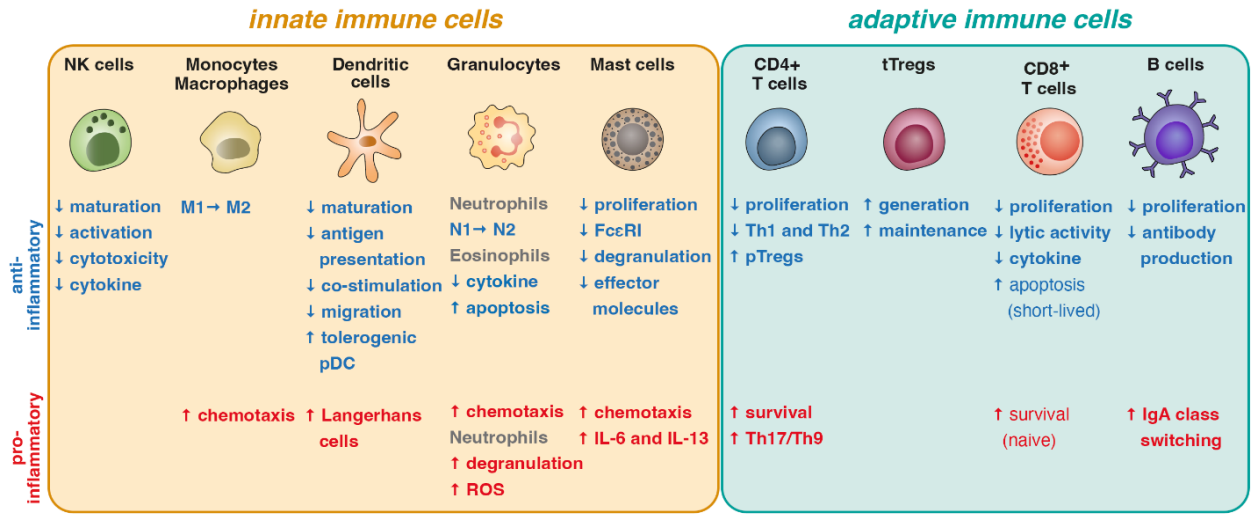

Figure 9: TGF-β1 effects on immune cell subsets. TGF-β1 regulates homeostasis, functions and differentiation of most innate and adaptative immune cells. For each cell type, anti-inflammatory effects of TGF-β1 are highlighted in blue, pro-inflammatory effects in red.

2.1.1. Innate immune cells

- **NK cells**

NK cells are innate lymphoid cells specialized in immune surveillance against tumoral and virus-infected cells. Recognition of target cells by NK cells is not specific of antigens, but rather relies on the balance between signals from activating and inhibitory NK receptors. Once activated, NK cells are capable of cytolysis by degranulation and release of perforin/granzyme, and produce proinflammatory cytokines and chemokines, among which INF-γ. TGF-β1 signals exert a general

inhibitory effect on NK cell maturation, activation and functions. Repression of NK cell maturation by TGF-β1 was demonstrated using mice lacking TGF-β signaling in innate cells. These mice express a dominant-negative TGF-β receptor under the control of a *CD11c* promoter (*CD11c*$^{dnT\beta RII}$ mice). They display a large pool of mature NK cells in the periphery and a reduced susceptibility to viral infection early after birth [108]. Several studies highlighted decreased expression of NKG2D (and NKp30) activating receptors on the surface of NK cells in response to TGF-β1. Finally, NK cytolytic functions and INF-γ production are also inhibited by TGF-β1 [109, 110].

- **Monocytes and macrophages**

Monocytes are produced in the bone marrow and circulate in the blood before going to tissues to differentiate into macrophages. Macrophages are a heterogeneous population often divided in an over simplistic way into pro-inflammatory M1 macrophages, activated by TLR ligands and inflammatory cytokines, and anti-inflammatory M2 macrophages, activated by IL-4 or IL-13. M1 macrophages clear pathogens and debris, whereas M2-macrophages are involved in the resolution phase of inflammation, favoring repair of damaged tissues and downregulating immune responses. TGF-β1 has a chemoattractant effect on monocytes, but not on intestinal macrophages. In the intestine particularly, TGF-β1 may induce trafficking of monocytes to the sites of inflammation and promote their differentiation into non-inflammatory macrophages that reside in the tissue [111]. TGF-β1 inhibits pro-inflammatory M1 macrophages via suppression of TLR signaling, a major pathway of microbial recognition. This feature is particularly manifest in mice lacking TGF-β signaling in monocytes and macrophages cells (*CD68*$^{dnT\beta RII}$). Macrophages from these mice are not sensitive to the TGF-β1-mediated inhibition of cytokine production upon TLR activation and are more susceptible to dextran sulfate sodium (DSS)-induced colitis. This highlights a key role of TGF-β1 in controling monocytes and macrophages and regulating intestinal inflammation [112].

- **Dendritic cells**

Dendritic cells (DCs) are professional antigen presenting cells that make the main bridge between innate and adaptative immunity. They initiate adaptative immune responses by presenting antigens to CD4 T cells on major histocompatibility class (MHC)-II molecules together with co-stimulatory signals (CD80 and CD86). DCs can present antigens in an immunogenic or tolerogenic manner, and thus modulate T cell activation, notably through cytokine secretion. This puts them in the foreground for the maintenance of central and peripheral tolerance under steady-state conditions. DCs are a heterogenous population widely distributed within tissues. Binding of the pattern recognition receptors (PRRs) by a pathogen associated molecular pattern (PAMP) induces the migration of the immature DC from the periphery to the tissue-draining secondary lymphoid organs, where they will encounter and activate T cells. During the migration process, DCs undergo maturation through processing of antigens, upregulation of MHC and costimulatory molecules, and formation of dendrites.

In vitro experiments revealed an effect of TGF-β1 signaling on virtually all these aspects of DC maturation. Depending on the context, TGF-β1 can promote or inhibit DC migration by regulating expression of chemokine receptors. It can also impair antigen presentation by differentiated DCs, by reducing expression of MHC-II and co-stimulatory molecules. In plasmacytoid DCs (pDCs), TGF-β1 induces expression of the immunosuppressive enzyme indoleamine 2,3 dioxygenase (IDO) thus resulting in a tolerogenic phenotype of pDCs. Taken together, TGF-β1 would thus favor immature and tolerogenic phenotypes that induce hyporesponsive T cells [110, 113]. However, not all these effects could be confirmed in mice lacking TGF-β signaling in DCs (*CD11c*^{Cre} *Tgfbr2*$^{fl/fl}$), which showed similar expression of MHC-II and co-stimulatory molecules than their WT counterparts. Interestingly, these mice display an alteration of Treg homeostasis and die from a systemic autoimmune disorder, showing a clear link between TGF-β signaling in the myeloid lineage and Tregs [114]. Similarly, partially reduced TGF-β signaling in the myeloid lineage (*CD11c*^{dnTβRII} mice) results in the development of autoimmune encephalomyelitis [115]. Several studies described a subset of mucosal CD103^{+} DC population that promotes tolerance by inducing pTreg differentiation from naïve CD4 T cells [116, 117], likely via integrin-mediated activation of TGF-β1 [57, 118]. Finally, *Tgfb1*$^{-/-}$ mice lack Langerhans cells, a specialized DC population localized in the epithelia, suggesting that TGF-β1 is required for differentiation of this specific DC population [119].

- **Granulocytes**

Granulocytes, also known as polymorphonuclear leukocytes, are characterized by the presence of dense granules in their cytoplasm that allows to classify them into neutrophils, eosinophils, and basophils. TGF-β1 effects on these cells remain poorly understood. TGF-β1 was first described to induce chemotaxis of both neutrophils and eosinophils and stimulate degranulation and the production of ROS by neutrophils under some conditions. Others described an inhibition of cytokine production and increased apoptosis of human eosinophils in response to TGF-β1 signals. Finally, TGF-β1 would lead the differentiation of neutrophils towards the “N2” anti-inflammatory phenotype, to the detriment of “N1” pro-inflammatory neutrophils [110].

- **Mast cells**

Mast cells play a key role in allergy responses but also in wound healing, tissue repair, and infections. Activation of mast cells arises upon binding of an antigen that crosslinks IgE molecules bound to FcεRI receptors, resulting in degranulation and release of inflammatory mediators such as IL-6 and IL-13. As for granulocytes, TGF-β1 induces chemotaxis and suppresses or promotes mast cell functions. It inhibits proliferation, FcεRI expression, degranulation, production of several effector molecules [110] and IL-33 mediated activation [120]. In contrast to this broad inhibition of mast cell activation, exogenous TGF-β1 added to murine mast cells in culture promotes IL-6 expression (by resting mast cells) and IL-13 production (upon IgE-mediated activation) [121, 122].

2.1.2. Adaptative immune cells

The adaptative immune system develops antigen-specific responses that evolve over the course of a lifetime thanks to immunological memory. It is composed of two major cell types: T lymphocytes and B lymphocytes.

- **T lymphocytes**

T cells comprise helper $CD4^+$ and cytolytic $CD8^+$ T cells that recognize peptide antigens presented by MHC-II or MHC-I molecules, respectively. They require IL-2 for their proliferation. The lethal multi-organ inflammation displayed by TGF-β1 knockout (KO) mice is fully recapitulated in mice with a conditional deletion of the *Tgfbr2* gene in all T cells (*CD4*^{Cre} x *Tfgbr2*$^{fl/fl}$ mice) [123]. Moreover, the lethal phenotype was abrogated by crossing *Tgfb1*$^{-/-}$ mice with mice lacking MHC-II and thus $CD4^+$ T cells, or lacking β2 microglobulin and thus MHC-I and $CD8^+$ T cells [124, 125]. Together, these mouse models demonstrate the central, key role played by TGF-β1 in controlling T cell mediated inflammation.

T lymphocytes differentiate from hematopoietic progenitors in the thymus, with each T cell clone acquiring the ability to express a functional and unique antigenic T cell receptor (TCR) thanks to recombination of gene segments in the *TCRA* and *TCRB* genes. So-called "naïve" $CD4^+$ and $CD8^+$ T cells then egress from the thymus (a primary lymphoid organ) and circulate in the periphery. When first exposed to their cognate antigen in secondary lymphoid organs, naïve $CD4^+$ and $CD8^+$ T cells will differentiate into various types of effector T cells, which include $CD4^+$ T helper lymphocytes (Th1, Th2, Th9 and Th17), $CD8^+$ cytotoxic T lymphocytes (CTLs), and $CD4^+$ pTregs. All these subsets are regulated by TGF-β1, whether for their differentiation, homeostasis, or effector functions.

Thymic development

T cells precursors are produced in the bone marrow and transit to the thymus (and hence are called "thymocytes"). There, they undergo maturation and selection before reaching the periphery. During maturation, T cell progenitors begin expressing an αβ TCR generated by random recombination of *TCRA and TCRB* gene segments, as well as $CD4^+$ and $CD8^+$ co-receptors. These double-positive $CD4^+CD8^+$ T cells subsequently undergo positive and negative selection. Positive selection retains T cells with TCRs that have sufficient affinity for self-peptide:MHC complexes to induce signals required for survival and maturation, while negative selection eliminates cells harboring TCRs with excessively high affinity for self-peptide:MHC complexes. Negative selection forms the core of central tolerance. After positive selection, double-positive T cells lose one of the co-receptors and reach the periphery as single-positive naïve $CD4^+$ or $CD8^+$ T cells.

TGF-β1 signaling in double-positive thymocytes induces IL-7Rα expression. IL-7 is a key cytokine that promotes the survival, proliferation and differentiation of immature thymocytes [126]. Intrathymic TGF-β1 signaling also sustains the development of regulatory cells, including tTreg. The generation of tTreg occurs after CD4 single-positive lineage specification and requires TCR, CD28 co-stimulation and cytokines. It was previously believed that the high-affinity TCR signal of self-reactive T cells that escaped negative selection was the main driver for inducing Treg cell differentiation. However, subsequent studies have shown that tTreg can derive from developing $CD4^+$ thymocytes with a broad range of self-reactivity [127, 128], challenging the notion that TCR signal alone is the determining factor for tTreg cell generation and giving way to a greater role for cytokines. The precise role played by TGF-β1 in commitment to the tTreg lineage remains debated. Initially, TGF-β1 was considered dispensable for tTreg generation because *Tgfb1*$^{-/-}$ and *CD4*^{Cre}x*Tgfbr2*$^{fl/fl}$ mice exhibit a normal tTreg frequency in the thymus at ~ 10 days of age, even though they show reduced frequencies of Tregs in the periphery [123, 129, 130].

It was then observed that *CD4*^{Cre}x*Tgfbr2*$^{fl/fl}$ neonates aged 3 to 5 days exhibited a transient lack of tTreg in their thymus [131]. Similarly, mice with a conditional deletion of *Tgfbr1* in T cells (*Lck*^{Cre}x*Tgfbr1*$^{fl/fl}$) also displayed lethal inflammation accompanied by a temporary deficiency in Tregs during the same neonatal period (3 to 5 days) [132]. These studies indicate that TGF-β1 signaling play a role in tTreg development during this period critical for the initial generation of tTreg cells in wt mice. The early decrease in tTreg was associated with an imbalance in the expression of anti- and pro-apoptotic proteins, resulting in an increased tTreg apoptosis [131, 132]. However, this early deficiency in tTreg was later recovered around 10 days of age, leading to an even higher tTreg population than in control mice by 3-4 weeks of age, while the systemic inflammatory syndrome develops. This restoration was attributed to a long-lasting enhancement in activation and proliferation of tTregs, overcoming the early transient increase in apoptosis. Finally, it is also postulated that the lack of TGF-β signaling during thymic Treg development could result in a reduced diversity in the Treg repertoire in the periphery [133].

Homeostasis

In the periphery, TGF-β1 regulates T cell homeostasis by regulating their proliferation, survival and apoptosis. TGF-β1 has been shown to inhibit $CD4^+$ and $CD8^+$ T cell proliferation during T cell priming, particularly by suppressing the transcription of the IL-2 gene and its production. It also affects the cell cycle by inhibiting the expression of various cell-cycle regulators and upregulating cyclin-dependent kinase inhibitors [42, 110]. Activated T cells appear to become less sensitive to this proliferation inhibition as their expression of TGF-βRII decreases [134, 135]. The increased IL-7Rα induced by TGF-β1 during the thymic development of naïve $CD4^+$ T cells appears to support the survival of $CD4^+$ T cells bearing low-affinity T cell receptors in the periphery. This is evident in *Tgfb1*$^{-/-}$ mice which exhibit an altered diversity of TCR repertoire in the periphery but not in the thymus [130, 136, 137]. Similarly, the sensitivity to TGF-β1 signaling in $CD8^+$ T cells varies

depending on their TCR affinity [110, 130]. Finally, the pro-apoptotic function of TGF-β1 has been highlighted by studying apoptosis in short-lived effector $CD8^+$ T cells during the contraction phase after *Listeria* monocytogenes infection [4].

Differentiation

Inhibition of Th1 and Th2 differentiation

Differentiation into the Th1 lineage is driven by IL-12 and INFγ, which activate STAT4 and STAT1 to induce the expression of the transcription factor T-bet and launch the production of INF-γ. One of the significant effects of TGF-β1 on T cells is to impede Th1 cell differentiation as demonstrated by the phenotype of mice lacking TGFRII specifically in T cells, who develop a wasting disorder early in life due to a massive T cell expansion and enhanced Th1 responses. In addition to block TCR signaling, TGF-β1 downregulates the expression of IL-12Rβ2, STAT4 and T-bet, resulting in the loss of production of INF-γ [42, 110].

TGF-β1 also hampers differentiation into Th2 lineage. IL-4 guides naïve T cell progenitors towards the Th2 lineage, characterized by the expression of transcription factor Gata-3 and the production of IL-4, IL-5 and IL-13. Th2 cells modulate the activity of other immune cells to maintain tissue homeostasis and control allergic inflammation. Similar to its effect on Th1 differentiation, TGF-β1 directly downregulates the key transcription factor Gata-3. Additionally, SMAD signaling induces the transcription factor SOX4, which not only downregulates Gata-3 but also inhibits Th2 cytokine production [42, 110].

Induction of pTreg, Th17, Th9 and tissue-resident memory T cells

In addition to repressing the conversion of naïve $CD4^+$ T cells into Th1 and Th2 cells, TGF-β1 fosters the differentiation of the anti-inflammatory pTreg and pro-inflammatory Th17 and Th9.

Naive $CD4^+$ T cells in the presence of TGF-β1, IL-2 and retinoic acid (RA) can differentiate into a second type of Tregs that acquire their regulatory phenotype in the periphery. They are named pTreg. The requirement of TGF-β1 for pTreg differentiation is clearer than for tTreg, as mice lacking both Smad2 and Smad3 develop fatal inflammatory diseases with reduced Foxp3 expression in $CD4^+$ T cells in the periphery. This phenotype was less severe in single *Smad2* or *Smad3* KO mice, suggesting that Smad 2 and 3 may have redundant functions in pTreg differentiation [138]. RA is a vitamin A metabolite that can be secreted by DCs and macrophages to promote pTreg induction in the intestine, lung, and skin by increasing the binding of SMAD3 to an enhancer region of the Foxp3 gene required for TGFβ-mediated pTreg induction (CNS1) [42]. However, mice lacking RARα have normal numbers of Tregs [139], suggesting a dispensable role of this metabolite in pTreg induction. The generation of pTreg has been shown to be particularly important in the intestine and other

peripheral organs, as mice specifically lacking pTregs develop inflammation of the intestine, lung and liver [140, 141].

The synergy between TGF-β1 and IL-6 induces the expression of the transcription factor RORγT, IL-23R and IL-21 to drive differentiation towards Th17 cells, which produce IL-17 and IL-22. IL-23 and IL-1β are additional cytokines required for full differentiation into Th17. Th17 mostly resides in the mucosa where their production of IL-17 and IL-22 induces inflammation and neutrophil recruitment. They notably protect from extracellular fungi and bacteria [142]. However, Th17 could also be implicated in immune diseases such as multiple sclerosis. Indeed, the overexpression of TGF-β1 in T cells in Experimental Autoimmune Encephalomyelitis (EAE), a mouse model of multiple sclerosis, exacerbates the inflammation of the central nervous system (CNS) and increases Th17 numbers [143]. Conversely, mice with T cells that have a reduced ability to signal through TGFβ-1 (*CD4*$^{dnT\beta RII}$) are less sensitive to EAE induction and display a reduced number of Th17 [144].

The combination of IL-4 and TGF-β1 induces the transcription factor PU.1, hence shifting differentiation from Th2 towards Th9 cells, yet another subset of inflammatory CD4$^+$T cells, that produce IL-9. Despite the production of the immunosuppressive cytokine IL-10, Th9 cells are considered effector cells that can drive inflammation in specific contexts, such as in gastrointestinal infection by helminths [145, 146].

Finally, the impact of TGF-β1 on differentiation is not restricted to CD4$^+$ T cells. TGF-β1 also induces the expression of integrin $\alpha_E\beta_7$ (CD103) on the surface of CD8$^+$T cells, allowing them to bind to E-cadherin present in epithelial cell junctions. This protein promotes the residence of T cells in tissues and facilitates the establishment of tissue-resident memory T cells [147].

Effector functions

Effector functions do not escape to the regulation by TGF-β1. In CD8$^+$ T cell, SMADs collaborate with the transcription factor ATF1 to downregulate the expression of Infg, Gzma, Gzmb, Prf1 and Faslg thereby directly inhibiting the entire cytotoxic program [148]. This is evident in T cells isolated from mice lacking *Tgfbr2* specifically in their T cells, which display heightened effector phenotypes and functions, such as increased expression of the receptor KLRG1 and production of granzyme-B and INF-γ [149, 150].

- **B lymphocytes**

After developping from hematopoietic progenitors in the bone marrow, naïve B cells enter the circulation and circulate in secondary lymphoid organs, where they can encounter their cognate antigen and undergo a complex maturation process, which includes isotype class switching. In line with its pleiotropic effect on all immune cells, TGF-β1 regulates various aspects of B lymphocyte biology. Mice with a B-cell-specific deficiency in TGF-β1 signaling (*CD19*^{Cre} x *Tgfbr2*$^{f/fl}$) do not show significant signs of autoimmunity, but their B cells display a more activated and hyperresponsive phenotype, resulting in the production of anti-dsDNA antibodies [151]. TGF-β1 exerts a cytostatic effect on B cells by decreasing the expression of the cell-cycle regulator cyclin A and inactivating the cell-cycle-dependent kinase Cdk2 [110]. This anti-proliferative effect was confirmed by an increased BrdU incorporation in splenic B cell from mouse with deficiency in TβRII expression compared to WT B cells. Moreover, these mice showed B cell hyperplasia in Peyer's patches [151]. TGF-β1 globally decreases immunoglobulin synthesis. Strikingly, it regulates immunoglobulin class switching to favor the production of IgA, which provide an important defense mechanism at mucosal barriers. In line with this, *CD19*^{Cre}x*Tgfbr2*$^{fl/fl}$ mice exhibit elevated total Ig levels in the serum, but a drastic reduction in serum and mucosal IgA levels.

2.2. TGF-β1 in inflammation and infection

Inflammation is an immune reaction triggered upon tissue injury or infection by pathogens such as viruses, bacteria, parasites and fungi. Its purpose is to clear the source of disturbance and facilitate the repair of damaged structures. The general mechanism of acute inflammation relies on an immediate reaction of local innate cells (macrophages, dendritic cells, neutrophils, mast cells) that release various inflammation mediators (cytokines, chemokines, vasoactive agents) to recruit and activate more immune cells, notably neutrophils and lymphocytes, to the site of infection or injury. To avoid excessive reactions that may cause collateral damage, inflammation ends with a resolution phase followed by healing. In some cases, the inflammatory inducer cannot be eliminated, leading to persistent chronic inflammation.

The initiation mechanism and the cells involved subsequently depend on the trigger. To effectively combat the invader, two main distinct pathways are generally established, called type 1 and type 2 immune responses. It is worth nothing that physiological inflammation can also occur in absence of infection or injury, triggered by various conditions as obesity, aging, cancer or sleep deprivation [152]. The type 1 response protects against intracellular microbes through the activation of phagocytosis and activation of $CD8^+$ cytolytic T cells (T_c1), $CD4^+$ Th1 cells, NK cells and type 1 innate lymphoid cells (ILC1) that produce INF-γ. Type 1 cytokines also include IL-2, IL-12 and tumor necrosis factor, which generate acute microvascular response, edema and recruitment of neutrophils and monocytes. On the opposite, type 2 immunity favors a strong humoral response to ensure the integrity of epithelial barriers and protect against helminth parasites or noxious environmental substances. The primary immune cells are Th2, ILC2, Tc2, which notably secrete IL-4, IL-5 and IL-13. These type 2 mediators contribute to the recruitment of eosinophils, mast cells, basophils and promote IgE production by B cells. Finally, a third type of inflammation is led by Th17 cells, ILC3, and neutrophils and characterized by IL-6, IL-17, IL-22 and IL-23. This immune response aims to protect mucosa from extracellular bacteria and fungi.

2.2.1. TGF-β1 in inflammation

To control the magnitude of acute inflammation, appropriate inflammatory responses require a delicate balance between pro- and anti-inflammatory effects. The main anti-inflammatory signals consist of TGF-β1, IL-10 and glucocorticoids [152]. TGF-β1 channels inflammatory response by its numerous effects on immune cells such as inhibition of Th1, Th2, cytolytic $CD8^+$T cells, NK cells, and macrophages thereby preserving host tissues from excessive inflammatory responses. In addition, TGF-β1 induces the development of Tregs, which further regulate the magnitude and duration of inflammation. In contrast, it is important to note that TGF-β1 also exerts pro-inflammatory effects by promoting the differentiation of Th17 cells. Finally, excessive activity of TGF-β1 can contribute to chronic inflammation by facilitating persistence of pathogens.

2.2.2. TGF-β1 in infection

Circulating levels of latent TGF-β1 increase during infection by many pathogens. Particular attention should be noted to the fact that this does not directly reflect an increase in TGF-β1 activity. Moreover, every immune response must generate regulatory components to dampen and terminate the process. Thus, increased TGF-β1 (or Tregs) during an infectious episode may reflect either a host homeostatic mechanism or a strategy developed by pathogens to suppress immunity. Pathogens have co-evolved to circumvent immune responses, and manipulation of TGF-β1 counts among these escape tactics. Whether by activation of host latent TGF-β1 or by mimicry stimulation of the signaling pathway, TGF-β1 can be exploited by pathogens to facilitate their entry, replication, or persistence in the host by weakening immunity. Yet, TGF-β1 can also protect the host from infection or from its consequence. The following paragraphs will provide a few representative examples of this dual role of TGF-β1. For a more comprehensive overview, the reader is referred to the excellent review from Rick Maizels [153].

- **TGF-β1 in viral infection**

Whether through direct inhibition of NK cells, $CD8^+$ T cells, suppression of neutralizing antibodies production, or induction of Tregs, TGF-β1 induces generalized immunosuppression in many viral infections. TGF-β1 is well-documented for its detrimental effects on NK and T-cell immunity during chronic viral infections. As mentioned in the section dedicated to the effects of TGF-β1 on NK cells, the blockade of TGF-β1 signaling in these cells reduces neonatal susceptibility to viral infection [108]. In the same line, persistent TGF-β1 signaling during chronic lymphocytic choriomeningitis virus (LCMV) infection in mice inhibits $CD8^+$T cells and NK cells. Infection of CD4-TGFβRII^{dn} mice (which exhibit decreased TGF-β1 signaling in T cell) resulted in reduced apoptosis and increased cytotoxicity of $CD8^+$ T cells ensuing in a rapid virus eradication and the generation of an effective memory T cell response [154]. However, in WT mice, blocking of TGF-β or its receptor before infection or during the early memory phase only resulted in a modest increase in antiviral T cells without enhancing antiviral response [155, 156].

During acute viral infection, TGF-β1 could protect the host from excessive immunopathology. This is illustrated in infections caused by *Influenza* viruses, which have the ability to activate TGF-β1 through the enzymatic activity of neuraminidase [157]. Inhibition of TGF-β1 using a pan-TGF-β neutralizing antibody results in increased morbidity of infected mice, indicating a protective role of TGF-β1 in limiting excessive immune reactions during infection [158] and promoting optimal influenza-specific mucosal responses [159].

In humans, serum levels of TGF-β1 increase during certain infections, such as hepatitis B virus (HBV) or hepatitis C virus (HCV), or human immunodeficiency virus (HIV) infections. In the case of hepatitis viruses, higher systemic levels of TGF-β1 are associated with a worse outcome in hepatic viral infections and contribute to liver fibrosis [160-162]. Whether this increased production is a

result of a homeostatic process or an active strategy employed by the virus, TGF-β1 can promote a decreased immune environment favorable to viral reproduction. For instance, infection by hepatitis B virus leads to elevated levels of TGF-β1 which hinders the function and proliferation of NK cells [163].

- **TGF-β1 in bacterial infection**

Different bacteria have the ability to induce the production of TGF-β1 in order to turn immune responses to their own advantage. In the case of vaginal *Neisseria gonorrhoeae* infection, the induced production of TGF-β1 inhibits the development of Th1 and Th2 cells while promoting Th17 cell development [164]. Similarly, inhibition of Th1 and Th2 responses has been observed in a mouse model of gonococcal infection, where the administration of the blocking anti-TGF-β antibody during the primary infection accelerated clearance by favoring the emergence of Th1 and Th2 responses and the establishment of immune memory [165, 166]. Induction of Th17 response is also observed during infections by the mucosal pathogen *Citrobacter rodentium* [167, 168] and the oral infection by *Yersinia enterocolitica* [169]. In other cases, TGF-β1 induces the development of Tregs, such as during *Helicobacter pylori* infection where the T cell response is also skewed towards hyporesponsive Th1 cells [165]. Similar increases in Tregs and alterations of T cell effector responses are observed in chronic infections caused by Mycobacterium tuberculosis.

- **TGF-β1 in parasitic infection**

Parasites have also evolved mechanisms to exploit TGF-β1 and establish an immunosuppressive environment suitable to chronic infections. Several helminth parasites have been reported to produce TGF-β homologues acting on mammalian receptors [170, 171]. Furthermore, the murine intestinal helminth *Heligmosomoides polygyrus* is able to produce TGF-β mimic proteins, which show no sequence similarity to the host cytokine, but are able to induce TGF-β signaling. This signaling goes along with an expansion of Treg population and is critical to the survival of *H. polygyrus* in its murine host, as the use of the TβRI inhibitor SB431542 decreases worm burden [172, 173]. Patients with onchocerciasis and lymphatic filariasis, both caused by helminth parasites, exhibit T cell hyporesponsiveness, which can be reversed with anti-TGF-β antibodies [174, 175].

During acute infection of protozoans such as *Leishmania* and *Trypanosoma cruzi*, TGF-β1 has been shown to suppress macrophage function and promote parasite replication [176-178]. Furthermore, during *Leishmania* infection, TGF-β signaling in NK cells hinder INF-γ secretion and subsequent skewing of $CD4^+$ cells into a Th1 phenotype that contributes to protection against the infection [179].

In malaria, caused by Plasmodium parasites, TGF-β1 may have a dual role according to the doses and the timing of infection [180]. During early infection, TGF-β1 could slow parasite replication and control excessive Th1 response. This notion gain support from the observation that administration

of neutralizing antibody against TGF-β aggravated Plasmodium infection and inflammation while increasing serum levels of TNF-α and INF-γ [181, 182]. Conversely, high doses of recombinant TGF-β1 have been associated with the inhibition of the development of Th1-mediated parasite clearance [183]. Finally, TGF-β1 inhibition enhances activity of CD8+ T cell and protection against reinfection in mice [184].

2.3. TGF-β1 in wound healing and fibrosis

TGF-β1 activity is enhanced acutely during wound healing and coordinates the tissue repair process (Fig.10). Upon tissue injuries, damaged blood vessels activate platelets which could be the first provider of active TGF-β1. Depending on the context, other possible sources are the ECM, activated macrophages, epithelial cells, endothelial cells, fibroblasts and mast cells. Along with other cytokines, TGF-β1 attracts and regulates mast cells, neutrophils and macrophages launching the early inflammatory phase that decontaminates the wound, joined by T cells in a later phase of inflammation. Upon activation, macrophages themselves begin to produce TGF-β1. These first inflammatory mediators secrete chemokines attracting a new set of cells involved in the resolution phase: vascular endothelial cells, pericytes, epithelial cells and fibroblasts [185].

Besides its role in recruitment of inflammatory cells and regulation of macrophages activity, TGF-β1 also promotes angiogenesis and vessel maturation. TGF-β1 plays a key role in the restoration of the matrix by stimulating matrix biosynthesis and inhibition of matrix degradation. A key step of matrix biosynthesis is the stiffening of the reconstructed ECM, made of newly deposited collagen fibers. This remodelling is driven by fibroblasts that produce and generate traction force on the new collagen fibers. As long as the resistance of ECM exceeds the force generated by the fibroblasts, TGF-β1 levels raises and converts fibroblasts (or other cell types as epithelial cells, endothelial cells, mesenchymal stem cells) into highly contractile myofibroblasts that drive the stiffening and stabilization of scar tissue. In addition, TGF-β1 stimulates activated myofibroblasts to synthetize collagen (type I, III, IV) and other ECM components. Finally, TGF-β1 inhibits matrix degradation by repressing the expression of genes encoding protease inhibitors, such as plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitors of metalloproteinases (TIMP) [185, 186].

As soon as the damage is repaired, tissue remodelling is turned off. To terminate these activities, myofibroblast population undergoes massive apoptosis while TGF-β1 stores get emptied. But in some pathological conditions such as chronic inflammation, autoimmune reactions or allergic responses, apoptotic clearance mechanisms fail and lead to an excessive repair called fibrosis. Fibrosis is characterized by accumulation of ECM components resulting in loss of physiological architecture of the tissue and eventually impaired organ functions. Apart from some conditions previously mentioned, the initiating trigger of fibrosis remains unknown for many fibrotic diseases. TGF-β1 levels remains chronically high during fibrosis and becomes a pro-fibrotic factor by continually stimulating the same cells programs while physiological healing is over [187].

TGF-β is likely involved in human fibrotic conditions such as systemic sclerosis, where patients treated with pan TGF-β1, -β2 and -β3 neutralizing antibody display decreased fibrosis-associated biomarkers [188]. Of note, TGF-β2 and -β3 isoforms could also be involved in this process but have been poorly studied so far [189].

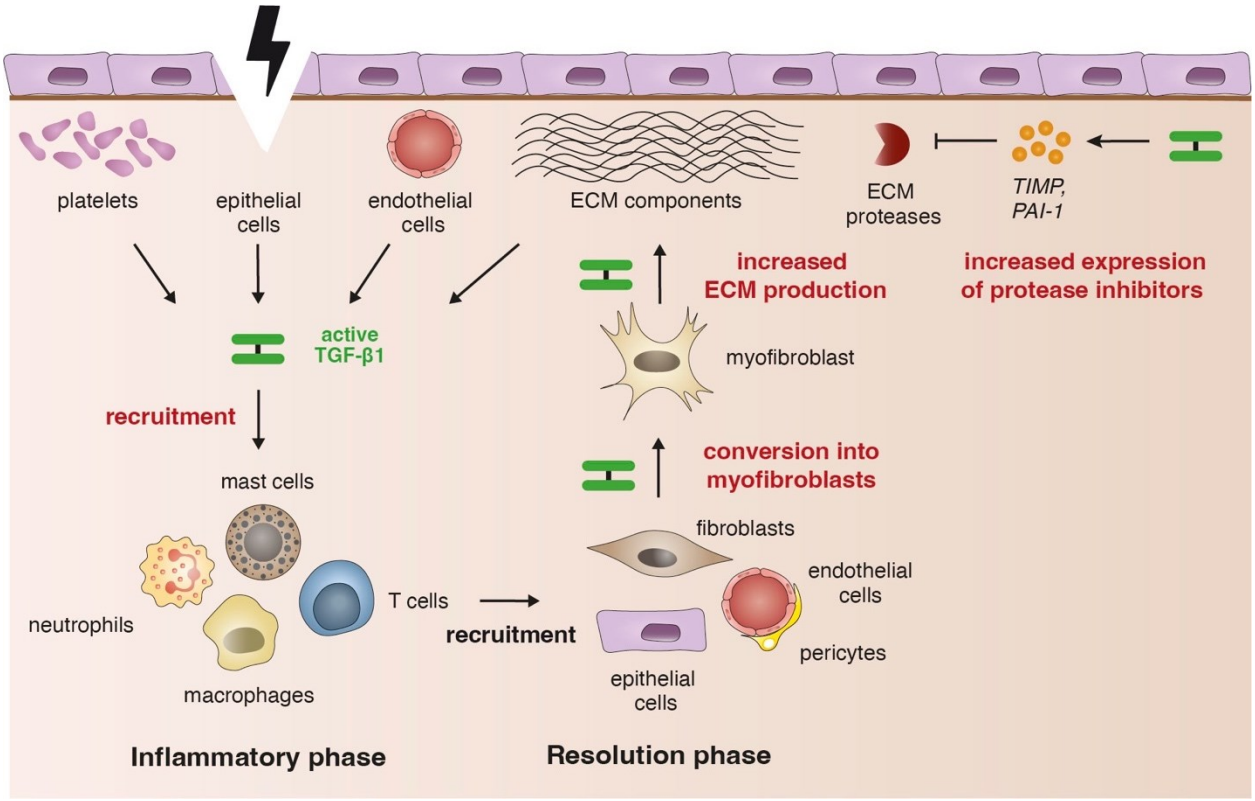

Figure 10: Origin and roles of TGF-β1 in matrix remodeling and tissue repair. Damaged cells or cells activated upon injury such as platelets, epithelial or endothelial cells secrete active TGF-β1. TGF-β1 recruits immune cells evolved in the inflammatory phase. During resolution phase, TGF-β1 stimulates matrix biosynthesis by inducing the conversion of different cells into myofibroblasts and by stimulating the production of extracellular matrix (ECM) components by these cells. Finally, TGF-β1 inhibits matrix degradation by inducing gene expression of protease inhibitors such as plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitors of metalloproteinases (TIMP). Adapted from Sara Lecomte's thesis.

2.4. TGF-β1 in immune tolerance, and auto-immune or chronic inflammatory diseases

Immune tolerance is the state of unresponsiveness of the immune system to a specific antigen, whether it is a self-antigen or a harmless antigen from the environment. Central and peripheral tolerance mechanisms ensure that the immune system does not mount a response against these antigens, thus preventing autoimmune reactions and allergies to food or harmless environmental antigens. Central tolerance is established in the thymus or bone marrow, where autoreactive developing lymphocytes are either deleted, inactivated, or differentiated into Tregs. Negative selection plays a significant role in this process by eliminating autoreactive T cells from the T-cell repertoire. On the other hand, peripheral tolerance is the mechanism by which lymphocytes that have escaped negative selection in the primary lymphoid compartment are inactivated or deleted in the periphery.

The inflammatory disorders observed in *Tgfb1*$^{-/-}$ and *CD4*^{Cre} x *Tfgbr2*$^{fl/fl}$ mice highlighted the crucial role of TGF-β1 signaling in T cell tolerance [1, 2, 123, 130]. TGF-β1 acts directly on effector T cell populations and through induction of regulatory T cells [190, 191], which are essential for maintaining immune tolerance [140]. The specific effects of TGF-β1 on major immune cell types were extensively described earlier in this section (2.1.1). The following lines will focus on its role in specific diseases to illustrate its diverse and complex effects.

2.4.1. Inflammatory Bowel Disease

The gastrointestinal tract is continuously exposed to various foreign antigens derived from food and commensal flora. The intestinal immune system must strike a delicate balance between protecting the body from harmful pathogens and tolerating the presence of beneficial commensal bacteria and food antigens. Disruption of this homeostasis exposes the intestine to chronic and excessive inflammation known as inflammatory bowel disease (IBD). The exact etiology of IBD is still unknown but is believed to involve a combination of factors such as host genetic susceptibility, immune response dysregulation, environmental triggers, and alterations in the luminal microbiota.

TGF-β1 contributes to immune tolerance to luminal bacteria. In healthy individuals, the phosphorylation of SMAD3 in intestinal T cells is upregulated compared to peripheral T cells [192]. Mouse models have unveiled the importance of TGF-β1 signaling in dendritic cells, T cells, macrophages, and epithelial cells. Impairment of TGF-β signaling in T cells and dendritic cells results in spontaneous colitis, characterized by massive infiltration of activated lymphocytes and a reduced number of Tregs in the periphery [114, 123, 130, 193]. Mice with reduced TGF-β signaling in macrophages (*CD68*^{dnTbRII}) do not develop spontaneous colitis but exhibit susceptibility to DSS-induced colitis, which is associated with increased production of the pro-inflammatory IL-33 by macrophages [112, 194]. Similarly, disruption of TGF-β signaling in the intestinal epithelium results

in increased susceptibility to DSS-induced colitis due to impaired regeneration of the intestinal mucosa [195]. The main cell types producing TGF-β1 in this context are Tregs, DCs, myofibroblasts and epithelial cells [196].

Impaired TGF-β signaling has also been reported in patients with IBD and may contribute to the development of the disease. Some patients with Loeys–Dietz syndrome, an autosomal dominant disorder caused by heterozygous mutations in *Tgfbr1* or *Tgfbr2*, develop early-onset IBD [197, 198]. In colonic lamina propria mononuclear cells from IBD patients, phosphorylation of SMAD3 was found to be downregulated and SMAD3 has been identified as one of the loci associated with IBD susceptibility [199, 200]. The decreased phosphorylation of SMAD3 may be a consequence of elevated levels of inhibitory SMAD7, which have been reported in the intestinal mucosa and lamina propria lymphocytes of IBD patients [192, 199]. This could explain the uncontrolled inflammation observed in IBD despite the high levels of TGF-β1 in intestinal tissues induced during the disease [196].

The inhibition of SMAD7 with a specific antisense oligonucleotide (Mongersen, GED-0301) is currently tested in patients with active Crohn's disease, a form of IBD. Phase I and II trials have shown safety and efficacy, although these findings were not confirmed in a phase III trial [201, 202]. TGF-β downregulates the expression of integrin $\alpha_4\beta_7$ on T cells, which interacts with mucosal adhesion molecule-1 (MAdCAM-1) for gut homing. Vedolizumab, a gut-selective $\alpha_4\beta_7$ inhibitor, has been approved for clinical IBD therapy in the USA and Europe [203].

2.4.2. Allergic asthma

The airway mucosa is responsible for protecting the lungs from pathogens and other external components. It is composed of a variety of cell types, including epithelial cells, dendritic cells, macrophages, and T cells. Loss of T cell tolerance towards harmless environmental antigens can initiate allergic rhinitis or allergic asthma, which are considered hypersensitivity reactions driven by type 2 immunity characterized by the Th2-IgE-mast cell pathway [204, 205].

Epithelial cells play a major role in the initiation of allergic asthma. Upon allergen exposure, they produce several cytokines and chemokines, including IL-33, IL-25, and TSLP, which contribute to Th2 responses. These cytokines cause the accumulation of a high number of eosinophils in the airway wall, mucus overproduction, synthesis of IgE by allergen-specific B cells, smooth muscle contraction, and airway narrowing. In allergic asthma, persistent inflammation leads to airway remodeling and airway hyper-responsiveness. Airway remodeling involves a complex series of events including smooth muscle thickening, goblet cell hyperplasia, deposition of extracellular matrix protein, and vascular remodelling, all of which result in reduced lung function [205, 206].

The importance of TGF-β1 signaling in allergic asthma is highlighted by numerous investigations associating polymorphisms in the promoter of *TGFB1* with a higher risk of asthma [207-213]. Additionally, patients with Loeys-Dietz syndrome, who carry loss-of-function mutations in TGFβR-I or TGFβR-II, frequently develop allergic diseases including asthma [214]. Increased TGF-β1 levels or signaling pathway components have been observed in bronchoalveolar lavage fluid or mucosal biopsies from patients with allergic rhinitis or asthma, and these levels are further increased following airway allergen challenge [215-218]. In the airways, TGF-β1 can be produced by structural cells of the airway wall including epithelial cells, fibroblasts, endothelial cells, and smooth muscle cells, as well as inflammatory cells infiltrating the bronchial mucosa such as eosinophils and macrophages [206]. TGF-β stands central in asthma pathogenesis as it possesses both important immunomodulatory and fibrogenic activities. As a results, it has been extensively studied and reviewed to decipher its implication in airways inflammation and remodeling processes [205, 219-221].

- **Immunomodulatory roles of TGF-β1 in asthma**

Despite numerous studies on the topic, the exact roles played by TGF-β1 in the inflammatory events of asthma remain enigmatic, as it can exert both pro and anti-inflammatory functions, leading to variable outcome across studies. This variability is well illustrated by systemic neutralization of TGF-β1, 2 and 3 using the pan-TGF-β blocking antibody, which has variable effects on inflammation and airway hyperactivity depending on the experimental design, likely reflecting differential cellular sources being targeted [222-225]. In contrast, intratracheal delivery of TGF-β1 has been shown to suppress allergen-induced inflammation [226].

TGF-β1 displays anti-inflammatory actions by regulating lymphocyte homeostasis, notably by inhibiting Th2 cell responses and promoting the differentiation of Treg cells. The reduction of systemic TGF-β1 expression in heterozygous *Tgfb1*$^{-/+}$ mice and the overexpression of the inhibitory SMAD7 in T cells both resulted in enhanced type 2 inflammation in the OVA-induced asthma model [227, 228]. The adoptive transfer of OVA-specific CD4^{+} T cells overexpressing TGF-β1 reversed type 2 inflammation and subsequent airway hyperresponsiveness in an antigen-specific manner [229]. In addition to reducing Th2 inflammation, TGF-β1 has been shown to prevent house dust mite (HDM)-induced allergic reactions in mouse lungs by inducing pTregs [230]. Furthermore, the therapeutic administration of antigen–specific splenic tTregs in chronic allergic airway OVA-induced model ameliorated many asthma features, including lung eosinophilia, Th2 infiltration, mucus hypersecretion, and peribronchial collagen deposition. Nevertheless, it was unable to reverse established airway hyperreactivity. This reduced inflammation went along with reduced expression of total IgE, IL-5, IL-13, and even TGF-β1 [231]. Several studies report a defect in Treg number among patients suffering from allergic rhinitis or asthma, using Foxp3^{+} expression as a reflection of Treg [232-235]. It should be noted that Foxp3 expression is not sufficient to identify Tregs in humans, as other activated T cells can also transiently express Foxp3. A more precise definition of human

Tregs requires the measurement of demethylation of a specific intronic region of *FOXP3 [236-238].* Similarly, a subpopulation of B cells secreting IL-10, IL-35 and TGF-β1, known as regulatory B cells (Bregs), is also impaired in terms of number or function in allergic asthma and allergic rhinitis [239].

In contrast, TGF-β1 has the potential to promote inflammatory cell types such as Th9, Th17 or ILC2, which have been more recently implicated in the pathogenesis of asthma. Administration of Th9 cells to mice has been shown to induce asthma features such as bronchial hyperresponsiveness, eosinophilia, and mucus production, similar to the transfer of Th2 cells [240]. Various mouse studies have identified IL-9 as a factor that contributes to several features of asthma, including the recruitment of inflammatory cells to sites of inflammation, generation and proliferation of mast cells, goblet cell hyperplasia and mucus production [241-243]. Increased expression of IL-9 has been observed in bronchoalveolar lavage fluid and PBMCs from patients with allergic asthma [244, 245]. However, no direct link with TGF-β1-induced production of IL-9 has been drawn so far, as Th9 can also be induced through TGF-β-independent mechanisms [246, 247]. It is worth nothing that Th9 are not the sole producers of IL-9 in asthmatic patients, as highly differentiated Th2 cells [245], eosinophils, and neutrophils [241] can also produce this cytokine.

ILC2 are innate lymphoid cells with a cytokine profile similar to Th2 cells. Though, in contrast to Th2 cells, TGF-β1 promotes the development and functions of ILC2 [248, 249]. Bronchiolar epithelial cells have been shown to produce IL-33 and TGF-β1, which act in concert to recruit and activate ILC2 [248]. Apart from the classical allergic asthma associated with Th2 cytokines and eosinophil infiltration, there exist distinct physiopathologies and phenotypes, called endotypes. Late-onset and severe forms of asthma have been associated with a Th1/Th17 profile characterized by neutrophilic infiltration [250]. IL-17A has been described to promote the contraction of airway smooth muscle cells and fibroblast proliferation, although Th17 cells are not the only cell type able to produce this cytokine [251, 252]. One study showed that TGF-β, activated by $\alpha_v\beta_8$ integrin on DCs, could favor IL-17A production by CD4+T cells following peripheral allergen sensitization and airway challenge in mice [252].

- **TGF-β1 in airway remodeling**

Collagen deposition, smooth muscle cells proliferation, goblet cell hyperplasia, and mucus production are all features of airway remodeling that have been found to be reduced by anti-TGF-β treatment in at least one of three independent experiments studying the inhaled ovalbumine (OVA)-induced allergic asthma model [223-225]. Airway remodeling was also decreased by the administration of TGF-βRI kinase inhibitor in the OVA-induced asthma model in mice and rats [131, 253]. However, TGF-β1 does not appear to play a critical role in the generation of HDM-induced airway remodeling, as WT mice treated with the TGF-β1 neutralizing antibody and SMAD3 KO mice developed features of airway remodeling to the same extent as HDM-exposed WT littermate control animals [222], again underlying the context-specific role of TGF-β1. The profibrotic properties of

TGF-β1 may also depend on its signaling pathway. Recent research has shown that in fibroblast from asthmatic patients, the profibrotic Smad2/3 signaling pathway was enhanced while the antifibrotic Smad1/5/(8)9 pathway was significantly diminished compared to fibroblasts from healthy individuals [254].

2.4.3. Multiple sclerosis

Multiple sclerosis (MS) is a demyelinating auto-immune disease of the central nervous system (CNS). The etiology is not completely defined but is influenced by genetics, environment and viral infections. Many information on the complex physiopathology of multiple sclerosis has been gathered from the study of the mouse model of Experimental Autoimmune Encephalomyelitis (EAE). The permeabilization of the blood brain barrier allows infiltration of auto-reactive T cells, followed by B cells, neutrophils and monocytes [255]. This cellular infiltrate produces antibodies and pro-inflammatory cytokines that activates pro-inflammatory microglia and peripheral macrophages, largely responsible of demyelination. This pro-inflammatory environment is notably characterized by INF-γ, IL-17 and granulocyte macrophage colony-stimulating factor (GM-CSF) [256, 257]. Among infiltrating T cells, Th1, Th17 and $CD8^+$T cells are the most implicated effectors (Fig.11).

The role played by TGF-β1 in the physiopathology of multiple sclerosis is not clear. Early experiments performed by independent groups indicated a protective effect of the administration of exogenous TGF-β1 in the EAE mouse model [258-260]. However, concerns remain about its role in the physiopathology as TGF-β1 may promote both differentiation of deleterious Th17 and protective Tregs [133]. *In vitro*, naïve $CD4^+$ T cells differentiate in Th17 under stimulation in presence of IL-6 and TGF-β1 but such differentiated Th17 failed to induce EAE, an observation which led to the emergence of the “non-pathogenic Th17” concept [261, 262]. Th17 cells exhibit a high degree of plasticity and further exposure to IL-23 may be required to convert these non-pathogenic Th17 into pathogenic Th17 [262]. Moreover, TGF-β1 could be dispensable in this induction of pathogenic Th17 as naïve T cells differentiated with a cocktail of IL-6, IL-23 and IL-1β also manage to induce EAE. These pathogenic Th17 cells co-express RORγT and T-bet resulting in Th17/Th1 cells that produce both IL-17 and INF-γ, while non-pathogenic Th17 differentiated under IL-6 and IL-17 lack T-bet expression. Interestingly, similar Th17/Th1 cells were found in the gut of patients with Crohn’s disease or in the joints of those with arthritis [263-265]. Thus, the cytokine environment is of primary importance for the differentiation of pathogenic Th17 cells and may exclude TGF-β1 as it is known to repress T-bet expression in Th1 cells.

Disruption of the Teff/Treg equilibrium may be involved in multiple sclerosis development. Immunoregulatory cells such as Tregs are thought to be protective by controlling inflammation and promoting good remyelination. Many studies point to a Treg defect in MS patients but the origin is less clear [133, 266]. Patients with MS showed a decreased $CD4^+$ $CD25^+$ $FOXP3^+$ number, but should be confirmed with the measurement of demethylation of the specific intronic region of

FOXP3 [267] as stated earlier. Other studies described default of migration into the CNS, impairment of functions or decreased T cells repertoire in MS patients and myasthenia gravis [133, 268]. TGF-β signaling in the peripheral blood cells of MS patients may be altered but their response to exogenous TGF-β1 was intact [269]. Numerous studies on naïve $CD4^+$ T cells from MS patients identified miRNA targeting TGF-β signaling, potentially explaining the alteration of Treg development and functions [133].

In a later phase of inflammation, M1 macrophages would switch to an anti-inflammatory M2 phenotype, followed by inflammation resolution and tissue repair [270-272]. Whether TGF-β1 directly influences this switch is not known but TGF-β1 might play a direct role in remyelination of the lesions by promoting oligodendrocytes maturation [273-275]. Microglial cells expressing LRRC33 were shown to activates latent TGF-β1 by interacting with integrin $\alpha_V\beta_8$ expressing cells. *Lrrc33*$^{-/-}$ mice have reactive microglia, ascending paraparesis and loss of myelinated axons leading to death by 5 months [22]. This observation underlies the role of TGF-β1 in the immune homeostasis within the SNC.

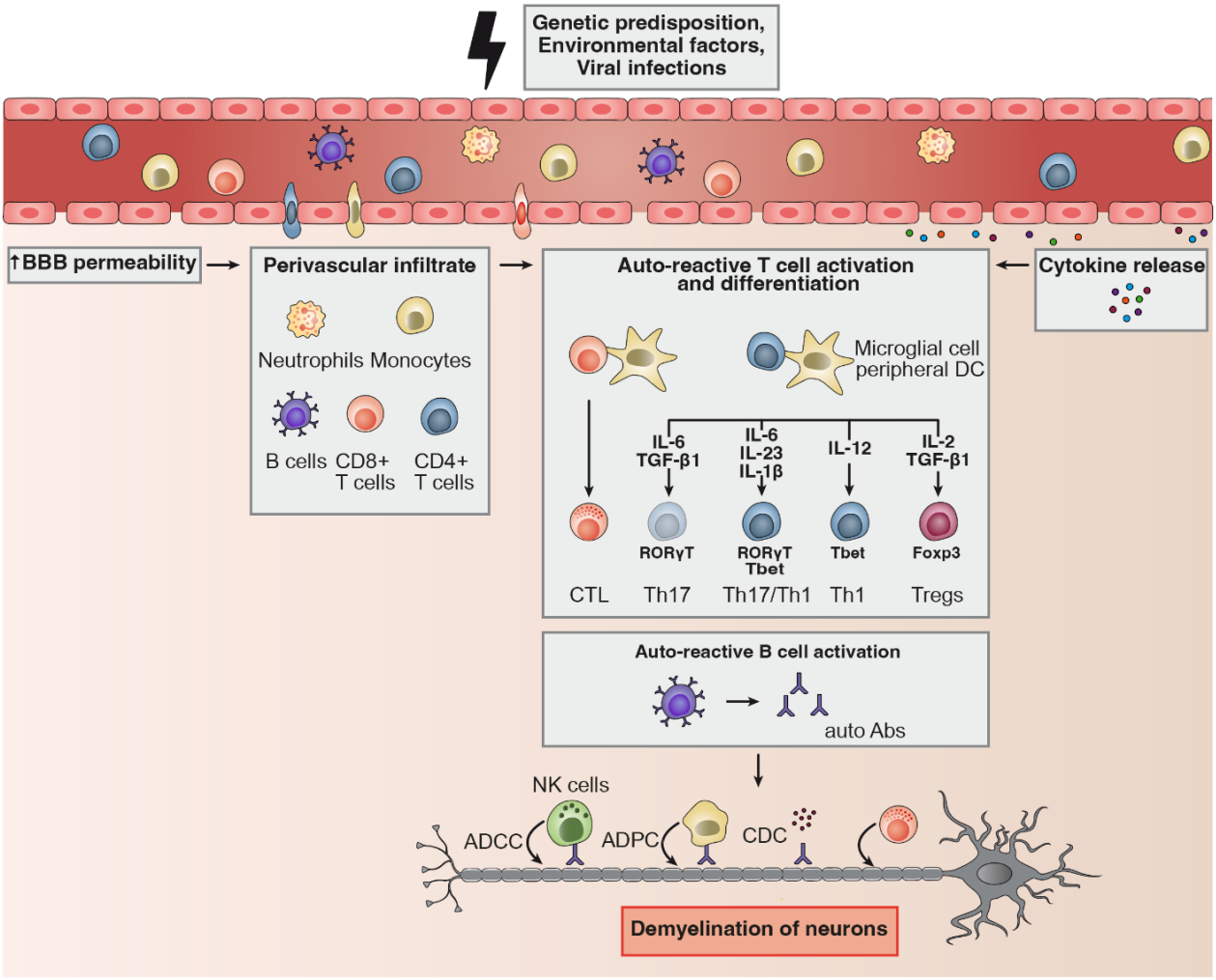

Figure 11: Physiopathology of multiple sclerosis. A complex interplay of genetic predisposition, environmental factors and viral infections leads the development of a pro-inflammatory environment and increased the permeability of the blood brain barrier (BBB), allowing infiltration of various immune cells. Within the CNS, autoreactive T cells become activated and differentiate according to the cytokine milieu. The activated T cells secrete cytokines such as IL-17 and INF-γ, which further activate microglial cells and peripheral DCs. In parallel, autoreactive B cells are also activated and produce autoreactive antibodies (auto Abs) that, in combination with phagocytic cells, largely contribute to the destruction of myelin sheath. ADCP, antibody-dependent cellular phagocytosis; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity.

2.4.4. Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder affecting multiple organs that is characterized by the generation of autoantibodies against nucleic acids. The pathogenesis of SLE starts with an imbalance between production and elimination of apoptotic material. This generates an inflammatory environment, favouring excessive auto-reactive T and B cell activation and loss of tolerance against self-antigens. Activated B cells produce anti-nuclear Abs (ANAs), leading to accumulation of immune complexes in multiple organs, that promotes type I INF inflammation and organ damages (Fig.12). Kidney is one of the main target organs in SLE, and lupus nephritis is one of the most important causes of morbidity and mortality [276].

Both total (latent + active) and active TGF-β1 levels were reported as decreased in the serum of SLE patients by comparison to healthy individuals [277, 278], and serum TGF-β1 levels were inversely related to disease activity [277-279] and severity of renal damages [280]. Of note, during the coagulation process that leads to serum formation, platelets become activated, which is not the case for plasma. Thus, the serum levels of active and total TGF-β1 might more accurately reflect the state of platelet activity rather than the circulating TGF-β1 itself [97]. Platelets have been reported to be aberrantly activated in SLE patients and to contribute to its pathogenesis, notably by activating complement and subsequent apoptotic cell clearance. However, the potential impact this aberrant activation of platelet on TGF-β1 production has not been investigated [281]. More recently, a decrease in plasma levels of total TGF-β1 has also been associated with SLE disease and lupus nephritis [282-285], providing further evidence of reduced cytokine circulation among affected patients.

Several studies have highlighted an impairment of TGF-β1 activity in SLE patients as shown by decreased active TGF-β1 produced by lymphocytes isolated from SLE patients [286] and a deficient expression of TGF-β signal transduction elements (TGFβ-RI, Smad2, Smad3 and Smad7) in T cells could be observed in certain cases [285]. Interestingly, lower TGF-β1 circulating levels were associated with a decrease in circulating $CD4^+CD25^+Foxp3^+$ cells [287], and *FOXP3* variants were associated with higher susceptibility to develop SLE [288, 289]. On the opposite, another *FOXP3* variant showed a protective effect in susceptibility to SLE associated with higher levels of plasmatic TGF-β1 [288]. However, a review of studies on quantitative and functional deficiencies of Tregs in SLE patients emphasized discrepant results about the decreased number and functions of Tregs in SLE patients, probably due to the heterogeneity of the disease and the difficulty to define and quantify human Tregs precisely [290].

The protective role of TGF-β1 in SLE was also confirmed in mouse models. Production of autoantibodies characteristic of lupus, such as anti-DNA or anti-Sm ribonucleoprotein antibodies, were identified in *Tgfb1*$^{-/-}$ mice [291]. On the contrary, in WT mice, immunization with an anti-DNA mAb induces a transient breakdown of B-cell tolerance that is rapidly controlled by TGF-β-

producing T cells [292]. Lupus-prone mice have reduced expression of TGF-β1 in lymphoid tissues and TGF-β1-producing T cells, what predisposes them to immune dysregulation and autoantibody production [293]. Administration of a vector that encodes TGF-β1 enhances survival and reduces disease severity in a murine model of lupus [294].

Tregs also were shown to be protective in murine models of lupus. Adoptive transfer of *ex vivo* isolated or *in vitro* expanded Tregs were both shown to suppress B cell responses [295] and protect from murine lupus [295-297]. B cell themselves could produce active TGF-β1, preventing occurrence of spontaneous autoimmunity during aging. Indeed, mice lacking GARP specifically on B cells developed a spontaneous lupus-like disease with aging [103]. This is in line with unpublished data of the laboratory showing that mice that carry a B cell- or a Treg- specific deletion of the *Garp* gene are prone to develop SLE-like symptoms upon aging or in response chronic inflammation induced by INFa electroporation, respectively (Zhang et al, in preparation).

Altogether, these studies suggest a beneficial role of TGF-β1 in the control of SLE pathogenesis. However, in lupus nephritis and other glomerular diseases that are characterized by excessive ECM synthesis, the pro-fibrotic properties of TGF-β make it a critical tissue injury factor contributing to local fibrogenesis and subsequent glomerular lesions [298, 299]. In contrast with the low levels of TGF-β1 detected in the serum and plasma of SLE patients, TGF-β1 levels are increased in the urine of patients with renal damages [287]. The expression of the three isoforms of TGF-β and the receptors were increased in glomeruli and the tubulointerstitium tissues from patients with renal diseases characterized by ECM accumulation, including in lupus nephritis [300, 301]. The kidney of lupus-prone mice displays increased levels of TGF-β1 and increased mRNA and protein expressions of TGF-β signaling pathway. These levels correlate with the extent of chronic lesions that represent local tissue fibrosis [293]. TGF-β1 can affect many renal cells, including mesangial, endothelial cells, epithelial cells (podocytes), tubular epithelial cells and fibroblasts, mainly by favoring collagen synthesis, EMT or apoptosis [298]. TGF-β blockade with the pan anti-TGF-β antibody could reduce fibrotic lesions without exacerbating inflammation in treated mice [293]. However, it displayed limited efficacy when tested in patients suffering from chronic kidney disease [302].

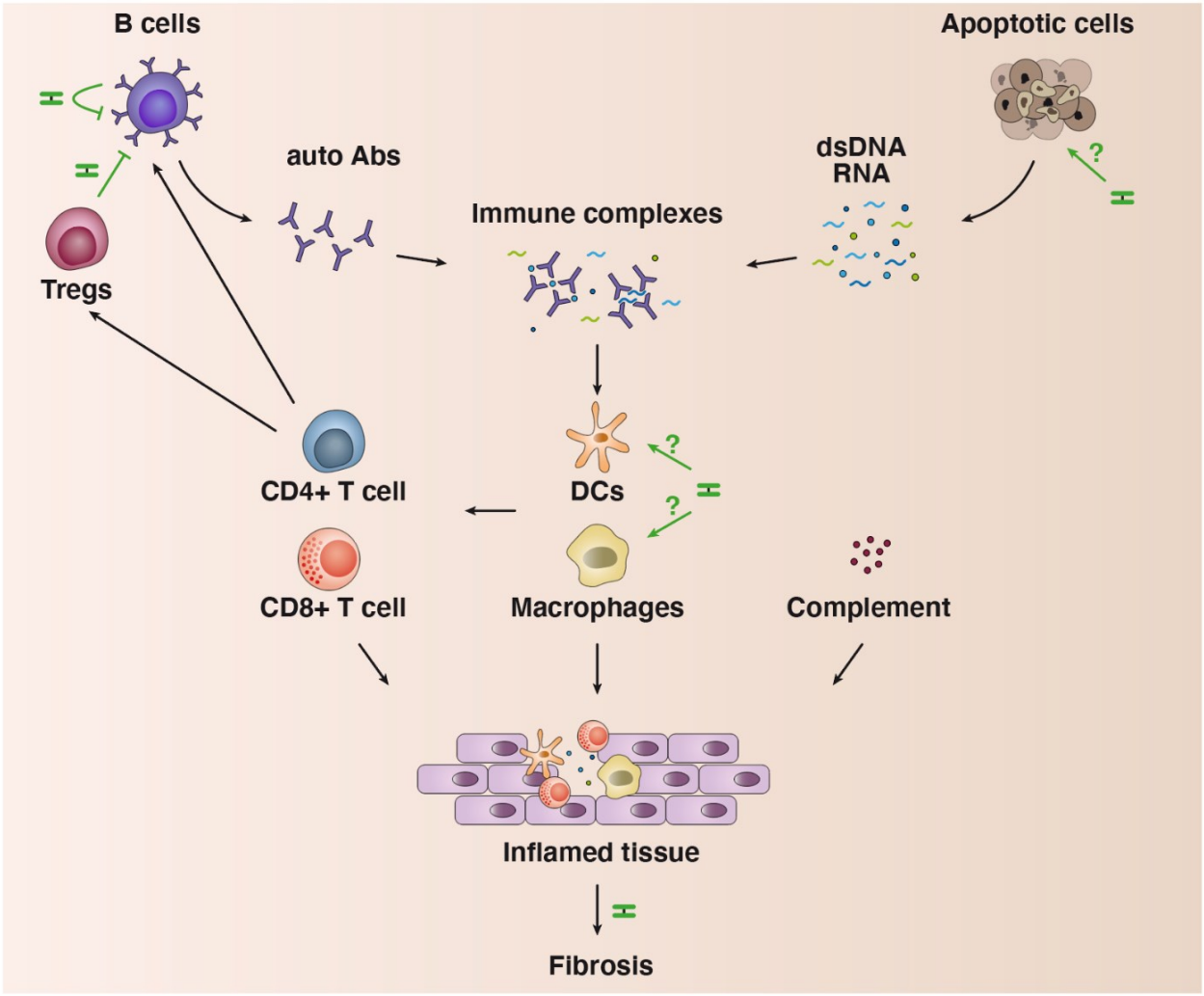

Figure 12: Potential roles of TGF-β1 in SLE physiopathology. An imbalance between apoptotic cell production and the efficient clearance of apoptotic material, along with loss of B cell tolerance leads to formation of immune complexes. These immune complexes activate the complement system resulting in the activation and recruitment of macrophages, DCs and T cells. The immune cells, along with autoantibodies and complement contribute to tissue inflammation. TGF-β1 may play a role in controlling the physiopathology of SLE by reducing B cell activity and promoting differentiation of Tregs. In later stages, TGF-β1 may increase fibrosis of inflamed tissue. Additionally, TGF-β1 might have effects on apoptosis, DCs or macrophages, although further studies are needed to investigate these aspects (adapted from [303]).

2.5. TGF-β1 in Graft-versus-Host disease

Graft-versus-Host-Disease (GvHD) is a severe complication of allogeneic hematopoietic cell transplantation (allo-HCT), a curative treatment for many patients suffering from malignant and non-malignant hematological diseases. In malignant diseases, the efficacy of allo-HCT relies notably on the so-called Graft-versus-Tumor (GvT) effect, an alloimmune response that occurs post-transplantation and results in the destruction of residual recipient tumor cells by allo-reactive T cells from the donor. However, the allo-reactive T cells contained in the graft can also induce extensive damage to the normal, non-tumoral tissues of the recipient, leading to a life-threatening Graft-versus-Host Disease (GvHD). In spite of improvements in histocompatibility, conditioning regimens, prophylaxis and treatment, GvHD remains a major impediment to the use of allo-HCT, which is nevertheless considered the standard of care for selected hematological diseases. Current research focuses on the challenging task of minimizing excessive GvHD without compromising the desired GvT effect [304].

Based on time of occurrence, physiopathology and clinical manifestations, GvHD can be divided into two distinct syndromes. Acute GvHD (aGvHD) is characterized by strong inflammatory responses mainly targeting the gastrointestinal tract, lung, liver and skin. Chronic GvHD (cGvHD) can affect almost any organ, exhibiting characteristics reminiscent of autoimmune disorders and fibrosis. Generally speaking, aGvHD occurs within 100 days post-transplantation, while cGvHD arises beyond this empirical threshold [305].

2.5.1. Acute GvHD

The physiopathology of aGvHD is generally depicted into a three-steps process: the induction phase, the activation phase, and the effector phase [306]. The induction phase starts prior to cell transplantation: patients receive a conditioning regimen, typically involving chemotherapy combined or not with total body irradiation [307] (Fig.13). The purpose of conditioning is to reduce tumor burden or eradicate tumor cells and facilitate the successful engraftment of the donor hematopoietic cells. However, conditioning also results in damage to host tissues and the integrity of the intestinal epithelium. These damages lead to release of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, …) as well as PAMPs and DAMPs from host cells and gut microbiome. This environment initiates activation of host and donor APCs, including non-hematopoietic APCs such as gut epithelial cells [305, 308]. In turn, activated APCs present major and minor histocompatibility allo-antigens to donor T cells, which leads to their activation, subsequent proliferation and differentiation, and to the activation phase. T cells are the main immune effectors of aGvHD, as total T-cell depletion from the grafts prevents aGvHD. Of note however, use of T-cell depleted grafts does not improve overall survival in allo-HCT because of slow immune system recovery and frequent leukemia relapse [305, 308-310]. Both $CD4^+$ and $CD8^+$ T cells are implicated in the physiopathology of aGvHD, as they are individually able to induce aGvHD in mouse models. Patients transplanted

with graft depleted from $CD4^+$ or $CD8^+$ T cells suffer from less severe GvHD [311-313]. The predominant immune responses driving aGvHD are Th1/Tc1 and Th17/Tc17. In mouse models, T-bet deficient donor cells fail to induce a lethal GvHD while ROR-γT deficient T cells are still able to induce a lethal GvHD, albeit delayed [314-316]. The activation phase classically takes place in the secondary lymphoid organs but can also occur in the targeted organs where non-hematopoietic APCs are activated by the conditioning. For example, epithelial cells from the gastrointestinal tract express MHC class II and co-stimulation molecules due to the inflammatory environment following conditioning [317]. Once activated, donor T cells acquire effector functions (hence the effector phase), migrate and infiltrate the target organs where they exert their cytotoxic activity or enroll other immune cells such as neutrophils, NK or macrophages. This results in amplification of damages and increased release of pro-inflammatory cytokines, nourishing a self-perpetuating reaction.

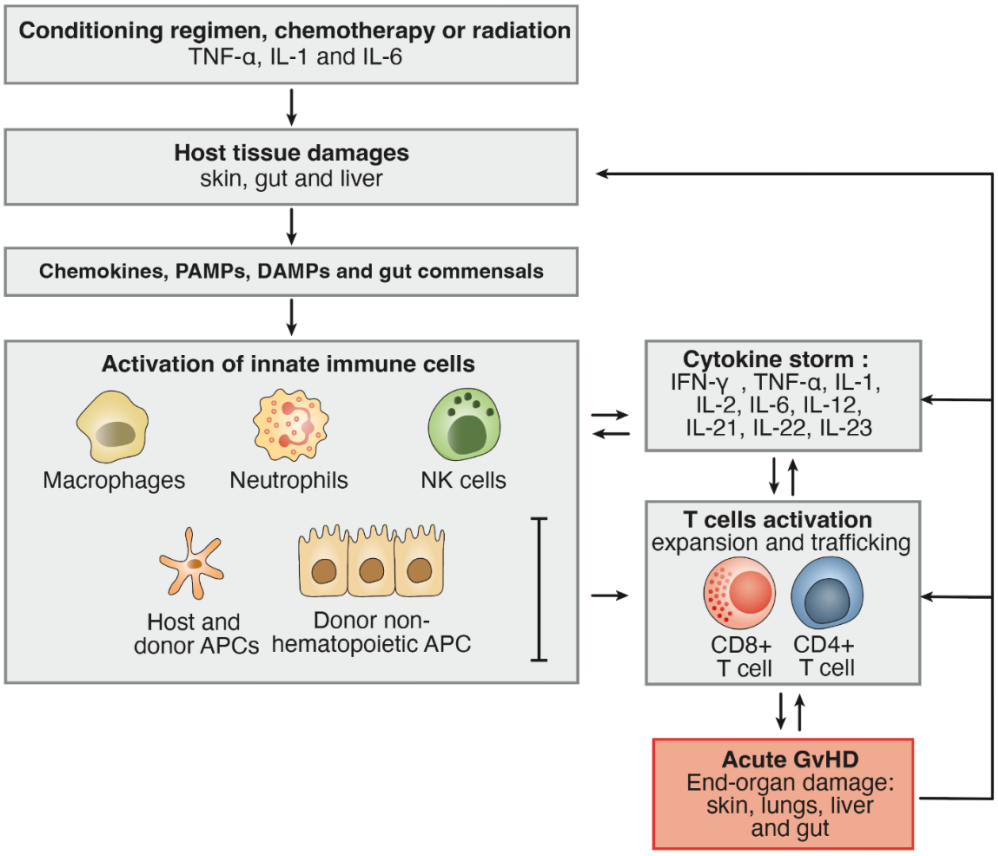

Figure 13: Acute GvHD pathogenesis. Initiation of aGvHD often involves conditioning regimen and host tissue damages that activate innate immune cells through the release of chemokines, pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and gut bacteria. Activated innate immune cells release massive inflammatory cytokines, a process called “cytokine storm”. Activated host and donor antigen presenting cells (APCs), along with pro-inflammatory cytokines, activate T cells that in turn expand and migrate to target organs and further reinforce the cytokine storm. Effector T cells are the main drivers of end-organ damages that generate a feedback loop that perpetuate the process.

- **TGF-β1 in aGvHD**

Most experimental evidence point towards a protective effect of TGF-β1 against aGvHD, quite expectedly given the dominant immunosuppressive functions TGF-β1 on most immune cells i [318, 319]. In an MHC-mismatched mouse model of GvHD, administration of the pan TGF-β neutralizing antibody increased allo-reactive donor T cell proliferation, resulting in increased aGvHD severity. This study suggests that allo-reactive T cells are a main source of TGF-β, underlying the importance of autocrine TGF-β signals in limiting T cell activation [320]. TGF-β signaling through SMAD3 was also shown to protect from aGvHD by controling Th1- and neutrophil- mediated cytotoxicity [321]. In a xenogenic model of GvHD induced by transfer of human PBMCs in immunodeficient NSG mice, co-transfer of human Tregs delayed the occurrence of xenogenic GvHD, unless TGF-β1 activation was blocked with anti-GARP:TGF-β1 or neutralized with anti-TGF-β mAbs [17]. TGF-β1 also favors differentiation of DCs in a tolerogenic phenotype [322], which in turn induce pTreg through TGF-β1 production [65, 323]. Exposure of DCs to TGF-β1 prior to their *in vivo* administration favors this tolerogenic DCs and prevent aGvHD in mice [323].

A few pieces of evidence confirm a benefic role for TGF-β1 in patients. Two studies observed a fall in TGF-β levels in the plasma or serum of HCT patients before the onset of aGvHD [324, 325]. These decreases were correlated with a decrease in circulating $CD4^+CD25^+$ T cells [326]. Skin biopsies from patients with aGvHD display reduced levels of TGF-β mRNA transcripts compared to biopsies from patients with cGVHD [327]. Similarly, pre-transplantation levels of SMAD3 transcripts in donor T cells was shown to predict for the occurrence of both aGVHD and cGVHD in patients [328]. Some studies highlighted association between aGVHD incidence and polymorphisms in the *Tgfb1* or *Tgfbr2* genes [328-330].

2.5.2. Chronic GvHD

From damages caused by the conditioning regimen to recruitment of Th1/Tc1 and Th17/Tc17, the early inflammation initiating cGvHD rely on the same mechanisms as aGvHD. Thereafter, the biological mechanisms underlying cGvHD are not as well understood as those leading to aGvHD, and both patients and mouse models can present heterogeneous phenotypes rarely recapitulated in a single individual [331].

A key hallmark of cGvHD is the dysregulation of T cell and B cell immunity, ensuing from damages caused by early inflammation (Fig.14). Besides naïve and mature donor T cells, the graft also contains donor T lymphoid progenitors that are supposed to migrate and achieve their development in the host thymus, becoming donor mature T cells that are tolerant against recipient self-antigens. However, the conditioning regimen and the allo-reactive T cells also damage the host thymus, leading to a failure of central tolerance mechanisms and promoting production of auto-reactive T cells and impairing production of tTregs [332-335]. This impact of impaired thymopoiesis on cGvHD

is notably sustained by the fact that reducing thymus injury by administration of keratinocyte growth factor results in an amelioration of cGvHD [336, 337].

Growing evidence also points to an impaired B cell homeostasis in cGvHD even if the underlying mechanisms have not yet been fully elucidated. Abnormalities of B cell reconstitution is notably characterized by decreased numbers of B cell progenitors and naïve B cells, increased representation of memory B cells, and decreased frequency of regulatory B cells [331, 338]. The implication of B cells in cGvHD pathogenesis is demonstrated by the beneficial outcome of rituximuab, a B-cell-depleting monoclonal antibody (mAb) used in patients with cGvHD refractory to steroid, the first line treatment [339]. Patients with active cGvHD display a delayed B cell reconstitution and high level of B-cell-activating-factor (BAFF), a cytokine essential for B cell survival and maturation. In absence of sufficient numbers of naïve and circulating transitional B cells neutralizing BAFF, this elevated level of the cytokine protects allo- and auto-reactive B cells from negative selection and is associated with auto-antibody production in cGvHD [338, 340, 341]. Dysregulated B cell homeostasis is also characterized by aberrant germinal center (GC) B cell expansion. T follicular helper cells (Tfh) are essential for germinal center formation, where antibody generation and class switching of mature B cells occur. Production of high-affinity class-switched antibodies by B cells activate immune responses and can cause severe damage to target tissues by activating complement or ADCC [342, 343]. Patients with active GvHD also have a low frequency of circulating Tfh, attributed to increased homing to secondary lymphoid organs. These Tfh display a highly activated profile and increased ability to promote immunoglobin secretion and maturation [344]. Different studies identified allo- or auto- antibodies production in cGvHD patients and Ig deposition has been found in liver and lung tissues from human biopsies [345-348]. This production of auto- and allo-antibodies contributes to tissue damages and fibrosis in cGvHD.

A hallmark of cGvHD is the development of sclerotic lesions that can develop in almost every organ and results from aberrant tissue repair and fibrosis. Th17, TGF-β and PDGF are thought to be the main mediators involved in this sclerotic process. Chronic inflammation driven by antibodies and Th17 activates macrophages that produces TGF-β, PDGF and MMPs. These mediators activate fibroblasts which produce excessive extracellular matrix collagen fibers, responsible of the thickening characterizing fibrosis encounter in cGvHD [349-353].

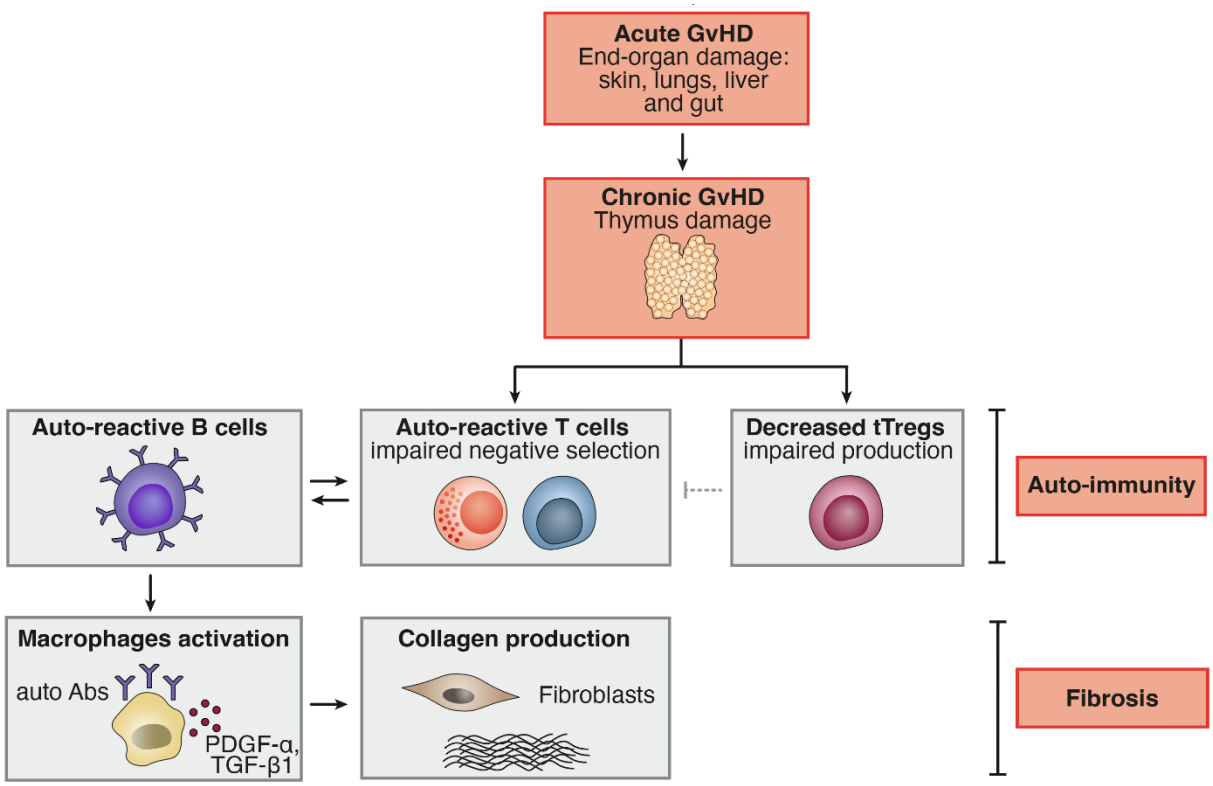

Figure 14: Chronic GvHD pathogenesis. Acute inflammation damages the thymus, leading to circulation of auto-reactive T cells and decreased tTreg production. For reasons that remain elusive, B cell homeostasis is also dysregulated and results in auto-reactive B cells producing auto-antibody, which activates macrophages and contribute to fibrosis. Auto-immune and fibrotic features are key hallmarks of cGvHD.

- **TGF-β1 in cGvHD**

Due to its implication in fibrosis, TGF-β is incriminated as a major contributor to cGvHD pathogenesis. Indeed, the blockade of TGF-β in a cGvHD model with the pan TGF-β neutralizing antibody decreases TGF-β production by myeloid cells and reduces skin, GI and lung fibrosis [320, 354]. Mouse sclerotic skin biopsy specimens contained high levels of *Tgfb1* gene transcripts compared with aGvHD skin lesions, an observation that corroborates a similar study on skin biopsies from patients [327].

Besides this clear effect in promoting fibrosis, the role of TGF-β1 in the other components of physiopathology is less clear. A correlative study has shown that the development of cGvHD is associated with increased levels of serum TGF-β1 [355]. In contrast to this increased serum level, gene array analyses have shown that decreased expression of TGF-β signaling components in donor $CD4^+$ and $CD8^+$ T cells correlates with increased cGVHD incidence in recipients [328]. Several studies in mouse models and cGvHD patients suggest a protective role of Tregs in cGvHD [356-359].

No formal link with TGF-β1 activation has been established but it is well accepted that Tregs depends at least partially on this cytokine for T cell suppression [360], so it is reasonable to think that TGF-β1 delivered by Tregs may be protective in acute and chronic GvHD. Finally, aGvHD and chronic inflammation are two known risk factors for the development of cGvHD that can be limited by TGF-β1. Thus, the context in which TGF-β1 is produced and its target cells may have radically different outcomes on cGvHD. While TGF-β1 appears to protect against early and chronic inflammation primarily mediated by T cells, it would be detrimental in the process of fibrosis driven by myeloid cells.

2.5.3. Graft-versus-Tumor effect (GvT)

The success of allo-HCT for the treatment of hematological malignancies rely on the Graft versus Tumor effect (GvT), an immune-mediated reaction by engrafted donor T cells against tumour cells. However, immunosuppressive therapies used to reduce acute and chronic GvHD compromise GvT and thus increase the risk of relapse. Current research focuses on the difficult challenge to minimize excessive GvHD without abrogating the desired GvT effect [304].

GvT effect mostly relies on donor allo-reactive T cells that recognize and eradicate recipient tumoral cells through recognition of minor histocompatibility antigens or, less frequently, haematopoietic or leukaemia-specific antigens. Thus, a broad T cell inhibition compromises the GvT effect. Mounting a specific GvT response may require fewer T cells or other T cell subsets than GvHD [304]. Mouse models and preclinical studies support a role for naïve T cell in inducing mostly GvHD, as opposed to central memory T cells which would exert mostly GvT activity [336]. These observations have motivated a first clinical trial where 138 patients with acute leukemia received peripheral blood stem cells that were depleted of naïve T cell from HLA-matched related or unrelated donors. These allografts resulted in very low incidences of severe acute GvHD and no cGVHD, without apparent increased risk of relapse or non-relapse mortality [361]. However, a recent review highlighted the importance to further clarify the role of naïve T cells in GvHD, as these cells represent a heterogenic population. Indeed, cord blood grafts induce low rates of GvHD although the contain almost exclusively naïve T cells [362].

The co-infusion of allo-reactive T cells and Tregs, either freshly isolated or expanded *in vitro*, is one of the most promising therapy under study for acute and chronic GvHD [304]. Tregs are intensively studied for their potential to decrease GvHD through their inherent immunomodulatory functions. Treg numbers are decreased during GvHD, owing to a pro-inflammatory environment largely favoring other Th responses. In mouse models, blocking cytokines involved in GvHD pathogenesis such as IL-6 or IL-27 allowed for an increase of Treg reconstitution and attenuation of aGvHD [363, 364]. These results corroborate with the low incidence of aGvHD observed in a phase I/II trial with a prophylactic dose of tocilizumab, a neutralizing anti-IL-6 mAb [365]. Many studies in mouse models demonstrated the protective effect of adoptive transfer of Tregs against GvHD without

abrogation of the GvT effect, and Tregs could even contribute to GvT effect by cytolytic activities [366-370]. First clinical trials have demonstrated that Treg infusion during alloHCT can prevent GvHD without impairing immune reconstitution and GvT effect [371-376].

Th9/Tc9 cells, whose differentiations require IL-4 and TGF-β1, are emerging as another immunomodulating cell type with both pro- and anti-inflammatory actions able to control GvHD while promoting anti-tumor responses. Numerous studies have demonstrated the anti-tumor effect of Th9 both towards solid and hematological tumors [377-381]. The adoptive transfer of donor Th9 cells differentiated *in vitro* confirmed this antitumoral cytotoxicity while showing the absence of graft-versus-host disease, supporting Th9 cells as an attractive candidate for donor lymphocyte infusions to improve long-term tumor-free survival [381, 382]. However, the exact mechanisms underlying these effects and the relevance in patients remain to be established.

Finally, donor NK cells are also extensively studied for their potential in mediating a GvT effect. Indeed, the early reconstitution of NK cells after allo-HCT and their inherent cytotoxicity against transformed or virally infected cells make them particularly attractive candidates for adoptive transfer to inhibit both cancer relapse and infections [304]. Several encouraging studies confirmed the benefic effects of adoptive transfer of NK cells on GvT effect, notably in myeloid malignancies [383-385]. However, concerns about the potential contribution of NK cells to GvHD disease remain. Most clinical studies showed no marked exacerbation of GvHD or toxicities, but clinical evidence indicates that NK cells can in some cases promote GvHD. NK cells are generally thought to suppress GvHD by repressing alloreactive T cell responses but, in some contexts, their hyperactivation leads them to produce proinflammatory cytokines that sustain T cell mediated GvHD induction [386].

- **TGF-β1 in GvT**

The roles played by TGF-β1 in tumor biology have been extensively studied and ranges from tumor-suppression by cytostatic and pro-apoptotic activities in pre-malignant cells to tumor-promotion by favoring metastasis, cell survival, angiogenesis and immunosuppressive microenvironment in advanced cancer. Owing to these last roles favouring cancer progression, multiple anti-TGF-β1 biopharmaceuticals are being tested in clinical trials [318, 387]. TGF-β1 regulates key features of normal and malignant hematopoiesis. In normal hematopoiesis, TGF-β1 controls proliferation and maintains quiescence of hematopoietic stem cells (HSCs) [388-391]. To escape these cytostatic effects, hematologic tumor cells frequently develop genetic alterations in the TGF-β signaling pathway [392-396]. TGF-β1 is also described as promoting myeloid-biased HSCs subtype, a feature that could be diverted to redistribute HSCs subpopulations described in malignant hematopoiesis such as in chronic lymphocytic leukemia [396, 397]. An excessive TGF-β signaling could also contribute to the intense fibrotic reaction observed in myelofibrosis and nodular sclerosing Hodgkin disease [398, 399]. Finally, TGF-β1 has also been shown to impair T-cell

mediated immune clearance of EL4 lymphoma and B16 melanoma as mice expressing dnTbRII specifically in T cells could prevent the development of these malignancies [400].

Direct evidence of a deleterious or benefic effect of TGF-β1 on GvT effect is scarce. In the previously cited experiments of Hill and colleagues [320], antibody-mediated TGF-β1 neutralization in mice surprisingly attenuated the GvT effect against an injectable hematopoietic cancer while accentuating aGvHD. This finding is consistent with other studies sustaining a model in which severe inflammation occurring during acute GvHD promotes T cell dysfunction and death that can impede GvT effect [401-403]. However, a more targeted inhibition of TGF-β1 could be beneficial for the GvT effect, especially at the tumor cell-CTL synapse. In an allo-HCT mouse model, the abrogation of TGF-β1 secretion by breast cancer cells (via transfection of a *Tgfb1* antisens vector) resulted in tumor rejection [404]. On the other side of the synapse, inhibition of TGF-β1 signaling restricted to antigen-specific cytotoxic T cells represents a promising approach to block immune cells within the tumor environment. This was notably demonstrated in a model of Epstein-Barr virus-positive lymphoma, a TGF-β–rich B cell neoplasm, where engineered EBV specific T cells expressing dnTbRII displayed an enhanced anti-lymphoma activity compared to unmodified T cells [405, 406].

The potent anti-cancer effects exerted by NK cells mainly rely on cytotoxic mechanisms that are generally antagonized by TGF-β1 [407, 408]. In addition, TGF-β1 could hinder NK cells reconstitutions.
Paradoxically, NK cells activated *in vitro* with IL-2 protects from GvHD in a TGF-β dependent manner [409, 410]. Regulatory cells induced by TGF-β1, such as Tregs and tolerogenic DCs, could protect from aGvHD without impairing GvT effects. The tolerogenic DCs induced by TGF-β1 were also shown to protect mice from leukemia relapse [323].

OBJECTIVES OF THE WORK

OBJECTIVES OF THE WORK

Our laboratory's research centers on the role of the transmembrane protein GARP in TGF-β1 activation. A few years ago, we derived mAbs that block TGF-β1 activation specifically from GARP:TGF-β1 complexes on mouse or human cells. Solving the crystal structure of GARP:TGF-β1 bound by a Fab fragment from a blocking mAb yielded precious information on the epitope bound by the antibody and its mode of action. While this work elucidated for the first time the structure of latent TGF-β1 presented by GARP, the precise molecular events leading to activation of the cytokine were not completely resolved yet.

Using a blocking anti-human GARP:TGF-β1 mAb, we could show that GARP-mediated TGF-β1 activation was required for immunosuppression exerted by human Tregs in a model of xenogeneic GvHD in NSG mice. We also demonstrated the anti-tumor potential of blocking anti-mouse GARP:TGF-β1 mAbs in both solid and liquid murine cancer models. This compelling evidence prompted us to propose GARP as a promising target for cancer immunotherapy, and an anti-human GARP:TGF-β1 mAb is now tested in a phase I clinical trial ((ClinicalTrials.gov: NCT03821935))

While its excessive activity is deleterious in cancer, TGF-β1 is required to maintain immune homeostasis. The objectives of my research work were threefold:

1. Derive mAbs able to enhance TGF-β1 activation, rather than block it.

2. Use TGF-β1 activating mAbs to obtain insights into the structural changes leading to TGF-β1 activation.

3. Investigate the effects of increasing TGF-β1 activity in diseases characterized by excessive immune responses such as allo- or auto- immune diseases.

The article presented in the results section describes how we succeeded in obtaining such TGF-β1 activating mAbs, the mechanistic insights grasped from biochemical and structural analyses conducted using these mAbs, and their therapeutic efficacy in a murine model of Graft-versus-host disease.

RESULTS

Contribution of the authors

The article presented in the following section represents a collaborative effort, with my role as a co-first author being central in several key aspects. I took the lead in a substantial portion of the work, overseeing the phage display selection experiments for a subset of the monoclonal antibodies (mAbs) generated by arGEN-X. Additionally, I conducted TGF-β1 reporter assay experiments for both screening (depicted in Fig.1A-C) and diving into the molecular mechanisms of the mAbs (illustrated in Fig.2). The experiment highlighted in Fig.2B was executed by Séverine Wautier and Mathieu Jamez under the guidance of Camille Michiels to summarize in one experiment several experiments I performed. For mapping the epitope, I handled mutagenesis, transfections, and flow cytometry experiments, except for Ala-mutants provided by arGEN-X (depicted in Fig.1D-F and S1). The Cryo-EM structures' generation and analysis in Fig.3 and Fig.S2-S4 were carried out by Jan Felix and Savvas Savvides (VIB). I made the first observations, interpretations, and propositions on the molecular mechanisms of TGF-β1 activation illustrated in Fig.4. GvHD experiments were conducted by me, initially supervised by Mélanie Gaignage and with the final experiments assisted by Séverine Wautier (Fig.5-6 and Fig.S5-S7), who performed RT-qPCR procedures. I also verified the absence of contaminating TGF-β1 in the antibody preparations (Fig.S8).

Antibody-mediated TGF-β1 activation to treat auto- and allo-immune diseases

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SUMMARY

TGF-β1 is an immunosuppressive cytokine produced as a latent homodimer, in which mature TGF-β1 is encapsulated and kept inactive by the Latency Associated Peptide (LAP). Transmembrane protein GARP presents latent TGF-β1 on the surface of Tregs to enable activation and release of mature TGF-β1 by integrins. Here, we derived monoclonal antibodies (mAbs) that activate human or mouse TGF-β1 anchored on cells by a transmembrane protein. Biochemical and structural studies by single-particle 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-β1 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-β1 in vivo is achievable with mAbs, introducing new immunotherapeutic options for allo- or auto-immune diseases characterized by deleterious T cell activity insufficiently controlled by Tregs.

Keywords

Immunosuppression; immunotherapy; adaptive immunity; regulatory T cells (Tregs); cytokines; transforming growth factor beta 1 (TGF-β1); monoclonal antibodies; autoimmunity; immune-related diseases

INTRODUCTION

Due to the potent immunosuppression it exerts in mice and humans, production of Transforming Growth Factor β1 (TGF-β1) is tightly regulated at a post-translation level, prior to its binding to a hetero-tetrameric receptor composed of TβRI and TβRII chains. Mice with a germline deletion of the *Tgfb1* gene or a T cell-specific deletion of *Tgfbr2* die 3 to 4 weeks after birth from a lymphoproliferative multisystemic autoimmune syndrome, indicating that TGF-β1 activity in T cells is required to maintain immune tolerance.[1-3] Due to the widespread expression of the TGF-β receptor, TGF-β1 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-β1 is a tightly regulated multi-step process.[5] Translation of *TGFB1* mRNA leads to a precursor comprising a signal peptide followed by a 25 kDa amino-acid (aa) sequence called LAP (Latency Associated Peptide) and a 12.5 kDa sequence corresponding to the mature TGF-β1 cytokine. After signal peptide removal and interchain disulfide bond formation, the pro-TGF-β1 homodimer is cleaved by a furin convertase. The resulting LAP and mature TGF-β1 homodimers remain non-covalently associated, forming a complex called “latent TGF-β1” because LAP prevents binding of mature TGF-β1 to its receptor. Structural studies by X-ray crystallography revealed that the N-terminal a1 and a2 helixes and an intervening loop called the latency lasso act as a straitjacket embracing the extremities of the mature TGF-β1 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.[7] Whereas most cell types and virtually all immune cells produce latent TGF-β1, only very few release mature TGF-β1 from the LAP straitjacket. This process is referred to as “TGF-β1 activation”. It is required to induce autocrine or paracrine TGF-β1 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 TGF-β1 by a mechanism that requires transmembrane proteins GARP and integrin $\alpha_V\beta_8$.[8-11] In the endoplasmic reticulum of Tregs, latent TGF-β1 is covalently linked to the extracellular leucin-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-β1, which can then bind integrin $\alpha_V\beta_8$ via an RGD motif in the arm region of LAP. T cell receptor stimulation of Tregs induces surface expression of GARP:(latent)TGF-β1 complexes and $\alpha_V\beta_8$, leading to TGF-β1 activation and autocrine or paracrine signaling in Tregs and neighboring cells. Another LRR-containing

transmembrane protein called LRRC33 presents latent TGF-β1 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-β1 presented by GARP or LRRC33 to $\alpha_V\beta_8$ are not completely resolved yet. Most studies suggest exertion of mechanical forces on latent TGF-β1 to release mature TGF-β1. A major mechanistic hypothesis is that application of pulling forces on latent TGF-β1 in opposing directions would unroll the LAP straitjacket and release mature TGF-β1 in the extra-cellular 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-β1 for binding to the cytokine receptor.[15]
We previously derived anti-GARP:TGF-β1 monoclonal antibodies (mAbs) that block TGF-β1 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.[16-18] Phase 1 and 2 trials are currently ongoing to test blocking anti-GARP:TGF-β1 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-β1 activation and Treg immunosuppression, with the objective to develop therapeutic tools for 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-β1 and Fab fragments reveals that TGF-β1 activation by a mAb requires crosslinking of at least two membrane-bound GARP:TGF-β1 complexes on cells and a profound conformational change in latent TGF-β1, shedding light on the molecular events that occur during TGF-β1 activation by integrins *in vivo*.

RESULTS

Monoclonal antibodies that activate human or mouse TGF-β1 recognize epitopes close to the LAP homodimerization interface

We screened 134 mAbs directed against human or mouse GARP, GARP:TGF-β1 or latent TGF-β1 for their ability to activate TGF-β1 in a reporter assay. 293T cells were co-transfected to express surface human or mouse GARP:TGF-β1 complexes, *Firefly* luciferase under the control of a TGF-β1-responsive promoter, and *Renilla* luciferase to normalize for transfection efficacy. Luciferase activity was measured in transfected cells 18 hours after incubation with mAbs. Fourteen mAbs activated TGF-β1 in the assay (**Figure 1A-B**): six are directed against human latent TGF-β1 (LHT-4, 15, 18, 20, 22, 25), and eight against mouse latent TGF-β1 (LMT-2, 5, 6, 7, 9, 10, 11, 12). None are directed against GARP or GARP:TGF-β1 complexes, and none are cross-reactive against human and mouse TGF-β1. Activation occurred at mAb concentrations in the nanomolar range (**Figure 1C**). Because activating mAbs are not cross-reactive, we used human/mouse (h/m) TGF-β1 chimeras to map their epitopes. Most aa differences between human and mouse TGF-β1 are localized in LAP, downstream of a highly conserved region comprising helixes α1 and α2 and the latency lasso (**Figure 1D-E**). We constructed plasmids encoding hemagglutinin (HA)-tagged h/mTGF-β1 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 TGF-β1 in which a single residue was replaced by alanine allowed to further refine binding requirements. 293T cells were co-transfected to express wild type, chimeric or single-Ala mutant TGF-β1 in complex with mouse GARP and analyzed for binding to mAbs by flow cytometry (**Figure S1A-C**). 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 supplementary material show that all activating mAbs bind loops close to the LAP dimerization interface, previously described as critical to maintain TGF-β1 latency by keeping the LAP monomers welded together[7] (**Figure 1E**). To activate human TGF-β1, LHT-22 requires residues W207, L208, G211, G212, I214, E215 and F217. To activate mouse TGF-β1, LMT-12 requires residues L208 and D212 (**Figure 1F**). LHT-22 and LMT-12 epitopes in human and mouse TGF-β1, respectively, are very similar to each other and are not shared with non-activating mAbs.

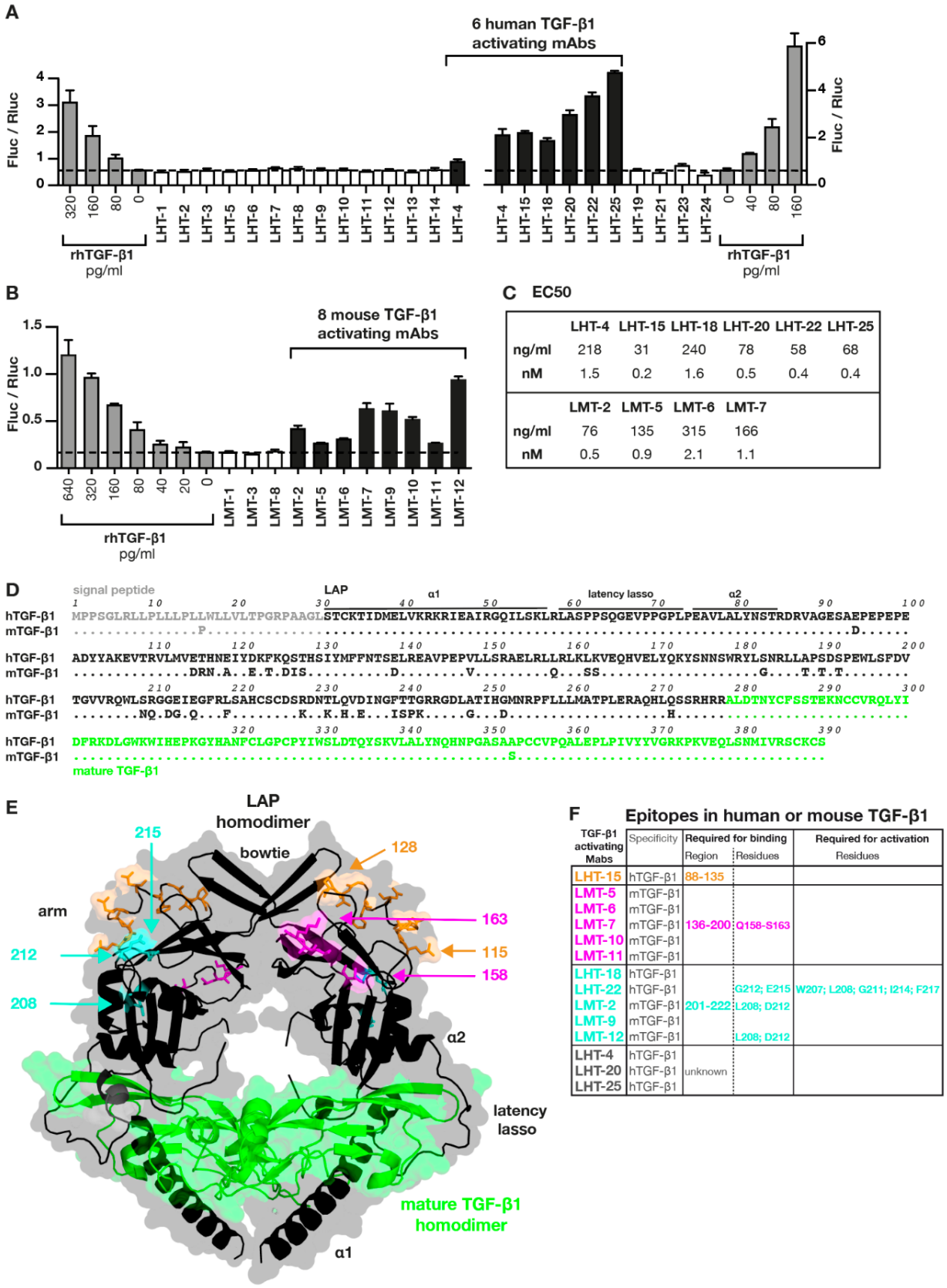

Figure 1. Human and mouse TGF-β1 activating mAbs bind close to the LAP dimerization interface

(A) 293T cells were co-transfected to express human GARP:TGF-β1 complexes, Fluc under the control of a *CAGA* element as a reporter for TGF-β1 activity, and Rluc as a transfection control, then incubated with the indicated mAbs (20 µg/ml) or serial dilutions of active rhTGF-β1. Bar graphs represent ratios of Fluc to Rluc activity (mean of triplicates + SD). Black bars highlight the 6 LHT activating mAbs (*i.e.* Fluc/Rluc in presence of the mAb > Fluc/Rluc in absence of rhTGF-β1 in ≥3 independent experiments). The dotted line shows the FLuc/Rluc ratio in absence of rhTGF-β1 or mAb. **(B)** Same as in (A), except that 293T cells were transfected with mouse GARP:TGF-β1. Black bars highlight the 8 LMT activating mAbs. **(C)** Half maximum effective concentrations (EC50) were measured by performing the reporter assay as in (A) or (B) with serial dilutions of mAbs. **(D)** Annotated alignment of h(uman)TGF-β1 and m(ouse)TGF-β1 sequences. Magenta box: region required for binding by mAbs LMT-5, 6, 7, 10, and 11. Turquoise boxes: residues required for binding and activation by LMT-12 and LHT-22. **(E)** Tridimensional structure of porcine latent TGF-β1.[7] Black: LAP. Green: mature TGF-β1. 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-β1 activating mAbs. Residues indicated as required for activation by LHT-22 are not required for binding.

Bivalent binding and membrane anchorage of latent TGF-β1 via GARP or LRRC33 is required for activation by mAbs

We tested 4 LMT antibodies as full length mAbs, $F(ab')_2$ or Fab fragments in the 293T reporter assay. They all activated mouse TGF-β1 when used as mAbs or $F(ab')_2$, but not as Fabs, indicating that bivalent binding is required to activate TGF-β1 (**Figure 2A**). 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-β1 on the Treg surface. We examined if membrane anchorage by GARP was required for mAb-mediated TGF-β1 activation. Reporter cells were transfected to express mouse TGF-β1 alone or in complex with full-length GARP ($GARP_{FL}$), GARP truncated from its transmembrane domain ($GARP_{\Delta TM}$), or LRRC33, another LRR-containing transmembrane protein known to covalently bind and present TGF-β1 on the surface of myeloid cells. As expected, large amounts of TGF-β1 were found in the lysates or supernatants of 293T cells transfected with TGF-β1 in comparison to controls, with high levels of surface TGF-β1 detected on cells co-transfected with $GARP_{FL}$ or LRRC33 (**Figure 2B**). Surface TGF-β1 was also detected on cells transfected with TGF-β1 alone, albeit at lower levels, indicating that molecule(s) other than LRRC33 or GARP can retain TGF-β1 on the 293T cell surface. Latent TGF-β1 was almost absent on the surface of 293T cells expressing $GARP_{\Delta TM}$, which secrete $GARP_{\Delta TM}$:TGF-β1 complexes in the extracellular space.[19] All mAbs tested (LMT-7, 9, 10 and 12) bound surface TGF-β1 whether it was presented by GARP, LRRC33 or the unidentified molecule(s) expressed in 293T cells. All mAbs activated TGF-β1 presented by $GARP_{FL}$, as expected, and most (LMT-7, 9 and 12) activated TGF-β1 presented by LRRC33. In contrast, none activated TGF-β1 expressed alone or with $GARP_{\Delta TM}$ (**Figure 2B**). These results show that mAbs activate latent TGF-β1 presented on the cell surface by a protein anchored in the membrane via a transmembrane domain.

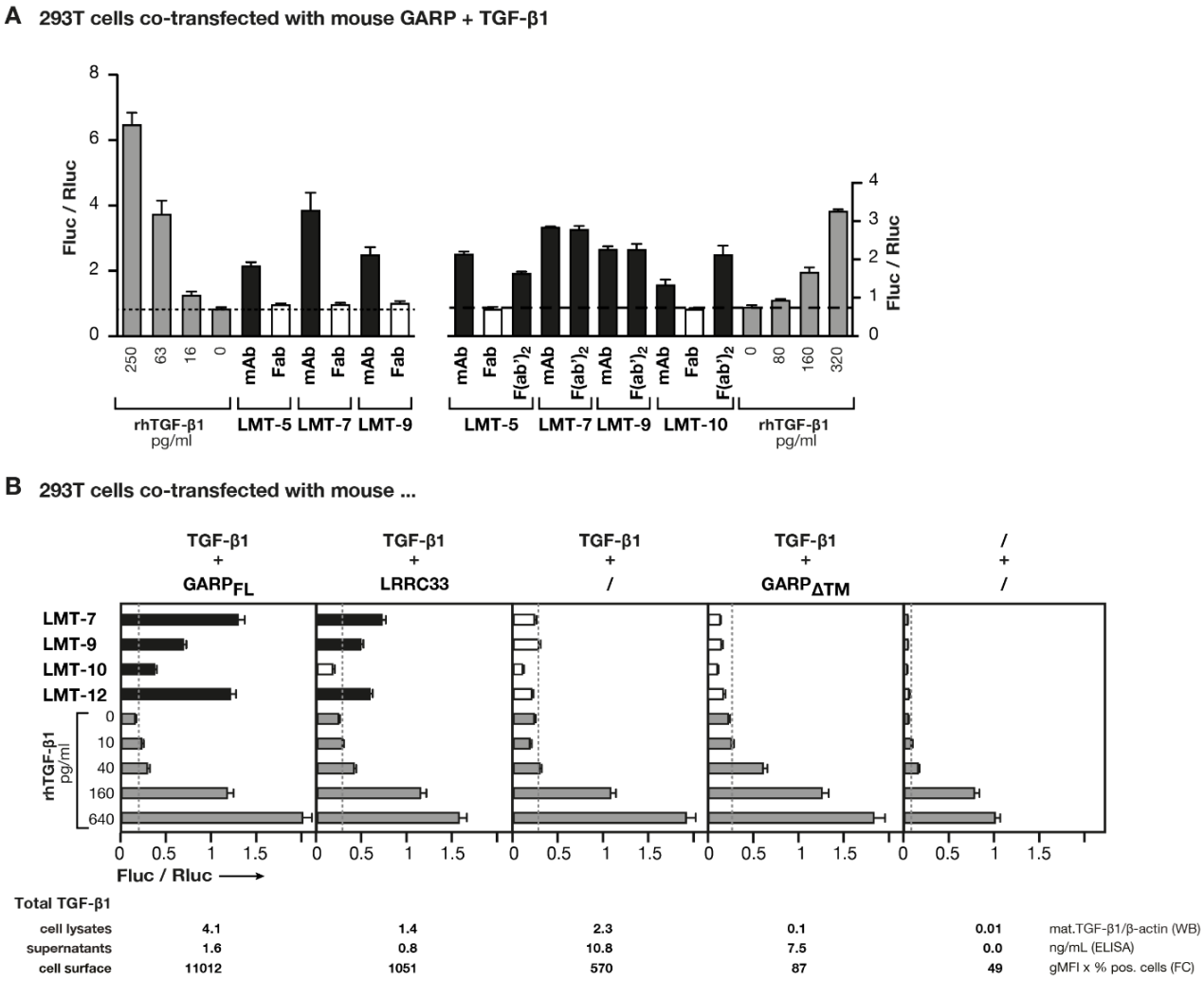

Figure 2. Bivalent binding to and membrane anchorage of latent TGF-β1 by a transmembrane protein are required for activation by mAbs. (A) Reporter assay as in **Figure 1B**, except that transfected cells were incubated with LMT antibodies under mAb, F(ab')$_2$ or Fab formats. Bar graphs represent ratios of Fluc to Rluc activity (mean of triplicate + SD). Black bars highlight antibody formats that activate TGF-β1. Dotted line shows FLuc/Rluc in absence of rhTGF-β1 or mAb. **(B)** 293T cells co-transfected with the indicated molecules were analyzed by Western Blot (top), flow cytometry (middle) and in a reporter assay after incubation with the indicated LMT mAbs (bottom).

Molecular and structural mechanism of TGF-β1 activation by mAbs

To obtain insights into the structural basis and mechanism of TGF-β1 activation by mAbs, we sought to determine the structure of human and mouse GARP:TGF-β1 in complex with Fab fragments derived from activating mAbs LHT-22 and LMT-12, respectively, by single particle cryo-electron 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-β1:LHT-22 complex, and a 20° tilt for the mouse GARP:TGF-β1:LMT-12 complex. The acquisition of tilted 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-β1:Fab complexes to a nominal resolution of 2.9 and 3.0 Å, respectively (**Figure S2, Figure S3, 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-β1 (i.e., one LAP_A/LAP_B and one mature TGF-$β1_A$/TGF-$β1_B$ dimer) and two LHT-22 or LMT-12 Fabs (**Figure 3A and B**). 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-β1. In both human and mouse GARP:TGF-β1: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. Therefore, a single GARP:TGF-β1 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-β1 complexes (**Figure 4**). The elucidated structural epitopes agree closely with our epitope mapping data (**Figure S1C-D**), such 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 α4 (136-142), and situates ˜20 Å away from the RGD motif that mediates recruitment of integrin αvβ8. 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-S2**).

TABLE 1

Cryo-EM data collection, refinement and validation statistics

	hGARP-lTGFβ1-Fab LHT-22 complex (EMDB-XXXXX) (PDB XXXX)	mGARP-lTGFβ1-Fab LMT-12 complex (EMDB-YYYYY (PDB YYYY)
Data collection and processing		
Magnification	60,000	60,000
Voltage (kV)	300	300
Electron exposure (e–/Å^2)	61.8	61.8
Defocus range (μm)	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		
Model resolution range (Å)	∞ - 3.20	∞ - 3.50
Map sharpening *B* factor (Å^2)	-87.4	-84.0
Model composition		
Non-hydrogen atoms	12,426	12355
Protein residues	1,609	1588
Ligands	NAG: 11, BMA: 2 MAN: 2	NAG: 8
B factors (Å^2)		
Protein	121.95	138.37
Ligand	93.28	99.16
R.m.s. deviations		
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
Ramachandran Z-score		
Whole	-1.22	-7.71
Helix	-1.38	-1.54
Sheet	-0.70	-0.59
Loop	-0.72	-1.34

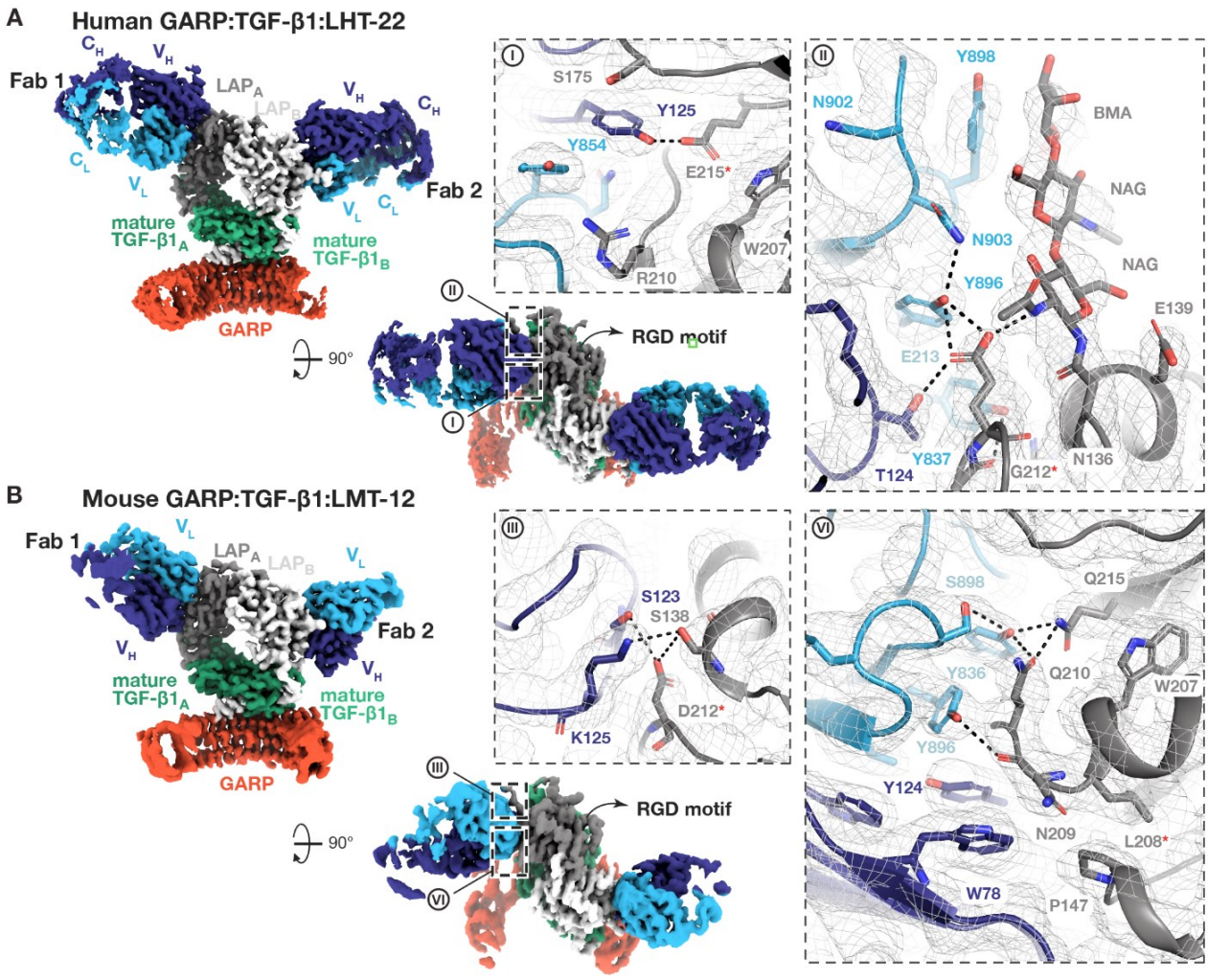

Figure 3. Cryo-EM structure of human and mouse GARP:TGF-β1 bound to Fabs from TGF-β1 activating mAbs. Annotated side and top views of GARP:TGF-β1:Fab complexes. Insets show zooms of the Fab binding epitopes. Structural models are shown as cartoons, with annotated residues shown as sticks, and hydrogen bonds are depicted as black dotted lines. Red asterisks indicate residues identified as binding hot-spots using TGF-β1 single Ala-mutants (**Figure S1**). **(A)** Human GARP:TGF-β1:LHT-22 complexes. **(B)** Mouse GARP:TGF-β1:LMT-12 complexes.

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 (r.m.s.d = 0.667 Å) (**Figure S4**). 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 3_{10} (η1) helixes, or "thumbs", of the mature TGF-β1 monomers, which are fixed by the α1 helixes 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 complex,[14] 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 different cells (in *trans*, **Figure 4B**) is not known. Finally, steric constraints imposed by the LAP α1 helix are poised to prevent binding of mature TGF-β1 to the TβRI receptor chain unless a profound conformational change in LAP and mature TGF-β1 occurs to unwind the LAP straitjacket (**Figure 4C**). The plethora of structural data at hand support such a mechanistic scenario.

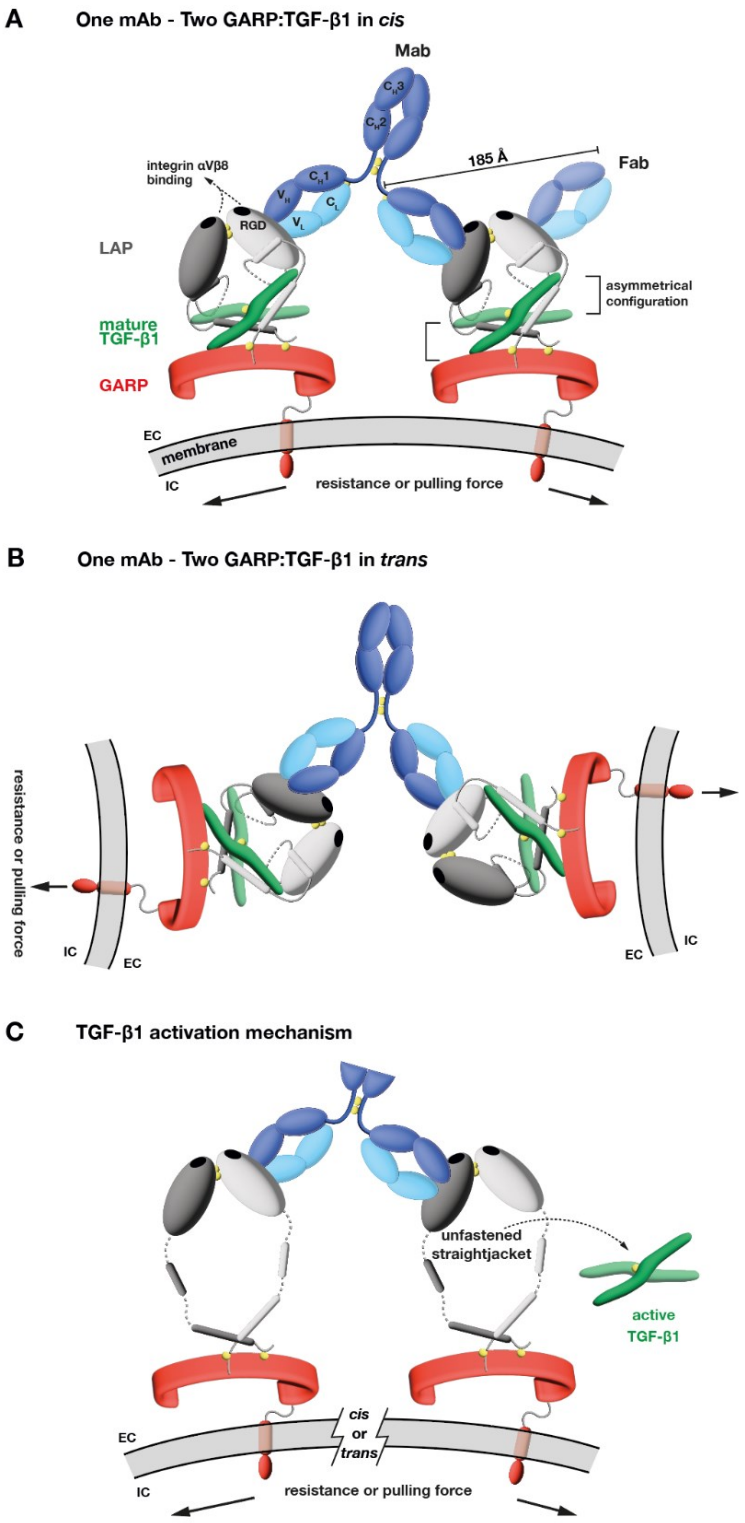

Figure 4. Molecular mechanism of TGF-β1 activation by mAbs. (A-B) Schematic representation of one activating mAb crosslinking two membrane-anchored GARP:TGF-β1 complexes located on the same cell (*cis*) or two adjacent cells (*trans*). **(C)** Resistance or pulling force exerted on two membrane-bound GARP:TGF-β1 complexes crosslinked by one mAb results in unfastening of the LAP straitjacket and release of active TGF-β1.

mAb-mediated TGF-β1 activation *in vivo* reduces disease severity and increases survival in mice with graft-*versus*-host disease (GvHD).

Given that a few cell types including Tregs in humans and mice are known to express GARP:TGF-β1 complexes at their surface, we sought to determine whether the mAbs presented herein could increase TGF-β1 activation and immunosuppression by Tregs in a mouse model of a disease characterized by excessive T cell activity that is insufficiently controlled by Tregs. 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 x DBA/2) recipients and treated the mice with TGF-β1 activating mAbs or isotype controls (**Figure 5A**). Antibodies were administered once a week, starting one day before cell transfer. H-2^b donor-derived T cells induced GvHD-like symptoms (GvHD score ≥1) within 10 to 20 days after transplantation in all recipient mice receiving control antibodies, leading to death of nearly all mice (43 of 44) by day 22 (**Figure 5B**). In contrast, approximately half of the mice treated with LMT-10 or LMT-12 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 LMT-10 or 12 developed GvHD-like symptoms early after cell transfer (day 9-20), indicating that donor alloreactive T cells had engrafted and that the therapeutic activity of the mAbs observed at later time points was not due to inhibition of donor T cell engraftment. Altogether, treatment with LMT-10 and 12 significantly improved survival by comparison to controls, with a median survival increased from 16 to 27 days in mice treated with LMT-10, and > 50% of mice surviving over 30 days in mice treated with LMT-12 (**Figure 5C**).

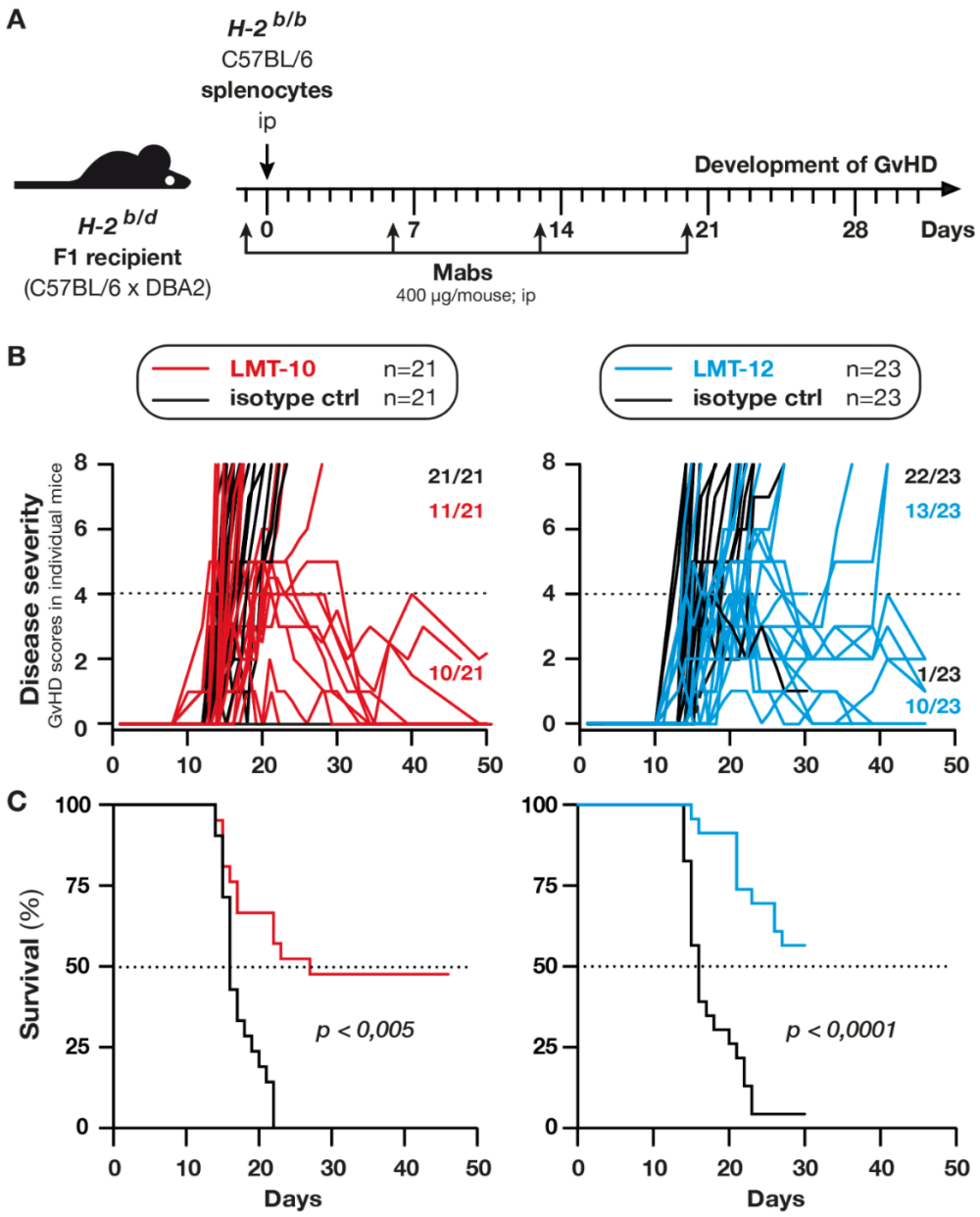

Figure 5. TGF-β1 activating mAbs reduce disease severity and increase survival in a mouse model of GvHD. (A) Schematic representation of the experimental design. H-2^{bd} F1 mice transplanted with H-2^{b} splenocytes (70-90x10^{6}) on day 0 were injected with TGF-β1 activating mAbs or isotype controls once a week, 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. Ratios above the dashed line indicate the proportion of mice dead from GvHD. Ratios below the dashed line indicate the proportion of mice alive 30-50 days after transplantation. **(C)** Kaplan-Meier plots representing the proportion of mice alive at the indicated days after transplantation. P values calculated using a Gehan-Breslow Wilcoxon test. Data pooled from 3 independent experiments.

Tregs are required for the therapeutic activity of TGF-β1 activating mAbs in mouse GvHD. The spleens of recipient mice were collected 9 days after transplantation to analyze cell composition by flow cytometry and gene expression by RT-qPCR. Similar numbers of recipient- and donor-derived $CD4^+$ and $CD8^+$ T cells were observed whether the recipients had received activating or controls mAbs, confirming that activating mAbs do not impair donor T cell engraftment (**Figure S5**). In line with the observed reduction in GvHD severity, mice receiving TGF-β1 activating mAbs had significantly reduced expression of *Tbx21* and *Gzmb1* and tended to express less *Infg* and *Prf1, s*uggesting reduced Th1 and CTL activity (**Figure S6**). In contrast, *Foxp3* expression and numbers of donor-derived Tregs ($CD4^+Foxp3^+$ cells) were significantly increased (~2-3 fold) in the spleen of mice treated with activating mAbs by comparison to controls (**Figure S5-S6**). Numbers of recipient-derived Tregs also tended to increase, but to a lower extent (~1.5-2 fold) and without reaching statistical significance (**Figure S5**).

Tregs are potent immunosuppressive cells that could mediate the therapeutic effects of TGF-β1 activating mAbs. To test this, we performed transplantation experiments using H-2^b *Foxp3*$^{DTR/DTR}$ or *Foxp3*$^{WT/WT}$ females as splenocyte donors and H-2^{bd} *Foxp3*^{DTR} or *Foxp3*^{WT} males as recipients. We injected diphteria toxin (DTx) to deplete Tregs from donor-, recipient- or both origins (**Figure 6** and **Figure S7**).[20] In all experiments as expected, donor splenocytes induced GvHD and LMT-12 increased survival when Tregs were not depleted (**Figure 6**: no DTx). In contrast, when both donor and recipient Tregs were depleted by DTx, the therapeutic activity of LMT-12 was lost, indicating that Tregs are required to mediate this effect (**Figure 6**: Exp. 1). Loss of therapeutic activity was also observed when recipient Tregs, but not donor Tregs, were depleted by DTx (**Figure 6**: Exp. 2 and 3, respectively). This could indicate that recipient, but not donor Tregs are required for therapeutic activity of the mAbs in mouse GvHD. However, it might also simply reflect the fact that recipient Tregs vastly outnumber donor Tregs (~$1.5x10^6$ per graft) in recipient mice, which are not irradiated in this model.

From the above, we conclude that TGF-β1 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.

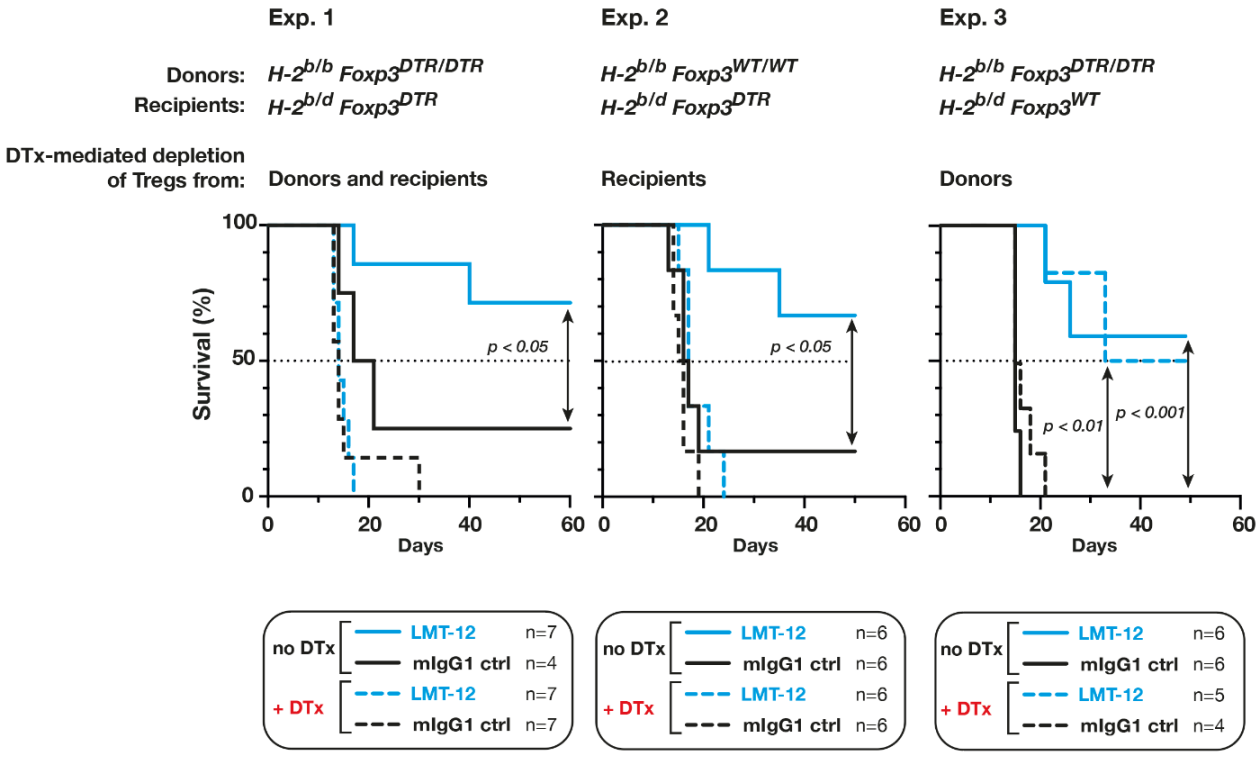

Figure 6. Tregs are required for the therapeutic effect of TGF-β1 activating mAbs. GvHD was induced in three independent experiments as described in Figure 5, by transplanting splenocytes from H-2^{b} *Foxp3*$^{DTR/DTR}$ or *Foxp3*$^{WT/WT}$ female donors to H-2^{bd} *Foxp3*^{DTR} or *Foxp3*^{WT} male recipients on day 0. DTx was injected to donors, recipients or both between day -1 and +1 to deplete Tregs from donor-, recipient- or both origins, as illustrated in **Figure S7**. Kaplan-Meier plots represent the proportion of mice alive at the indicated days after transplantation. P values calculated using a Gehan-Breslow Wilcoxon test (GraphPad Prism).

DISCUSSION

The ubiquitous expression of TGF-β receptors across all cells and the pleiotropic nature of signaling mediated by TGF-β1 imposes tightly regulated maintenance of TGF-β1 latency under homeostatic conditions. To date, all mAbs developed to therapeutically target TGF-β1 aim at blocking its immunosuppressive, pro-tumoral and pro-fibrotic activities in the context of cancer or fibrosis. The mAbs described herein do the opposite: they activate latent TGF-β1 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-β1 binding and activation by mAbs, or Fabs derived from these mAbs, 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-β1 activation by integrins in mammals. Three important observations need to be considered: *i)* in contrast to the activating mAbs, the corresponding Fabs cannot activate latent TGF-β1; *ii)* TGF-β1 activation by mAbs requires anchorage of latent TGF-β1 at the cell surface via transmembrane proteins such as GARP or LRRC33; and *iii)* the binding mode of two Fabs derived from an activating mAb to one GARP:TGF-β1 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-β1 *in vivo* by crosslinking two distinct latent TGF-β1 molecules presented at the cell surface by transmembrane proteins such as GARP or LRRC33.

A major implication of these observations in a therapeutic context, is that such activating mAbs will not activate soluble latent TGF-β1 circulating in the blood or other biological fluids but will only activate TGF-β1 presented at a high density at the cell surface. This allows for specific targeting and activation of latent TGF-β1 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-β1 on their surface and might serve as TGF-β1 reservoirs amenable to activation by therapeutic mAbs, activation of latent TGF-β1 by mAbs on the surface of endothelial cells was not sufficient for exerting therapeutic activity in the absence of Tregs in mouse GvHD experiments. Furthermore, this suggests that cell-free reservoirs of latent TGF-β1 deposited in the extracellular matrix via covalent binding to Latent TGF-β1 Binding Proteins (LTBPs) would not be amenable to mAbs-mediated activation. Indeed, 293T cells used in our analyses are known to express LTBPs[21] but they do not activate TGF-β1 in the presence of mAbs unless they are transfected with GARP or LRRC33. This also requires further confirmation. Nevertheless, the data

presented here suggest that activating mAbs can exert a potent immunosuppressive therapeutic activity *in vivo* in a Treg-dependent manner.

Another implication of our observations is more fundamental: high-affinity binding to the two LAP monomers from a single latent TGF-β1 molecule is not sufficient for TGF-β1 activation, even when binding occurs close to the RGD motifs and dimerization interface. Activation requires profound conformational changes to allow access of the mature TGF-β1 dimer to the TβRI:TβRII heterotetrameric receptor. These changes include not only unmasking mature TGF-β1 residues from the LAP latency lasso to allow interaction with TβRII, but also modification of the mature TGF-β1 helix α3 fixed by LAP α1 in a position precluding interaction with TβRI. Recently, elucidation of the cryo-EM structure of one αVβ8 ectodomain bound to a latent TGF-β1 dimer led to the suggestion that flexibility in TGF-β1 and integrin molecules in the complex is sufficient to expose mature TGF-β1 to a minimal TGF-β1 receptor comprising a single TβRI:TβRII heterodimer instead of the conventional TβRI:TβRII heterotetramer, without complete dissociation of mature TGF-β1 from LAP. The authors suggested that this would result in a minimal TGF-β1 signaling cascade.[15] However, helix α3 of mature TGF-β1 in that structural analysis is in the same position as that observed herein and in previously reported structures of latent TGF-β1, free or bound to GARP, LRRC33 or an antibody fragment.[6,14,22] As such this conformation is grossly incompatible with the proposed interaction between helix α3 in mature TGF-β1 and a TβRI receptor chain upon binding by a single αVβ8, due to severe steric clashes with helix α1 of LAP. Thus, we propose that TGF-β1 activation requires crosslinking of two membrane-bound latent TGF-β1 by one mAb, or alternatively, crosslinking of two membrane-bound integrins by one latent TGF-β1. This would allow the exertion of cellular forces in opposite directions leading to unwinding of LAP and profoundly modify the conformation of TGF-β1, as previously suggested.[13] Importantly, our proposed model is compatible with recent work by Duan et al, who reported a more efficient activation of TGF-β1 presented by GARP or LRRC33 upon binding of two, rather than one single αVβ8 molecule.[22] Our mechanistic proposal also implies that latent TGF-β1 reservoirs deposited in cell-free extracellular matrix would not be activated by soluble activating mAbs, avoiding massive release of active TGF-β1 in tissues that are not inflamed or infiltrated by immune cells.

Our data in a murine model of GvHD indicate that TGF-β1 activating mAbs may serve to treat patients with severe alloreactive immune responses observed during allogeneic bone marrow or solid organ transplantation. Several severe auto-immune 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. Auto-immune 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-β1 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-β1 activity.

Collectively, our findings pave the way to clinical trials with TGF-β1 activating mAbs to investigate a targeted immunosuppressive therapy for diseases characterized by excessive immune activity insufficiently controlled by Tregs or TGF-β1.

REFERENCES

1. Kulkarni, A.B., Huh, C.G., Becker, D., Geiser, A., Lyght, M., Flanders, K.C., Roberts, A.B., Sporn, M.B., Ward, J.M., and Karlsson, S. (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc. Natl. Acad. Sci. U S A *90*, 770-774. 10.1073/pnas.90.2.770.
2. Marie, J.C., Liggitt, D., and Rudensky, A.Y. (2006). Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. Immunity *25*, 441-454. 10.1016/j.immuni.2006.07.012.
3. Shull, M.M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., and et al. (1992). Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature *359*, 693-699. 10.1038/359693a0.
4. Travis, M.A., and Sheppard, D. (2014). TGF-beta activation and function in immunity. Annu Rev Immunol *32*, 51-82. 10.1146/annurev-immunol-032713-120257.
5. ten Dijke, P., and Arthur, H.M. (2007). Extracellular control of TGFbeta signalling in vascular development and disease. Nat Rev Mol Cell Biol *8*, 857-869. 10.1038/nrm2262.
6. Shi, M., Zhu, J., Wang, R., Chen, X., Mi, L., Walz, T., and Springer, T.A. (2011). Latent TGF-β structure and activation. Nature *474*, 343-349. 10.1038/nature10152.
7. Shi, M., Zhu, J., Wang, R., Chen, X., Mi, L., Walz, T., and Springer, T.A. (2011). Latent TGF-beta structure and activation. Nature *474*, 343-349. 10.1038/nature10152.
8. Edwards, J.P., Thornton, A.M., and Shevach, E.M. (2014). Release of active TGF-beta1 from the latent TGF-beta1/GARP complex on T regulatory cells is mediated by integrin beta8. J Immunol *193*, 2843-2849. 10.4049/jimmunol.1401102.
9. Stockis, J., Colau, D., Coulie, P.G., and Lucas, S. (2009). Membrane protein GARP is a receptor for latent TGF-beta on the surface of activated human Treg. Eur J Immunol *39*, 3315-3322. 10.1002/eji.200939684.
10. Stockis, J., Fink, W., Francois, V., Connerotte, T., de Smet, C., Knoops, L., van der Bruggen, P., Boon, T., Coulie, P.G., and Lucas, S. (2009). Comparison of stable human Treg and Th clones by transcriptional profiling. Eur J Immunol *39*, 869-882. 10.1002/eji.200838807.
11. Stockis, J., Lienart, S., Colau, D., Collignon, A., Nishimura, S.L., Sheppard, D., Coulie, P.G., and Lucas, S. (2017). Blocking immunosuppression by human Tregs in vivo with antibodies targeting integrin alphaVbeta8. Proc Natl Acad Sci U S A *114*, E10161-E10168. 10.1073/pnas.1710680114.
12. Qin, Y., Garrison, B.S., Ma, W., Wang, R., Jiang, A., Li, J., Mistry, M., Bronson, R.T., Santoro, D., Franco, C., et al. (2018). A Milieu Molecule for TGF-beta Required for Microglia Function in the Nervous System. Cell *174*, 156-171 e116. 10.1016/j.cell.2018.05.027.
13. Dong, X., Zhao, B., Iacob, R.E., Zhu, J., Koksal, A.C., Lu, C., Engen, J.R., and Springer, T.A. (2017). Force interacts with macromolecular structure in activation of TGF-beta. Nature *542*, 55-59. 10.1038/nature21035.
14. Liénart, S., Merceron, R., Vanderaa, C., Lambert, F., Colau, D., Stockis, J., van der Woning, B., De Haard, H., Saunders, M., Coulie, P.G., et al. (2018). Structural basis of latent TGF-beta1 presentation and activation by GARP on human regulatory T cells. Science *362*, 952-956. 10.1126/science.aau2909.

15. Campbell, M.G., Cormier, A., Ito, S., Seed, R.I., Bondesson, A.J., Lou, J., Marks, J.D., Baron, J.L., Cheng, Y., and Nishimura, S.L. (2020). Cryo-EM Reveals Integrin-Mediated TGF-beta Activation without Release from Latent TGF-beta. Cell *180*, 490-501 e416. 10.1016/j.cell.2019.12.030.
16. de Streel, G., and Lucas, S. (2021). Targeting immunosuppression by TGF-beta1 for cancer immunotherapy. Biochem Pharmacol *192*, 114697. 10.1016/j.bcp.2021.114697.
17. Bertrand, C., Van Meerbeeck, P., de Streel, G., Vaherto-Bleeckx, N., Benhaddi, F., Rouaud, L., Noel, A., Coulie, P.G., van Baren, N., and Lucas, S. (2021). Combined Blockade of GARP:TGF-beta1 and PD-1 Increases Infiltration of T Cells and Density of Pericyte-Covered GARP(+) Blood Vessels in Mouse MC38 Tumors. Front Immunol *12*, 704050. 10.3389/fimmu.2021.704050.
18. Lecomte, S., Devreux, J., de Streel, G., van Baren, N., Havelange, V., Schroder, D., Vaherto, N., Vanhaver, C., Vanderaa, C., Dupuis, N., et al. (2022). Therapeutic activity of GARP:TGF-beta1 blockade in murine primary myelofibrosis. Blood *141*, 490-502. 10.1182/blood.2022017097.
19. Wang, R., Zhu, J., Dong, X., Shi, M., Lu, C., and Springer, T.A. (2012). GARP regulates the bioavailability and activation of TGFβ. Mol. Biol. Cell *23*, 1129-1139. 10.1091/mbc.E11-12-1018.
20. Kim, J.M., Rasmussen, J.P., and Rudensky, A.Y. (2007). Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nat Immunol *8*, 191-197. 10.1038/ni1428.
21. Walton, K.L., Makanji, Y., Chen, J., Wilce, M.C., Chan, K.L., Robertson, D.M., and Harrison, C.A. (2010). Two distinct regions of latency-associated peptide coordinate stability of the latent transforming growth factor-beta1 complex. J Biol Chem *285*, 17029-17037. 10.1074/jbc.M110.110288.
22. Duan, Z., Lin, X., Wang, L., Zhen, Q., Jiang, Y., Chen, C., Yang, J., Lee, C.H., Qin, Y., Li, Y., et al. (2022). Specificity of TGF-beta1 signal designated by LRRC33 and integrin alpha(V)beta(8). Nat. Commun. *13*, 4988. 10.1038/s41467-022-32655-9.
23. Punjani, A., Rubinstein, J.L., Fleet, D.J., and Brubaker, M.A. (2017). cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. Nat. Methods *14*, 290-296. 10.1038/nmeth.4169.
24. Chen, D.S., and Mellman, I. (2013). Oncology meets immunology: the cancer-immunity cycle. Immunity *39*, 1-10. 10.1016/j.immuni.2013.07.012.
25. Cuende, J., Lienart, S., Dedobbeleer, O., van der Woning, B., De Boeck, G., Stockis, J., Huygens, C., Colau, D., Somja, J., Delvenne, P., et al. (2015). Monoclonal antibodies against GARP/TGF-beta1 complexes inhibit the immunosuppressive activity of human regulatory T cells in vivo. Sci Transl Med *7*, 284ra256. 10.1126/scitranslmed.aaa1983.
26. de Streel, G., Bertrand, C., Chalon, N., Lienart, S., Bricard, O., Lecomte, S., Devreux, J., Gaignage, M., De Boeck, G., Marien, L., et al. (2020). Selective inhibition of TGF-beta1 produced by GARP-expressing Tregs overcomes resistance to PD-1/PD-L1 blockade in cancer. Nat Commun *11*, 4545. 10.1038/s41467-020-17811-3.
27. Bruhns, P. (2012). Properties of mouse and human IgG receptors and their contribution to disease models. Blood *119*, 5640-5649. 10.1182/blood-2012-01-380121.
28. Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J.M. (1998). Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J *17*, 3091-3100. 10.1093/emboj/17.11.3091.

29. Naydenova, K., and Russo, C.J. (2017). Measuring the effects of particle orientation to improve the efficiency of electron cryomicroscopy. Nat. Commun. *8*, 629. 10.1038/s41467-017-00782-3.
30. Liebschner, D., Afonine, P.V., Baker, M.L., Bunkoczi, G., Chen, V.B., Croll, T.I., Hintze, B., Hung, L.W., Jain, S., McCoy, A.J., et al. (2019). Macromolecular structure determination using X-rays, neutrons and electrons: recent developments in Phenix. Acta Crystallogr. D. Struct. Biol. *75*, 861-877. 10.1107/S2059798319011471.
31. Sanchez-Garcia, R., Gomez-Blanco, J., Cuervo, A., Carazo, J.M., Sorzano, C.O.S., and Vargas, J. (2021). DeepEMhancer: a deep learning solution for cryo-EM volume post-processing. Commun. Biol. *4*, 874. 10.1038/s42003-021-02399-1.
32. Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and development of Coot. Acta Crystallogr. D. Biol. Crystallogr. *66*, 486-501. 10.1107/S0907444910007493.
33. Pettersen, E.F., Goddard, T.D., Huang, C.C., Meng, E.C., Couch, G.S., Croll, T.I., Morris, J.H., and Ferrin, T.E. (2021). UCSF ChimeraX: Structure visualization for researchers, educators, and developers. Protein Sci. *30*, 70-82. 10.1002/pro.3943.
34. Lin, Z., Akin, H., Rao, R., Hie, B., Zhu, Z., Lu, W., Smetanin, N., Verkuil, R., Kabeli, O., Shmueli, Y., et al. (2023). Evolutionary-scale prediction of atomic-level protein structure with a language model. Science *379*, 1123-1130. 10.1126/science.ade2574.
35. Kidmose, R.T., Juhl, J., Nissen, P., Boesen, T., Karlsen, J.L., and Pedersen, B.P. (2019). Namdinator - automatic molecular dynamics flexible fitting of structural models into cryo-EM and crystallography experimental maps. IUCrJ *6*, 526-531. 10.1107/S2052252519007619.

SUPPLEMENTAL FIGURE

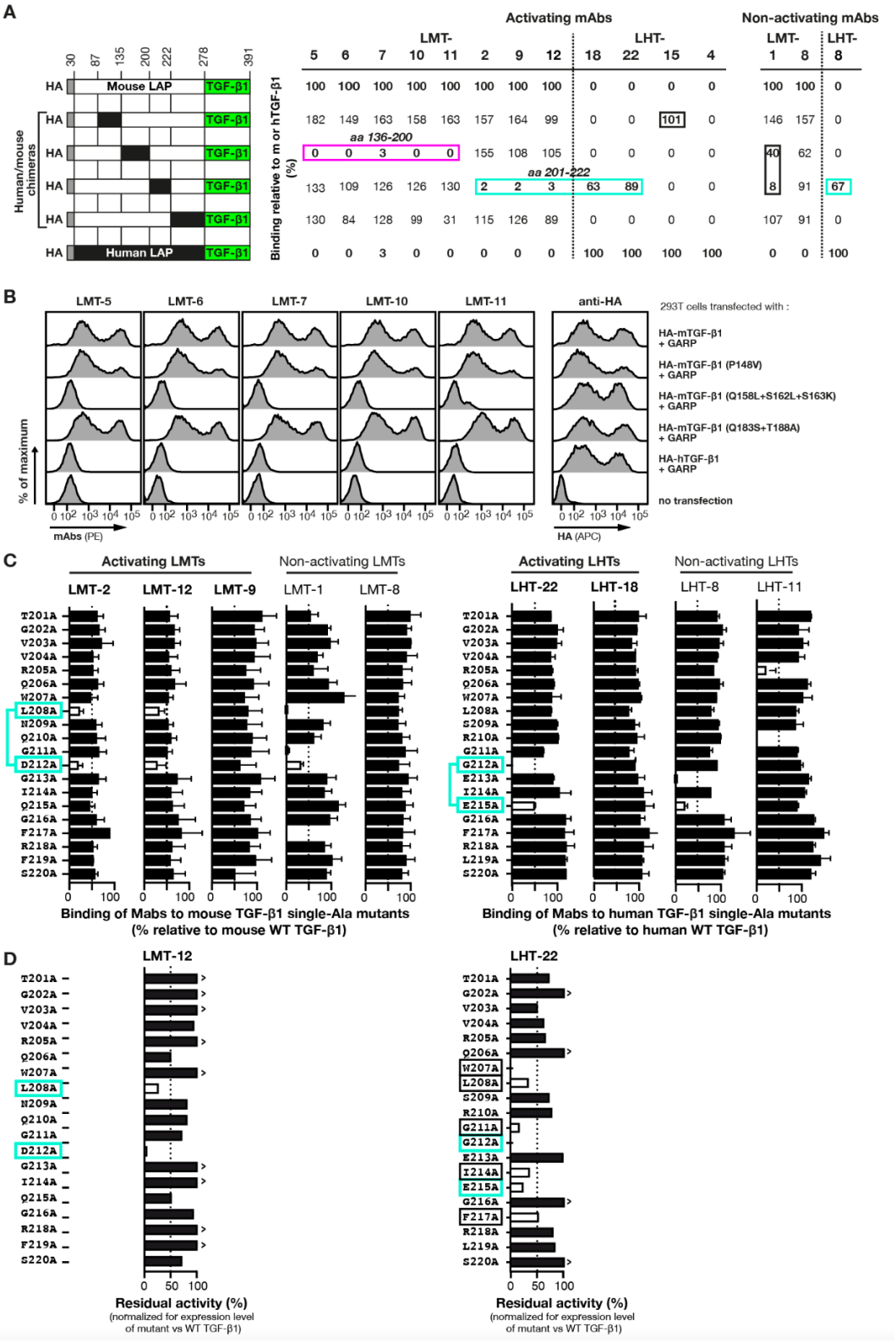

Figure S1. Mapping of epitopes recognized by TGF-β1 activating mAbs.

(A) Left: Schematic representation of h/mTGF-β1 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/mTGF-β1 chimera indicated on the left and analyzed for binding to mAbs by flow cytometry. Results are expressed as percent binding to the indicated h/mTGF-β1 chimera relative to mouse WT TGF-β1 for LMT mAbs, or human WT TGF-β1 for LHT mAbs. Magenta box: binding by 5 mouse TGF-β1 activating mAbs (LMT-5, 6, 7, 10 and 11) was lost when region 136-200 of mouse TGF-β1 was replaced by that of human TGF-β1. Turquoise box: binding by 3 other mouse TGF-β1 activating mAbs (LMT-2, 9 and 12) required aa from region 201-222. The same region was required for binding by 2 human TGF-β1 activating mAbs (LHT-18 and 22), as these were not able to bind h/mTGF-β1 chimeras unless region 201-222 was replaced by that of human TGF-β1. Black box: human TGF-β1 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-β1 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 TGF-β1 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-β1 (mean of 2 to 4 experiments + SD). White bars : < 50% binding. Binding by LMT-2 and LMT-12 was lost only when mouse TGF-β1 L208 or D212 were mutated to A, and binding by LHT-22 was lost only when human TGF-β1 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 TGF-β1 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-β1 (black boxes).

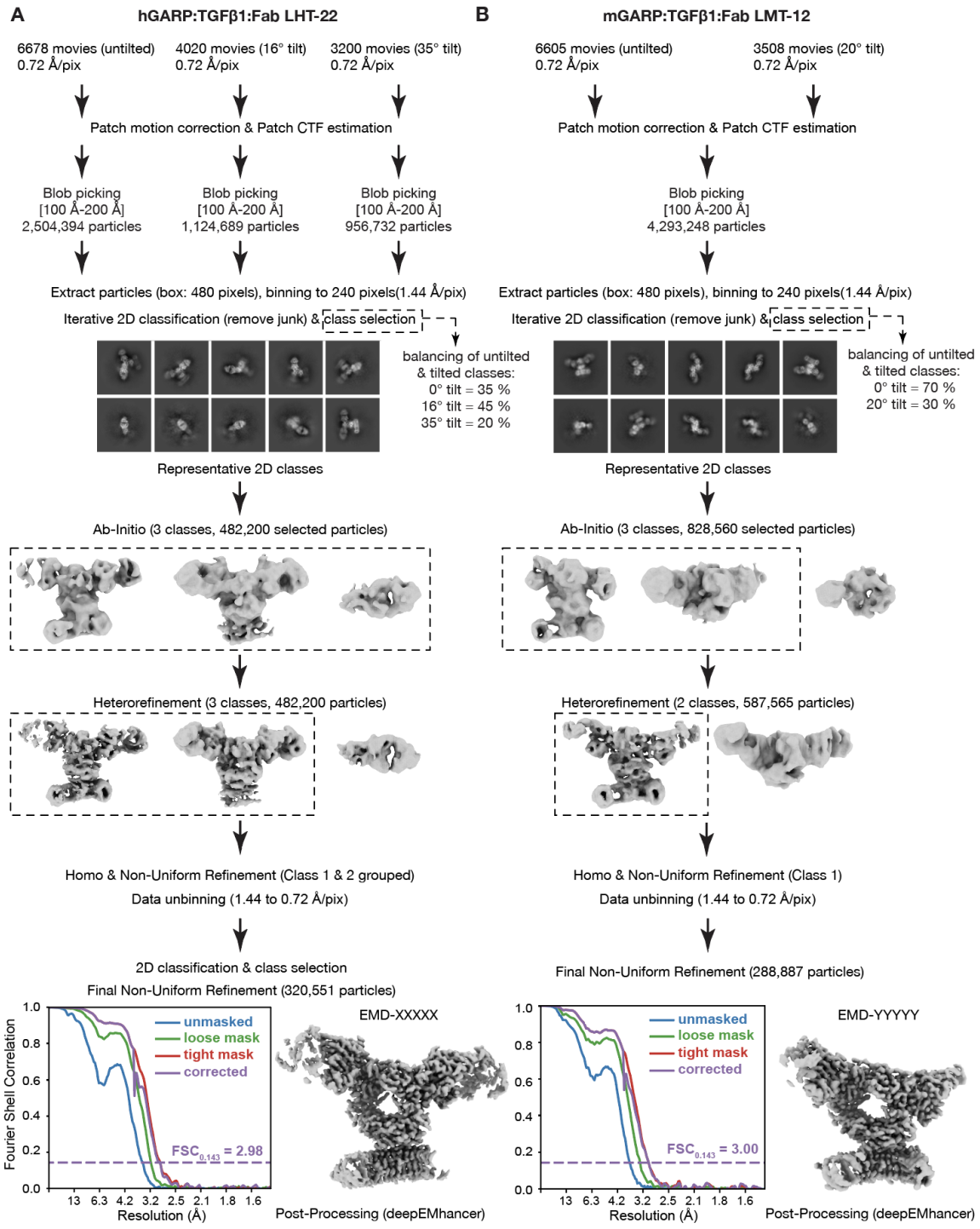

Figure S2. Cryo-EM data processing workflow of human (A) and mouse (B) GARP:TGF-β1:Fab complexes. Data processing was performed using CryoSPARC v4.2.1.[23] Gold-standard Fourier Shell Correlation (FSC) curves are shown (A and B, bottom) after applying either no mask (blue curve), a loose mask (green 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.[24] The estimated resolution at FSC = 0.143 (dotted purple lines) is shown for the corrected FSC (purple curve).

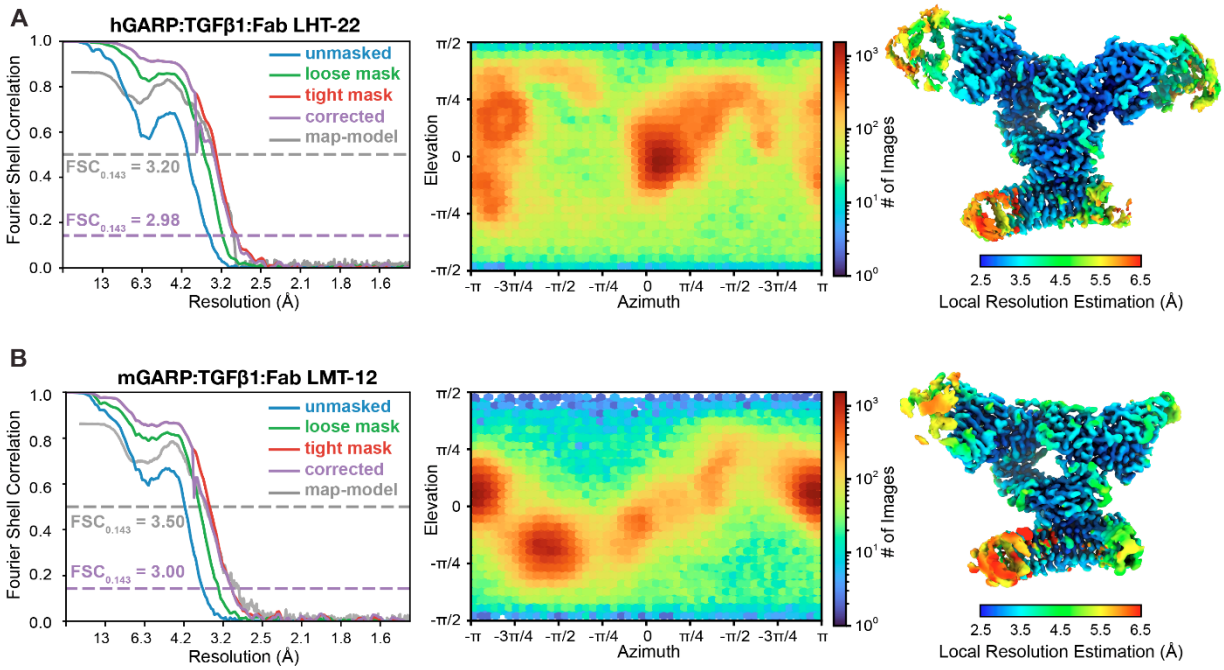

Figure S3. Gold-standard Fourier Shell Correlation (FSC) curves, particle orientation distribution plots and local resolution estimation of human (A) and mouse (B) GARP:TGF-β1:Fab complexes. FSC curves are calculated after applying either no mask (blue curve), a loose mask (green curve), or a tight mask (red curve) to both half maps. The purple curve depicts the corrected FSC calculated using an auto-tightened mask with correction by noise substitution.[24] 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.

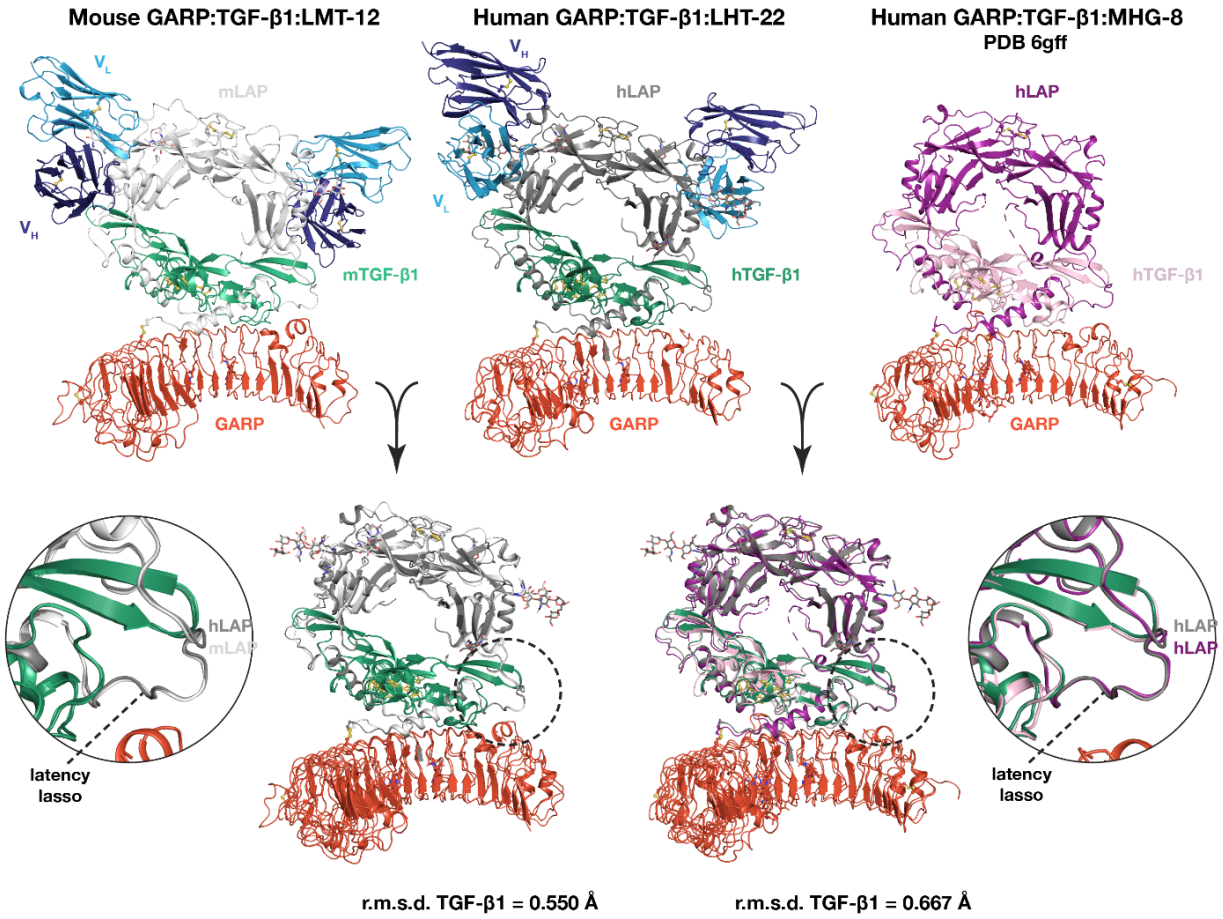

Figure S4. Structural alignment of GARP:TGF-β1:LHT-22, GARP:TGF-β1:LMT-12, and GARP:TGF-β1:MHG-8 complexes. (A-C) Cartoon representation of the mouse GARP:TGF-β1:LMT-12 complex (A), the human GARP:TGF-β1:LHT-22 complex (B) and the human GARP:TGF-β1 extracted from PDB 6GFF (C). (D) Structural alignment 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. Insets show a zoomed view of the latency lasso region of the 'straitjacket' domain of LAP.

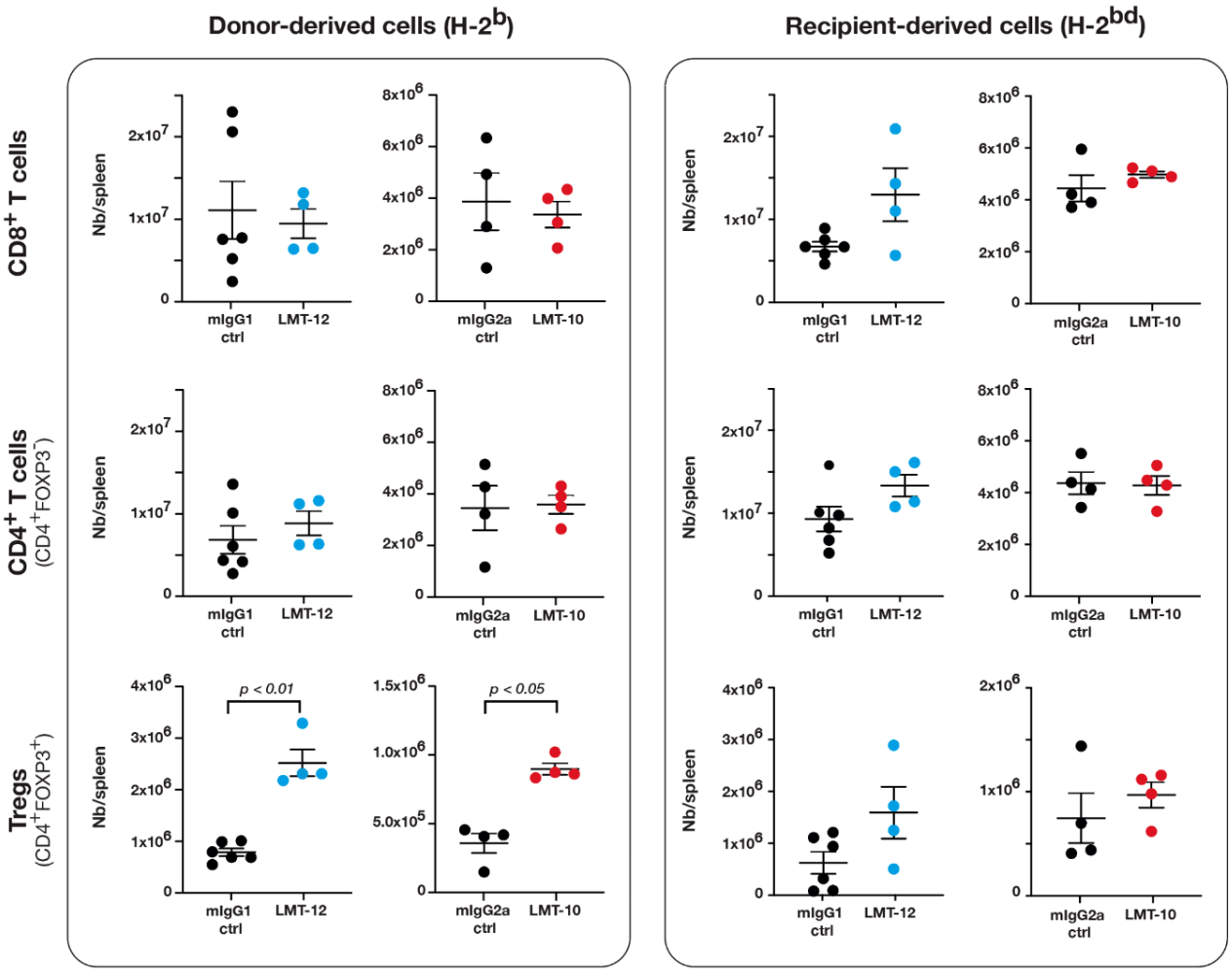

Figure S5. Treatment with LMT-10 or LMT-12 increases Treg numbers in spleens of transplanted mice undergoing GvHD. Spleens were collected from recipient mice on day 9 after transplantation. Splenocytes were counted and analyzed by flow cytometry with antibodies against CD4, CD8, FOXP3, H-2K^{d} and H-2K^{b} to evaluate numbers of Tregs, CD8^{+} and CD4^{+} T cells from donor (H-2^{b}) and recipient (H-2^{bd}) origin. Statistical analysis was performed using an unpaired two-tailed Mann-Whitney test.

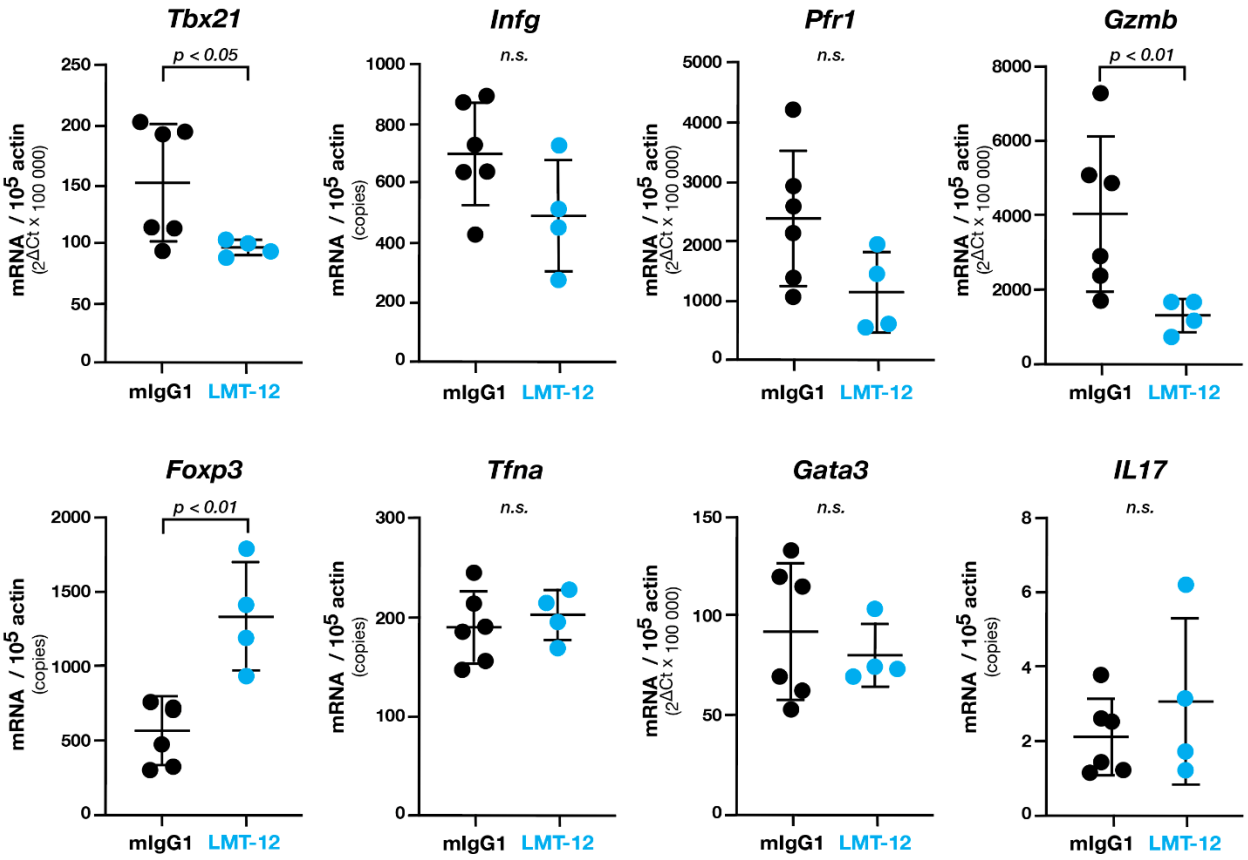

Figure S6. Treatment with LMT-12 increases expression of Foxp3 and reduces expression of genes characteristic of Th1 and CTL responses. Expression of the indicated genes in spleens collected from recipient mice on day 9 after transplantation was measured by RT-qPCR. Statistical analysis was performed unpaired two-tailed Mann-Whitney test.

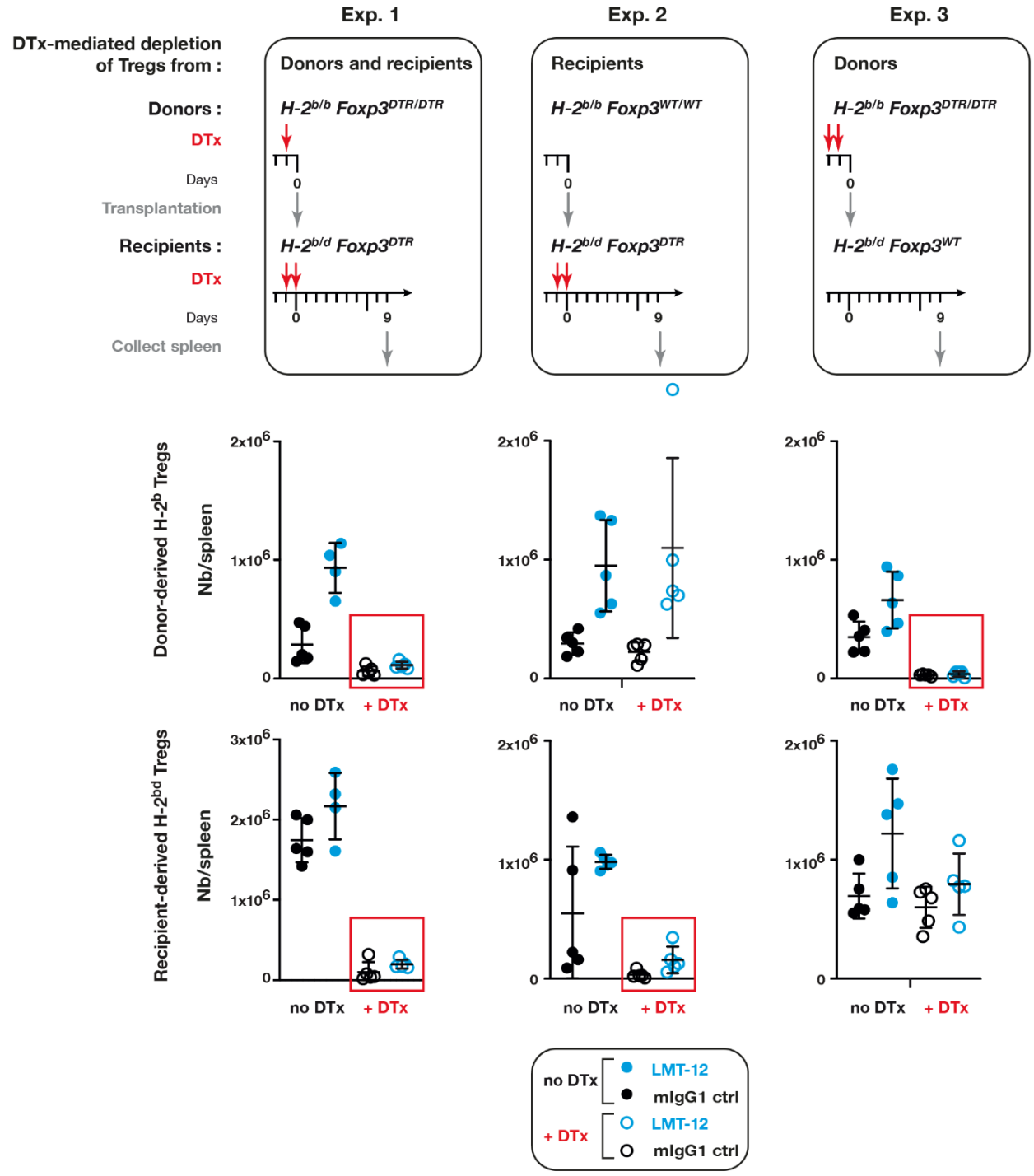

Figure S7. Depletion of Tregs from donor, recipient or both origins by injection of diphteria toxin
Splenocytes from recipient mice of experiments illustrated in Figure 6 were collected on days 1 and 9 after transplantation and analyzed by flow cytometry with antibodies against CD4, FOXP3, H-2K^{d} and H-2K^{b}. Depending on the genotypes of donors and recipients, DTx injections induced depletion of Tregs (CD4^{+}FOXP3^{+} cells) from donor (H-2^{b}), from recipient (H-2^{bd}) or from both donor and recipient origin.

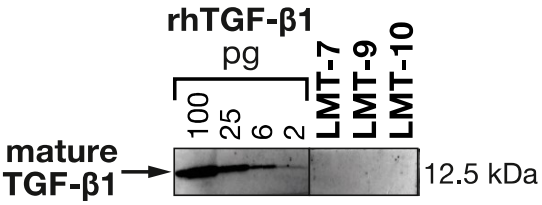

Figure S8. Absence of contaminating active TGF-β1 in antibody preparations
Western Blot analysis of mature TGF-β1 content in 25 µg of the indicated mAb preparations. The indicated amounts of rhTGF-β1 were used as references.

SUPPLEMENTAL TABLE 1

Interaction between human TGF-β1 (LAP) and Fab 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 834[NH1]	2.57	A:SER 209[O]
2	F:ASN 835[ND2]	3.30	A:SER 138[O]
3	F:ASN 835[ND2]	2.99	A:GLU 142[OE2]
4	F:TYR 837[OH]	2.95	A:GLY 212[O]
5	F:TYR 837[OH]	3.27	A:SER 138[OG]
6	F:ASN 855[ND2]	2.98	A:ARG 210[O]
7	F:TYR 896[OH]	3.08	A:GLU 213[OE1]
8	F:TYR 896[OH]	3.05	A:GLU 213[OE2]
9	F:TYR 837[OH]	2.72	A:ARG 141[NH1]

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 834 (H*)	1	A:SER 138 (H)
2	F:ASN 835 (H)	2	A:ARG 141 (H)
3	F:TYR 837 (H)	3	A:GLU 142
4	F:TYR 854	4	A:PRO 145
5	F:ASN 855 (H)	5	A:GLU 146
6	F:VAL 857	6	A:PRO 147
7	F:ALA 858	7	A:LEU 208
8	F:ARG 859	8	A:SER 209 (H)
9	F:TYR 896 (H)	9	A:ARG 210 (H)
10	F:ASN 903	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)
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 TGF-β1 (LAP) and Fab 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 836[OH]	3.11	A:GLN 210[OE1]
2	I:TYR 837[N]	3.40	A:ASP 212[O]
3	I:TYR 896[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 829	1	A:SER 138
2	I:SER 830	2	A:TYR 174
3	I:GLY 834	3	A:SER 175
4	I:GLY 835	4	A:ASN 176
5	I:TYR 836 (H*)	5	A:ASN 209 (H)
6	I:TYR 837 (H)	6	A:GLN 210 (H)
7	I:TYR 896 (H)	7	A:GLY 211
8	I:SER 898	8	A:ASP 212 (H)
9	I:SER 901	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		A:SER 138	(H)
2	H:TYR 52	(H)	A:ARG 141	(H)
3	H:SER 71	(H)	A:GLU 142	(H)
4	H:ILE 72		A:PRO 145	(H)
5	H:GLY 73		A:GLU 146	(H)
6	H:GLY 75	(H)	A:PRO 147	
7	H:SER 76		A:PRO 148	
8	H:TRP 78		A:LEU 149	
9	H:ASN 120		A:LEU 208	
10	H:SER 121		A:ASN 209	
11	H:ARG 122	(H)	A:GLN 210	
12	H:SER 123		A:GLY 211	
13	H:TYR 124	(H)	A:ASP 212	(H)
14	H:LYS 125	(H)	A:LEU 265	
			15 A:GLU 266	

*H: Hydrogen Bond

METHODS

Generation of mAbs directed against human and mouse latent TGF-β1

mAbs against GARP and GARP:TGF-β1 complexes were derived and described previously.[25,26]. 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 (hIgG1) or mouse (mIgG1, or mIgG2a$_{DANA}$) IgGs. mIgG1 and mIgG2a mIgG2a$_{DANA}$ exert few or no FcγR-dependent activities.[27]

TGF-β1 reporter assay

293T cells (±2.10^4) were plated in 96w plates the day before transfection. Unless otherwise specified, cells were transfected with plasmids encoding human or mouse GARP (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*[28], 50 ng/well), and Renilla luciferase under a constitutive promoter (*pRL-TK*, Promega, 1 ng/well) using TransIT-LT1 (Mirus Bio). 6 hours after transfection, transfected cells were incubated in the presence of mAbs (20 µg/ml) or with serial dilutions of active recombinant human TGF-β1 (rhTGF-β1). Firefly and Renilla luciferase activities were measured 24 hours after transfection using the Dual-Glo System (Promega). EC50 were calculated with GraphPad Prism software based on results obtained with serial dilutions of the mAbs.

Contamination of antibody preparations by active TGF-β1

The absence of co-purifying active TGF-β1 in antibody preparations was verified by Western Blot (**Figure S8**), by ELISA and in reporter assays. Western blots were performed in reducing conditions with a primary Ab directed against mature TGF-β1 (BD Pharmingen, clone X). ELISA (Thermofisher, Human/Mouse TGF beta 1 ELISA Ready-SET-Go! – 2nd generation) were performed directly or after acidification of samples to measure active or total TGF-β1 content, respectively. Reporter assays were performed by incubating mAbs with 293T cells transfected with *pCAGA-12-Fluc* and *pRL-TK* (but not *TGFB1*, *GARP* and *LRRC33)*. Active rhTGF-β1 served as controls in the assays. All mAb solutions used herein contained less than 0.8 pg of active TGF-β1 per µg of mAb.

Epitope mapping

Alignment of mouse and human protein sequences was performed using MacVector software. DNA sequences encoding the h/m TGF-β1 chimeras and single Ala-mutants were ordered at 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). 293T cells (5.10^5) were transfected in 6 well plates with plasmids encoding mouse *GARP* (1250 ng/well) and h/m TGF-β1 chimeras or single-Ala TGF-β1 mutants (1250 ng/well). 24 hours after transfection, cells were incubated with mAbs (5µg/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 (Biolegend) or an antibody against GARP:TGF-β1 complexes,[25,26] 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-β1.

Recombinant protein purification

Purified human and mouse GARP:TGF-β1 complex was 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-β1:Fab complexes, 4 µl 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 (BEDM, Brussels) equipped an Omega filter (JEOL) and a 6k × 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−/Å^2, 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-β1:Fab complex, a total of 6678 untilted movies were collected with a defocus of 0.8 – 2 µm. 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-β1: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.[29]

Cryo-EM Image Processing

Processing of uncorrected movies was performed in CryoSPARC v4.2.1.[23] 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 section 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,[30] and using deepEMhancer[31] to obtain maps for model building in Coot[32] and visualization in ChimeraX[33] and Pymol. A schematic overview of cryo-EM image processing can be found in Figure S7 and S8.

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 6gff[23] 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.[34] The resulting model was used as an input for automatic molecular dynamics flexible fitting using the

NAMDINATOR webserver.[35] 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.

Data Availability

Cryo-EM maps of human and mouse GARP:lTGFβ1:Fab complexes and corresponding structural models are deposited in the EMDB/PDB database.

Mice

C57BL/6, C57BL/6 *Foxp3*^{DTR}, F1 (C57BL/6 x DBA/2), F1 *Foxp3*^{DTR} (C57BL/6 *Foxp3*$^{DTR/DTR}$ x DBA/2) mice were bred at the SPF animal facility of the UCLouvain. C57BL/6 *Foxp3*^{DTR} were purchased at Charles River. As *Foxp3* is located on the X chromosome, F1 *H-2*$^{b/d}$ *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 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 and 2019/UCL/MD/032 at the UCLouvain.

Induction and scoring of mouse GvHD

Splenocytes (70-110.10^6) collected from the spleens of C57BL/6 female donor mice were injected i.p. in F1 (C57BL/6 x DBA/2) male recipients. Recipients received weekly *i.p.* injections (400 µg/mouse) of mAbs (LMT-10, LMT-12 or Motavizumab isotype control, under mIgG2a DANA or mIgG1 formats), starting one day before GvHD induction. In some experiments, diphteria toxin (DTx, 5 µg/mouse, i.e. ±25µg/kg) was administrated on the days indicated in figure S7. Mice were evaluated for the development of GvHD symptoms twice a week starting from day 0 until the development of first symptoms in one of the mice (usually around day 13), then daily thereafter (from days 13 to 20). GvHD scores were established based on weight loss (+1 or +2), posture (+1 or +2), reduced mobility (+1 or +2), 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 analyses of mouse splenocytes

Spleens were harvested immediately after euthanasia and mechanically dissociated. Homogenates were clarified through 70µm filters. After red blood cell lysis, single cell suspensions were counted on a Luna® cell counter with a live-dead cell marker, centrifuged, resuspended in PBS containing 2 mM EDTA and 1% FCS and clarified through 40µm filters prior to incubation with antibodies coupled to fluorochromes. Antibodies were directed against the following surface markers: B220, clone RA-6B2; CD4, clone GK1.5; H-2D^{d}, clone 34-2-12; H-2D^{b}, clone 28-8-6 (Biolegend) and CD8a, clone 53-6.7 (BD Pharmingen) in the presence of a viability dye (eBioscience) and anti-CD16/32 to block FcγRs (Biolegend). After staining for surface markers, cells 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 Fcg receptors. Cells were analysed on a FACS LSR Fortessa flow cytometer (BD Biosciences) and data were computed using the FlowJo software (Tree Star).

Statistical analyses

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

Differences between groups were compared using unpaired two-tailed Student's t test (for blood cell counts), or unpaired two-tailed Mann-Whitney test (for fibrosis scores and TGF-β1 concentrations in serum). Threshold for statistical significance: 0.05.

Differences between mice receiving TGF-β1 activating mAbs and mice receiving isotype control was tested by unpaired two-tailed Mann-Whitney test

Kaplan Meier plots representing the proportion of mice alive at the indicated days after transfer of splenocytes. P values calculated using a Gehan-Breslow Wilcoxon test (GraphPad Prism).

Comparisons of measurements taken at a single time point were performed using a two-tailed, non-paired, non-parametric Wilcoxon test. *Post-hoc* Tukey's test was performed to adjust for multiple comparisons. Numbers of experiments and numbers of mice (n) in the various experimental groups are indicated in the corresponding figure legend.

DISCUSSION AND PERSPECTIVES

1. How do antibodies activate TGF-β1?

1.1. What are the molecular changes induced by TGF-β1 activating antibodies?

Our work led to the identification of epitopes that are recognized by some TGF-β1 activating antibodies, but not by the non-activating antibodies that we tested. These epitopes were defined using two approaches: analysis of binding and activity of mAbs in presence of a set of mutations on the one hand, and cryo-EM with two Fab fragment of antibodies binding region 201-222 (LMT-12 and LHT-22) on the other hand. The convergence between the two techniques attests of the validity of using mutagenesis and binding analysis as a first approach to define epitopes. Therefore, the definition of the epitopes showed us that most TGF-β1 activating antibodies binds in the arm region of LAP, nearby the dimerization interface.

As shown in our article manuscript (Fig. 2), bivalency of is required for the activity of the antibodies, which are not active as Fabs. In line with this, we did not observe any change in the structure of latent TGF-β1 bound by Fabs derived from LMT-12 or LHT-22 by comparison to that of latent TGF-β1 bond by a blocking Fab (Fig. S4 of our article manuscript). Determination of the molecular changes occurring upon activation by mAbs would thus require solving the structure of latent TGF-β1 bound a F(ab)'2 or a full mAb. However, this would still not guarantee to visualize the active state of the complex as cryo-EM is performed in absence of cells that may bring other indispensable features required for activation, such as anchorage in a bilipid layer and/or force exerted via connection to the cytoskeleton. Indeed, we have not been able to show that TGF-β1 activating antibodies can activate recombinant latent TGF-β1 in solution, whether it is in complex with GARP or not (data not shown and Fig.2 of our article manuscript). In absence of the structure of TGF-β1 in an active state induced by binding to activating mAbs, we are left with two main hypotheses based on the epitopes identified.

1.1.1. Unrolling of LAP, like integrins $\alpha_V\beta_6$

Integrins bind the RGD motif located in the shoulder of LAP, close from the region bound by activating mAbs (Fig.3 of our article manuscript). Like integrins binding to RGD-containing ligands, antibodies generally interact with their target with high affinity, and in particular here, activating mAbs interact with latent TGF-β1 with affinities in the nanomolar range (Fig.1 of our article manuscript). TGF-β1 activation by integrins, like that by mAbs, require strong anchorage of latent TGF-β1 to a support, for example to a cell membrane via GARP. Indeed, in our TGF-β1 reporter assay, if the transmembrane domain of GARP is truncated, soluble GARP:TGF-β1 complexes are produced in the medium but no activation is observed in presence of a TGF-β1 activating mAbs (Fig.15, and Fig.2 of our article manuscript). Based on these common points, we could imagine

similar molecular changes occurring during activation by integrins or by mAbs. It is well accepted that integrin $\alpha_V\beta_6$ transmit pulling forces from the cytoskeleton through the arm of LAP to break the strong interaction between the fastener residues of LAP, which leads to elongation of the latency lasso and adjoining structures, ending up in the release of mature TGF-β1 from LAP. However, it is less clear what force could drive this fastener unsnapping and LAP elongation events in the cases of activation by integrin $\alpha_V\beta_8$ or TGF-β1 activating antibodies. In the case of $\alpha_V\beta_8$, cytoskeleton proteins interacting with the intracellular domain of β_8 and able to generate pulling forces transmitted to the extracellular part of the integrin still need to be identified. In the case of soluble mAbs, the only option we can imagine is that bivalent binding of one mAb to two GARP:TGF-β1 complexes could exert enough resistance to pulling forces exerted by two GARP:TGF-β1 complexes drifting away from one another. This could occur in *trans* when the GARP:TGF-β1 complexes are on two distinct cells, or in *cis* when the two GARP:TGF- β1 are on the same cell, with intracellular proteins linked to the cytoskeleton pulling in opposite directions on the intracellular domains of the two GARP molecules (Fig. 4 of our article manuscript). Mathieu Jamez, a PhD student in our laboratory, is currently testing whether a form of GARP truncated on the C-terminus of its transmembrane domain, which lacks only 14 intracellular aa but is still able to anchor to the cell membrane, can present TGF- β1 for activation by mAbs or by integrins.

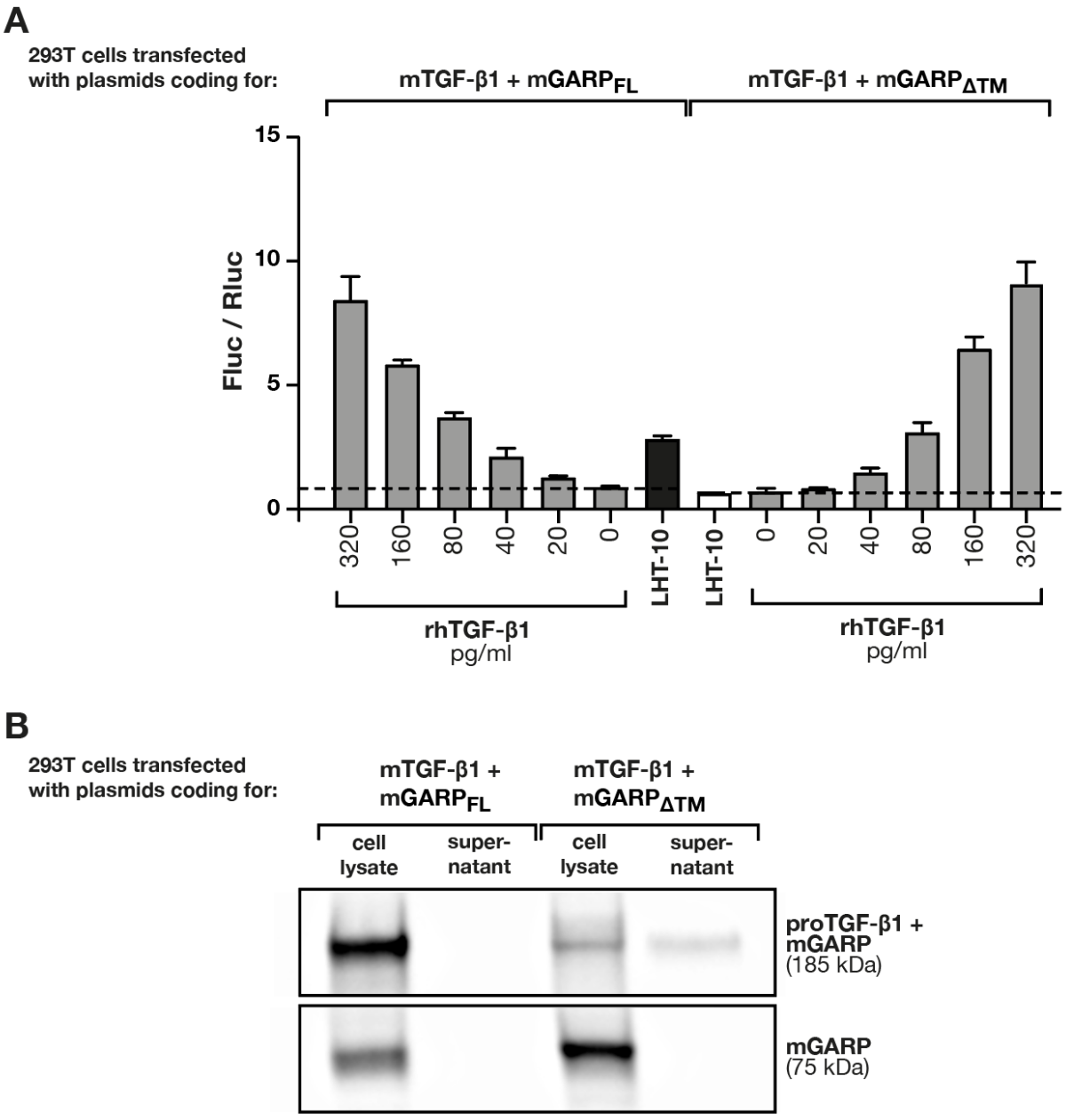

Figure 15: TGF-β1 activating mAbs do not activate TGF-β1 from soluble GARP: TGF-β1 complexes. **(A)** 293T cells were co-transfected to express mouse TGF-β1 in complex with GARP full lenght ($GARP_{FL}$), GARP truncated from its transmembrane domain ($GARP_{\Delta TM}$), Fluc under the control of a CAGA element as a reporter for TGF-β1 activity, and Rluc as a transfection control, then incubated with the indicated mAbs (20 µg/ml) or serial dilutions of active rhTGF-β1. Bar graphs represent ratios of Fluc to Rluc activity (mean of triplicates + SD). The dotted line shows the FLuc/Rluc ratio in absence of rhTGF-β1 or mAb. **(B)** Cell lysates and supernatant of 293T cells co-transfected with the indicated molecules were analyzed by Western Blot to assess the presence of GARP:TGF-β1 in both conditions.

1.1.2. Destabilization of dimerization interface

In this work, we identified two regions of LAP important for TGF-β1 activating mAbs : region 158-163 and 201-222. Several amino acids in the close vicinity of these epitopes are found mutated in patients suffering from Camurati-Engelmann disease (Figure 16), who display long bone and skull malformations associated to aberrant activation of TGF-β1. Among the amino acids mutated in this disease, the two cysteines forming the bowtie disulfide bond, C223 and C225, have the most predictable impact on the dimerization interface as they prevent the formation of the interchain disulfide bonds. This interface is further reinforced by a salt bridge between E169 and H222, that are also found mutated in patients suffering from this disease. R218 is an important amino acid that forms a cation-pi bond with Y171 and a salt bridge at the dimer interface with D226. Finally, R156 can also be mutated but its involvement in LAP stability is unknown. To date, no structural information is available for these mutants. Most likely, salt bridges and the cation-pi bond ensure a proper formation of the disulfide links between C223 and C225 [411]. These electrostatic bonds could also reinforce the dimerization interface held by the disulfide link once the protein is secreted. The binding of TGF-β1 activating mAbs in such close vicinity to amino acids involved in the stability of the homodimer may suggest that the bivalent binding by activating mAbs could destabilize this interface required to keep latency.

Whatever the molecular changes occurring in LAP and resulting in TGF-β1 activation, binding of the antibody *per se* is not sufficient to induce activation, highlighting the need of a physical force to drive TGF-β1 activation. But what kind of force could an antibody exert on the GARP:TGF-β1 complexes ?

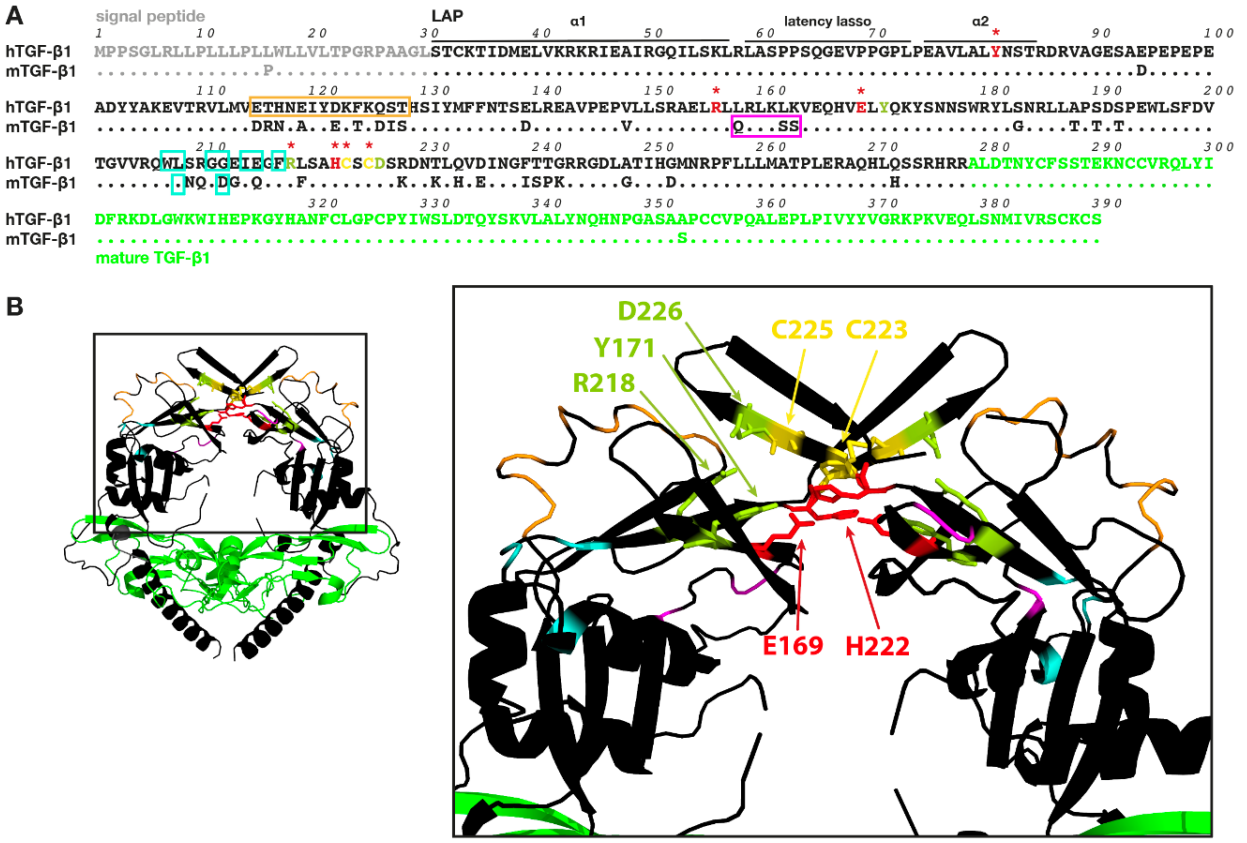

Figure 16: Proximity between epitopes bound by mAbs and CED mutations.
(A) Annotated alignment of h(uman)TGF-β1 and m(ouse)TGF-β1 sequences. Magenta box: region required for binding by mAbs LMT-5, 6, 7, 10, and 11. Turquoise boxes: residues required for binding and activation by LMT-12 and LHT-22. Orange box: residues required for binding and activation by LHT-15. Aa stabilizing dimerization interface are colored in green, yellow or red. Aa mutated in CED disease are indicated with *. **(B)** Tridimensional structure of porcine latent TGF-β1 [6]. Black: LAP. Green: mature TGF-β1. Color code as indicated in (A).

1.2. What would be the force driving TGF-β1 activation mediated by antibodies ?

Antibodies have an Fc portion that can be bound by Fc receptors. We could thus imagine a pulling force exerted on the Fc portion of the mAb by a cell expressing Fc receptors. However, the experimental conditions used in our work led us to exclude this hypothesis. Firstly, our screening and most of our assays were performed in 293T cells which, to the best of our knowledge, do not express Fc receptors. Secondly, activating antibodies were also active as F(ab)'2 fragment, which are devoid of the Fc portion. Eventually, the isotype used for *in vivo* experiments were chosen to limit interactions with Fc receptors.

As stated above, TGF-β1 activation by mAbs requires bivalent binding. The cryo-EM data suggests that binding by the two Fab fragments of one antibody molecule cannot not occur on a single LAP homodimer, but rather implies binding to two LAP homodimers. This results in cross-linking of at least two molecules of TGF-β1 in the presence of mAbs. How the cross-linking of latent TGF-β1 would provide sufficient force to deform LAP remains a mystery. As stated above, knowing that anchorage to the cell membrane via GARP was required in our experiments, we could assume that GARP itself may have a role in transmit this force. GARP displays a very short intracytoplasmic tail of 14 aa that has never been described as interacting with other proteins. In our laboratory, we tried to identify proteins that would participate to TGF-β1 activation in Tregs in collaboration with GARP and integrin $\alpha_V\beta_8$. IP-MS experiments was first performed to identify proteins interacting with GARP in Tregs. To this aim, lysates of stimulated human Tregs were immunoprecipitated with GARP and the co-immunoprecipitated proteins were analyzed by mass spectrometry [412]. Among candidate proteins that potentially interact with GARP, Filamin is an actin-binding protein known to connect some transmembrane proteins to the cytoskeleton [413]. More recently, we used the previously described TGF-β1 reporter assay with transfection of 293T cells with a library of cDNA from Tregs to identify new candidates that could participate to TGF-β1 activation (thesis work of Mathieu Jamez). Tubuline-α4A, a component of microtubules of eucaryotic cytoskeleton, was thus identified as potentiating activation in presence of GARP and integrin $\alpha_V\beta_8$ but no clear interaction with one of these proteins has been shown so far. Further investigations are thus needed to confirm the role played by these two candidates in TGF-β1 activation. Alternatively, mutant GARP truncated for its intracytoplasmic tail will be tested in our TGF-β1 reporter assays, as explained above.

2. Where do antibodies activate TGF-β1?

2.1. What is the source of TGF-β1 activated by mAbs *in vivo*?

TGF-β1 and its receptor are expressed by almost every cell in adult mammals. Therefore, understanding the sources from which TGF-β1 can be activated by TGF-β1 activating antibodies is a crucial point to anticipate potential adverse effects. Towards this aim, use of radiolabeled mAbs combined with imaging techniques could provide critical data on the pharmacokinetics and pharmacodynamics of activating mAbs [414]. However, as the sole binding of the antibody does not induce TGF-β1 activation, the information provided by this tool will give valuable information on the global distribution of the mAbs but not directly reflect the sites where activation occurs *in vivo*.

As stated in the first paragraphs of this discussion, we observed no signs of activation with soluble recombinant latent TGF-β1 (not shown) or soluble GARP:TGF-β1 complexes (Fig.15) activating antibodies were incubated with soluble recombinant latent TGF-β1 or GARP:TGF-β1 complexes. These observations suggest that circulating latent TGF-β1 is not expected to be activated by these antibodies. Instead, the requirement for anchorage to a support would restrict possible sources to TGF-β1 presented by LTBPs in the extracellular matrix, or TGF-β1 presented by GARP or LRRC33 at the surface of several cell types. In the model of GvHD used in this work, we could show a correlation between the protective effects of TGF-β1 activating mAbs and the presence of Tregs. Based on this observation, we hypothesized that this particular cell type could serve as a source of TGF-β1. To address that question, we constructed a bispecific antibody with one arm from a TGF-β1 activating mAb and the other arm binding GARP. As the Fab fragment of TGF-β1 activating mAbs is not able to activate TGF-β1, this approach restricts TGF-β1 activation to GARP expressing cells, among which are Tregs and endothelial cells. We could show that such a bispefic mAb can activate latent TGF-β1 in our TGF-β1 reporter assay (Fig.17). Whether a bispecific GARP/TGF-β1 activating mAb is as potent to protect from aGvHD will be assessed in the coming months. With this tool, we will learn if activation of TGF-β1 from GARP expressing cells is sufficient to protect from aGvHD. To go further in deciphering if Tregs play a particular role within the GARP expressing cells, we also plan to use *Foxp3*^{Cre} *GARP*$^{fl/fl}$ mice, who have no GARP at the surface of Tregs, in a model of aGvHD. We already have these mice in the C57Bl/6 background. Going further in this direction would either implicate to generate the equivalent mice in the DBA2 background or test this hypothesis in another model such as the “C57Bl/6 to Balb/C” aGvHD model, in which recipient mice are lethally irradiated before transplantation.

Specific activation of TGF-β1 from GARP expressing cells with the bispecific GARP/TGF-β1 activating mAbs could be ineffective to protect from aGvHD. Other bispecific antibodies could also be constructed to specifically target TGF-β1 presented by LRRC33 or by one of the LTBPs. This set of mAbs, allowing activation from restricted sources of TGF-β1, would provide incomparable tools to study the role of TGF-β1 in various disease conditions.

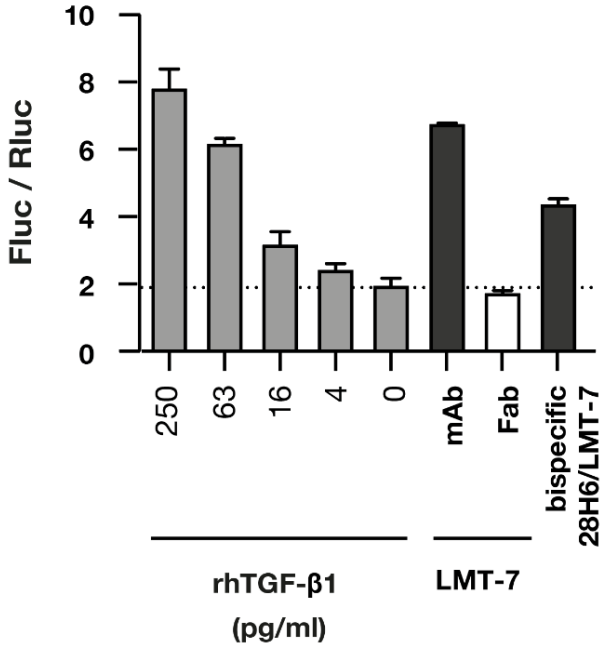

Figure 17: A bispecific GARP/TGF-β1 mAb activate TGF-β1 from GARP: TGF-β1 complexes in a reporter assay. 293T cells were co-transfected to express mouse GARP:TGF-β1, Fluc under the control of a CAGA element as a reporter for TGF-β1 activity, and Rluc as a transfection control, then incubated with the indicated mAb, Fab or bispecific mAb (20 µg/ml) or serial dilutions of active rhTGF-β1. Bar graphs represent ratios of Fluc to Rluc activity (mean of triplicates + SD). The dotted line shows the FLuc/Rluc ratio in absence of rhTGF-β1 or mAb.

2.2. What cells are targeted by activated TGF-β1?

To predict potential adverse effects, determination of the cells on which TGF-β1 acts is even more important. Knowing the very short half-live of the cytokine in serum and the high affinity and ubiquitous expression of its receptor, it is very likely that target cells are located in the immediate vicinity of the source from which TGF-β1 is activated. In other words, TGF-β1 activated by mAbs, like TGF-β1 “naturally” activated by integrins or other molecules, most probably acts in an autocrine or very short-distance paracrine manner. A way to address the question of the cellular targets of TGF-β1 activated by mAbs could be to analyze phosphorylation of Smad2 (pSmad2) at a single cell level resolution. Analysis of physiological levels of pSmad2 by flow cytometry has given poor results in our hands. In the laboratory, we recently tried to analyze pSmad2 signals among different cell types by digitalized multiplex immunofluorescence stainings of mouse and patient-derived tumor sections thesis work of Pierre Van Meerbeeck). However, the frequency and distribution of pSMAD2+ cells in tumor samples are vary variable from one sample to another. These types of analyses are probably difficult because they imply to catch a short-lived pSmad2 signal (15 min to a few hours) in vast array of different cells and tissues, requiring many set-up experiments to identify the good combination of antibodies and fluorochromes to identify cell types in each tissue.

3. TGF-β1 activating antibodies to treat diseases associated with immune dysfunction

3.1. Can TGF-β1 activating antibodies be used to treat Graft-versus-host diseases ?

3.1.1. TGF-β1 activating antibodies in aGvHD

In this work, we showed that the injection of TGF-β1 activating antibodies increases the survival and decreases the severity of aGvHD in a MHC mismatched model driven by C57Bl/6 $CD4^+$ and $CD8^+$ T grafted in B6D2 F1 mice. The protection was associated with an increase in Treg numbers, an encouraging observation that resonates well with the growing interest in this immunoregulatory population in the field of GvHD and auto-immune diseases. Among the numerous models of aGvHD, this model, also known as “Parent to F1”, is commonly used to mimic the clinical situations in which patients undergo nonmyeloablative or reduced intensity conditioning regimens. Therefore, the relevance of using TGF-β1 activating antibodies in patients receiving myeloablative radiation conditioning remains to be investigated. Indeed, the irradiation causes tissue damages and cytokine storm that generates a radically different immune environment that could influence the severity of aGvHD. We thus aimed to assess the efficacy of TGF-β1 activating antibodies in a commonly used model of full allogeneic aGvHD, in which lethally irradiated Balb/C ($H2^d$) mice receive T cell-depleted bone marrow cells complemented with $CD4^+$ and $CD8^+$ T cells from C57Bl/6 mice ($H2^b$). In this so-called “B6 to Balb/c” model, we did not observe a protective effect of TGF-β1 activating mAs (Fig.18). In the parent to F1 model, the mAbs lost their therapeutic effect in the absence of recipient Tregs. We are now trying to complement the graft with purified Tregs in the B6 to Balb/c model, to verify if the protective effect of Tregs can be increased by TGF-β1 activating mAbs.

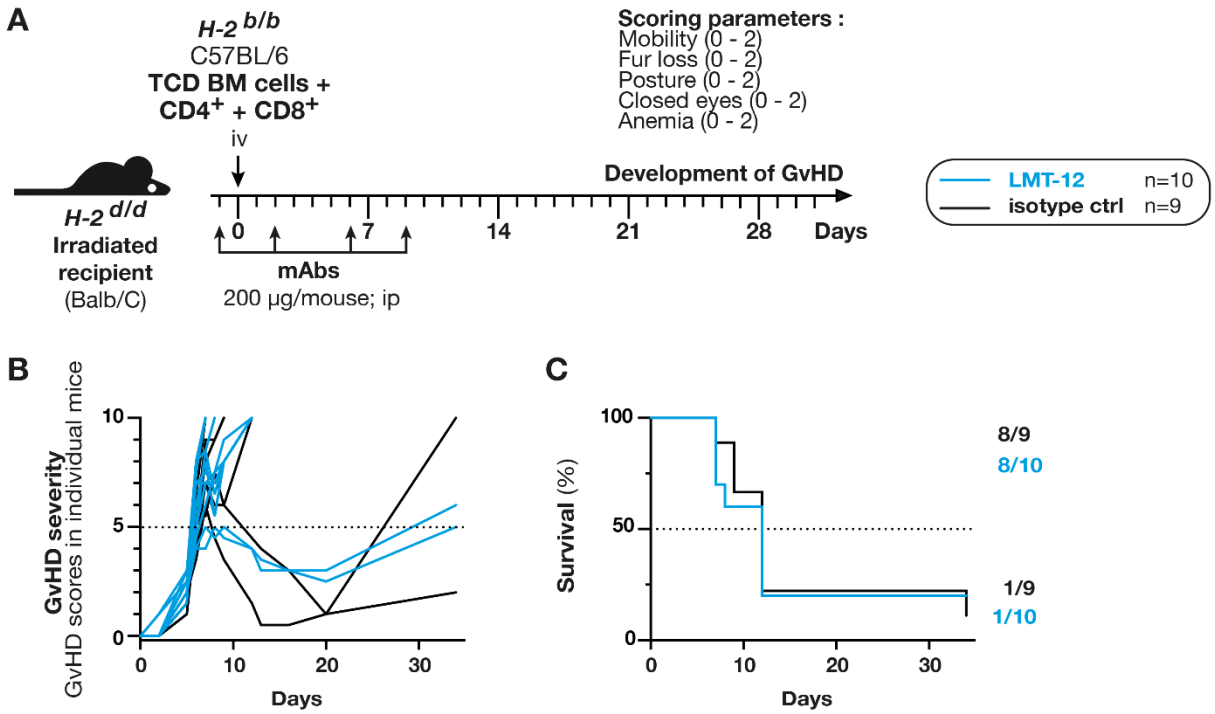

Figure 18: TGF-β1 activating mAbs do not protect from aGvHD in B6 to Balb/C.
(A) Schematic representation of the experimental design. H-$2^{d/d}$ F1 mice transplanted with 10^7 T cell depleted bone marrow (TCD BM) cells, 8.10^5 $CD4^+$ and 6.10^5 $CD8^+$ isolated from H-$2^{b/b}$ mice on day 0. Recipient mice were injected with TGF-β1 activating mAbs or isotype controls twice a week, starting on day -1. Mice were scored symptoms of GvHD as indicated (maximum score: 10; mice were sacrificed when reaching a score of 8). **(B)** Evolution of GvHD scores in individual mice. **(C)** Kaplan-Meier plots representing the proportion of mice alive at the indicated days after transplantation.

To get another clue on the cellular source of TGF-β1 activated by mAbs in GvHD, we also tested the effect of anti-human (LHT-22) and anti-mouse TGF-β1 activating antibodies (LMT-12) in a xenogeneic model of GvHD induced by transfer of human PBMCs into immunocompromised NOD/Scid/Il2rg$^{-/-}$ (NSG) mice, which lack functional T, B, and NK cells. Transfer of human PBMCs in NSG mice induce GvHD symptoms that can be attenuated by co-transfer of autologous Tregs [366] [415]. Our laboratory showed that the protective effect of human Tregs in this model is dependent on TGF-β1 activation from GARP:TGF-β1 complexes [17]. We thus hypothesized that TGF-β1 activating mAbs could potentiate the protective effect of Tregs in this model. We combined administration of autologous human Tregs with treatment with either LHT-22 (that activates TGF-β1 from human cells exclusively), LMT-12 (that activates TGF-β1 from mouse cells exclusively) or both antibodies. Unfortunately, we did not observe a beneficial outcome of the anti- human or mouse TGF-β1 activating antibodies, either used in monotherapy or in combination (Figure 19). Are human Tregs already at the maximum of their protective effect through TGF-β1 activation, or do the mAbs lack activity in this model? A potential sign of activity we could look for is increased splenic Treg numbers. However, even if we observe this, we still will not know why the mAbs do

not decrease GvHD severity. Another hypothesis would be that Tregs are not abundant enough to mediate therapeutic activity of the mAbs. We could test this by injecting low-doses of IL-2 in combination with the TGF-β1 activating mAbs. One of the difficulties of this model is the high variability in the type and intensity of GvHD induced by PBMCs and the protection given by Tregs, even when cells are isolated from the same individual but at different time points. We could thus imagine that in the combination of PBMCs and autologous Tregs that we used in this experiment, Tregs were already at their maximum potential of protection, while a beneficial effect of the mAbs could have been observed with a PBMCs/Treg combination from another donor.

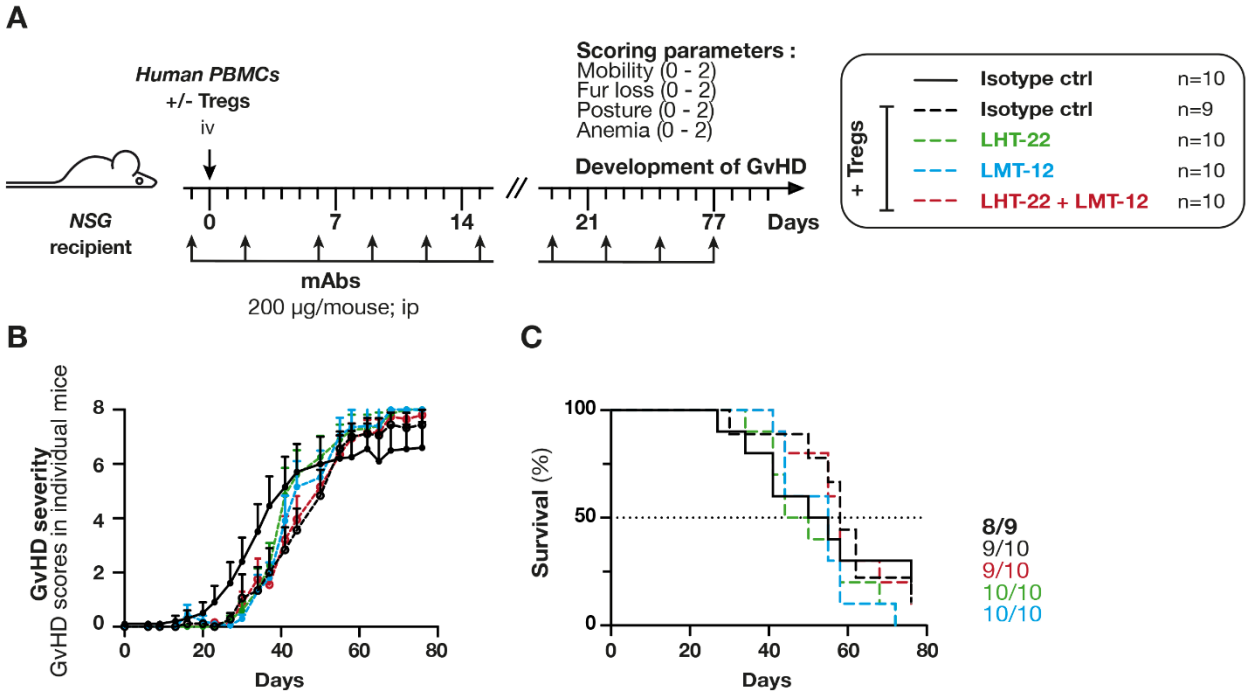

Figure 19: TGF-β1 activating mAbs do not protect from GvHD in a xenogenic model induced by transfer of human PBMCs. (A) Schematic representation of the experimental design. NSG mice transplanted with 3.10^6 human PBMCs supplemented or not with $1,5.10^6$ autologous Tregs on day 0. Recipient mice were injected with TGF-β1 activating mAbs or isotype controls twice a week, starting on day -1. Mice were scored for symptoms of GvHD as indicated (maximum score: 8; mice were sacrificed when reaching a score of 6). **(B)** Evolution of GvHD scores in individual mice. **(C)** Kaplan-Meier plots representing the proportion of mice alive at the indicated days after transplantation

3.1.2. TGF-β1 activating antibodies in cGvHD

Allo-HCT is not only complicated by the severe and acute form of GvHD, but also by a chronic form that generally occurs after aGvHD. In addition to T cells, cGvHD also involves B-cell stimulation, autoantibody production and systemic fibrosis leading to an auto-immune-like syndrome [416]. Based on the pro-fibrotic properties of TGF-β, we could fear that TGF-β1 activating antibodies would increase the risk of fibrosis in cGvHD. Indeed, blockade of TGF-β signaling with a pan-blocking antibody was shown to decrease severity of cGvHD in a model characterized by skin thickening, and GI and lung fibrosis [320]. The authors incriminated myeloid cells as producers of TGF-β1 in this context of cGvHD, while protective TGF-β1 would be produced by T cells during the acute phase of the disease, again underlying the importance of the context and timing of TGF-β1 production. Of note, TGF-β2 and 3 but not TGF-β1 may drive fibrosis, as described recently in a mouse model of lung fibrosis induced by bleomycin, and in patients suffering from idiopathic pulmonary fibrosis and nonalcoholic fatty liver disease [189].

One of the main physiopathological mechanisms described in cGvHD is the defect in thymic functions following damages caused by aGvHD and conditioning, resulting in failure to efficiently delete autoreactive T cells differentiating from donor HSCs, and reduced tTreg numbers or function. By increasing Treg numbers in the periphery during aGvHD, TGF-β1 activating mAbs could compensate for the reduction in tTregs that could mitigate cGvHD. TGF-β1 activation by mAbs could also control proliferation and auto-antibody production by B cells. TGF-β1 may also favors production of IgA antibodies, the consequence of which is hard to predict but may result in a better mucosal immune homeostasis. Finally, TGF-β1 activating mAbs decreased severity of aGvHD, which is itself a well described inducer of cGvHD. The mAbs may thus exert a dual role in cGvHD, depending on the context, and sending us back to the importance of determining the cellular sources and targets of TGF-β1 activated by our antibodies. Thus, a more extensive study on the cellular context (organ and cells), ongoing inflammation, the timing and the isoforms of TGF-β being produced during GvHD would illuminate the comprehension of the roles played by this cytokine, particularly in fibrotic processes.

Addressing these questions in mice is a tremendous challenge that will be at the core of a new PhD thesis in the laboratory, led by Séverine Wautier. The main impediment to overcome in the study of cGvHD is the great clinical heterogeneity and the ill-defined physiopathology that can hardly be recapitulated in a single mouse model. Her studies will start in a cGvHD model in which small numbers of $CD4^+$ and $CD8^+$ T cells from C57BL/6 mice are injected in BALB/C recipients (B6 → Balb/C). The advantage of this model is that it is characterized by a first phase of aGvHD to which most mice survive, causing thymic damages and ensuing in cGvHD development characterized by auto-antibody production, skin fibrosis, salivary gland tissue damages hair and weight loss [335]. With this model, we aim to decipher if TGF-β1 activating mAbs impacts thymic damages, Treg numbers or functions and clinical manifestations of cGvHD. To better define the source of TGF-β1

activated by mAbs, she will also use bispecific GARP/TGF-β1 activating mAbs in this model. If the activating mAbs display a pro-fibrotic activity, the timing of mAb administration could be adapted to focus on the acute phase of GvHD.

3.1.3. TGF-β1 activating antibodies in GvT and infections

In addition to investigating the therapeutic activity of TGF-β1 activating mAbs on cGvHD, we also aim to address the question of consequences of their use on the GvT effect. The potential outcome of increasing TGF-β1 activity is uncertain and could range from increasing the immunosuppressive environment which would be beneficial to tumor growth, to decreasing the severe aGvHD-related inflammation that promotes T cell dysfunction and apoptosis, hence preserving the GvT effect [318]. Several mouse models to study the GvT effect have emerged. In these models, small numbers of leukemic cells are infused in mice around the time of alloHCT transplantation [417]. Several leukemia/lymphoma cell lines have been modified to express a bioluminescent marker gene (generally American firefly luciferase gene) to track tumor growth by *in vivo* bioluminescence imaging in a noninvasive and quantitative way.

Finally, the cytostatic effects of TGF-β1 could jeopardize proper reconstitution of immune cells from the graft, a condition favoring both the progression of tumor cells and opportunistic infections. Such a decrease in reconstitution was not observed in our study. However, we cannot exclude that a broad immunosuppression exerted by TGF-β1 could represent a favorable ground for infections. As stated in the introduction, TGF-β1 also played a dual role in infections depending on the pathogen. On the one hand, TGF-β1 was shown to inhibit T cell-dependent viral clearance in the lung post-HCT, leading to severe pneumonitis [418]. On the other hand, TGF-β1 can also contribute to mucosal immune homeostasis by favoring IgA production by B lymphocytes or differentiation of $CD4^+$ T cells into Th17 effectors. In the lab, we previously show that the inhibition of TGF-β1 produced by GARP expressing cells did not alter innate or adaptative immune responses in a model of intestinal infection by the bacteria *Citrobacter rodentium* [102]. This suggests that TGF-β1 activated by GARP expressing cells do not play a role in immune responses to this infection, but does not exclude a role for TGF-β1 activated by other cells.

3.2. Can TGF-β1 activating antibodies be used to treat auto-immune disease?

Auto-immune diseases refer to conditions in which the immune system of an individual aberrantly attacks its own healthy tissues. First line treatment often relies on broad immunosuppression with corticoids that can induce many side effects. Research in the field aims to decipher the physiopathology of these complex diseases, which often intertwine genetical predisposition and environmental factors, in other to find new therapeutical targets. The role of TGF-β1 in these pathologies has been poorly studied so far and is often restricted to correlations with the levels of the circulating (latent) cytokine, genetic alterations or a global inhibition with the pan-blocking TGF-β antibody. Yet, TGF-β1 activation is a highly regulated process and the cytokine is acting in specific contexts that deserve deeper investigations. The TGF-β1 activating antibodies described in this work could help grasping the role played by this cytokine in the physiopathology of many auto-immune diseases and result in a potential new therapeutic tool in some of them. The following lines will focus on two well described immune diseases for which mouse models are available and that will be studied in the coming months by Emilie Dupré, a post-doc in our laboratory.

3.2.1. Systemic lupus erythematosus

The physiopathology of SLE involves an accumulation of apoptotic debris that lead to a loss in B and T cell tolerance to autoantigens and production of various anti-nuclear antibodies. Immune complexes generally accumulate in the kidney basement membranes, activate complement and lead to inflammation and kidney fibrosis. Patients suffering from SLE are generally treated with corticosteroids and immunosuppressive agents that are not always efficient and the generalized immunosuppression they induce represents an increased risk of infections. More targeted approaches are thus needed. Belimumab, an anti-BAFF (B cell-activating factor) mAb that reduces survival and activation of B cells, has been approved by the FDA for the treatment of patients with lupus nephritis. However, there is still an unmet need for novel, disease-specific, effective and safe treatment modalities [419].

Immunosuppressive functions of TGF-β1 would be very interesting to study in this context, as it may act on nearly every cell type contributing to the physiopathology. The triggering event of loss of tolerance in SLE is linked to an imbalance between apoptosis and clearance of debris, the etiology of which remains unclear. The roles of TGF-β1 on macrophages is poorly documented and has not been studied in the context of SLE. In intestinal inflammation, TGF-β1 would increase chemotaxis of monocytes, decrease pro-inflammatory responses by suppression of TLR signaling and favor “M2” phenotype that favor resolution phase of inflammation instead of clearance of pathogens. It would be interesting to see whether TGF-β1 activating mAbs would result in an increased in apoptotic debris accumulation or in a decreased inflammation. Similarly, evidence of the roles of TGF-β1 on dendritic cells is scarce. *In vitro* experiment suggests a decreased presentation of antigens and co-stimulatory molecules but these observations were not confirmed

in vivo. Thus, new insight on the role of TGF-β1 on innate immune cells could be grasped by studying TGF-β1 activating mAbs in SLE models.

The most interesting effects of TGF-β1 activating mAbs would be on adaptative immunity, and particularly on auto-reactive B lymphocytes that are at the core of SLE pathogenesis. Generally speaking, immunosuppressive effects of TGF-β1 could lead to decrease in their proliferation, survival and antibody production. In the context of SLE, TGF-β1 was described to restrain anti-nuclear antibody production [290-293], and the potential cells pointed out to produce TGF-β1 in this context are Tregs [293] and B cell themselves [103]. These two cell types share the common point of activating TGF-β1 via GARP. Thus, the bispecific GARP/TGF-β1 activating mAb would also be interesting to test in this context. TGF-β1 activating mAbs could decrease SLE by controlling B cells by a direct effect on B cells themselves, and/or by an indirect effect of increased Treg numbers or function. Of note, an increase in IgA production may occur, but would be expected to reinforce immune homeostasis at the mucosal barrier and should not interfere directly in SLE pathogenesis. Similar to cGvHD, long lasting inflammation of the disease ends up in fibrosis of targeted organs, mainly the kidney but also lungs, heart or joints. Due to the pro-fibrotic properties of TGF-β, particular attention will be paid to a putative increase in fibrosis following treatment with TGF-β1 activating mAbs. Thus TGF-β1 activating mAbs may be beneficial in the early steps of physiopathology by decreasing inflammation but may strengthen fibrosis in the advanced stages of the disease.

These questions will be assessed in a model of lupus-prone mice, the C57Bl/6 Sle123 model. This congenic mouse strain was developed by introgressing three lupus-susceptibility loci (*sle1*, *sle2* and *sle3*) identified in the NZM2410 lupus-prone strain onto the C57Bl/6 resistant genetic background [420]. These mice display a fully-penetrant and lethal disease recapitulating important features including, production of anti-nuclear antibodies, activation of polyclonal B and T cell and kidney fibrosis [421, 422].

3.2.2. Multiple sclerosis

Multiple sclerosis (MS) is a neurodegenerative auto-immune disease mainly driven by T cells, with macrophages as the final effector cells for the demyelinating process. Th1 cells were first incriminated as being mainly responsible of the disease, and then gave way to Th17 cells. Recent studies showed that Th17 is a heterogeneous and plastic cell population that is divided between encephalitogenic and non-encephalitogenic Th17 cells. Known to drive Th17 differentiation, TGF-β1 was first depicted as contributing to MS pathogenicity, whereas this emerging dichotomy between the two types of Th17 cells nuances its implications. In addition to IL-6, other cytokines such as IL-23 and IL-1β appear to orient differentiation towards pathogenic Th17 cells, while TGF-β1 may rather favor the non-pathogenic Th17 cells [262]. The exact role played by TGF-β1 in the pathogenicity of Th17 cells remains to be clarified. In contrast, TGF-β1 may sustain differentiation

of Tregs that regulate major aspects of the disease as inflammation and remyelination. Various studies point towards defective Tregs in MS patients and TGF-β1-activating mAbs could thus represent an interesting therapeutic option to correct this defect.

Eager to know the resultant effect of TGF-β1 on the Th17/Treg balance, we will inject TGF-β1 activating mAbs in mice with Experimental Autoimmune Encephalomyelitis (EAE), a well described murine model of MS. We also plan to inject mice with a TGF-β1 blocking antibody (SRK-181). We will first look at the clinical manifestation of the disease and at immune populations present in the blood, spleen and central nervous system (CNS). If an increase in Th17 cells is observed upon injection of TGF- β1 activating antibodies, we will try to decipher if they are pathogenic or not, by looking at clinical manifestations and expression of transcription factors, for example. We could also combine the TGF-β1 activating mAbs with antibodies neutralizing the IL-6 receptor, IL-23 or IL-1β, to see if we can shift the balance in favor of Treg differentiation. The final effectors of demyelination are cytolytic $CD8^+$T cells and macrophages. Macrophages of the CNS, also known as microglia, have been described to activate latent TGF-β1 by a mechanism requiring LRRC33 and integrin $\alpha_v\beta_8$. The role of TGF-β1 produced by this cell population may be studied with a bispecific TGF-β1 activating/LRRC33 antibody and may help in understanding the role that TGF-β1 plays during CNS inflammation and repair [22].

REFERENCES

1. Kulkarni, A.B., et al., *Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death.* Proc Natl Acad Sci U S A, 1993. **90**(2): p. 770-4.
2. Shull, M.M., et al., *Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease.* Nature, 1992. **359**(6397): p. 693-9.
3. Stockis, J., O. Dedobbeleer, and S. Lucas, *Role of GARP in the activation of latent TGF-beta1.* Mol Biosyst, 2017. **13**(10): p. 1925-1935.
4. Kelly, A., et al., *Regulation of Innate and Adaptive Immunity by TGFbeta.* Adv Immunol, 2017. **134**: p. 137-233.
5. Gauthy, E., et al., *GARP is regulated by miRNAs and controls latent TGF-beta1 production by human regulatory T cells.* PLoS One, 2013. **8**(9): p. e76186.
6. Robertson, I.B. and D.B. Rifkin, *Regulation of the Bioavailability of TGF-beta and TGF-beta-Related Proteins.* Cold Spring Harb Perspect Biol, 2016. **8**(6).
7. Rifkin, D.B., W.J. Rifkin, and L. Zilberberg, *LTBPs in biology and medicine: LTBP diseases.* Matrix Biol, 2018. **71-72**: p. 90-99.
8. Ollendorff, V., et al., *The GARP gene encodes a new member of the family of leucine-rich repeat-containing proteins.* Cell Growth Differ, 1994. **5**(2): p. 213-9.
9. Kobe, B. and A.V. Kajava, *The leucine-rich repeat as a protein recognition motif.* Curr Opin Struct Biol, 2001. **11**(6): p. 725-32.
10. Tran, D.Q., et al., *GARP (LRRC32) is essential for the surface expression of latent TGF-beta on platelets and activated FOXP3+ regulatory T cells.* Proc Natl Acad Sci U S A, 2009. **106**(32): p. 13445-50.
11. Stockis, J., et al., *Membrane protein GARP is a receptor for latent TGF-beta on the surface of activated human Treg.* Eur J Immunol, 2009. **39**(12): p. 3315-22.
12. Wang, R., et al., *Identification of a regulatory T cell specific cell surface molecule that mediates suppressive signals and induces Foxp3 expression.* PLoS One, 2008. **3**(7): p. e2705.
13. Probst-Kepper, M., et al., *GARP: a key receptor controlling FOXP3 in human regulatory T cells.* J Cell Mol Med, 2009. **13**(9B): p. 3343-57.
14. Dedobbeleer, O., et al., *Cutting Edge: Active TGF-beta1 Released from GARP/TGF-beta1 Complexes on the Surface of Stimulated Human B Lymphocytes Increases Class-Switch Recombination and Production of IgA.* J Immunol, 2017. **199**(2): p. 391-396.
15. Vermeersch, E., et al., *The role of platelet and endothelial GARP in thrombosis and hemostasis.* PLoS One, 2017. **12**(3): p. e0173329.
16. de Streel, G., et al., *Selective inhibition of TGF-beta1 produced by GARP-expressing Tregs overcomes resistance to PD-1/PD-L1 blockade in cancer.* Nat Commun, 2020. **11**(1): p. 4545.
17. Cuende, J., et al., *Monoclonal antibodies against GARP/TGF-beta1 complexes inhibit the immunosuppressive activity of human regulatory T cells in vivo.* Sci Transl Med, 2015. **7**(284): p. 284ra56.
18. Carrillo-Galvez, A.B., et al., *Mesenchymal stromal cells express GARP/LRRC32 on their surface: effects on their biology and immunomodulatory capacity.* Stem Cells, 2015. **33**(1): p. 183-95.
19. Barbet, R., et al., *Comparison of Gene Expression in Human Embryonic Stem Cells, hESC-Derived Mesenchymal Stem Cells and Human Mesenchymal Stem Cells.* Stem Cells Int, 2011. **2011**: p. 368192.

20. Li, Y., et al., *Hepatic Stellate Cells Inhibit T Cells through Active TGF-beta1 from a Cell Surface-Bound Latent TGF-beta1/GARP Complex.* J Immunol, 2015. **195**(6): p. 2648-56.
21. Wong, K., et al., *Mice deficient in NRROS show abnormal microglial development and neurological disorders.* Nat Immunol, 2017. **18**(6): p. 633-641.
22. Qin, Y., et al., *A Milieu Molecule for TGF-beta Required for Microglia Function in the Nervous System.* Cell, 2018. **174**(1): p. 156-171 e16.
23. Ma, W., et al., *LRRC33 is a novel binding and potential regulating protein of TGF-beta1 function in human acute myeloid leukemia cells.* PLoS One, 2019. **14**(10): p. e0213482.
24. Hinck, A.P., T.D. Mueller, and T.A. Springer, *Structural Biology and Evolution of the TGF-beta Family.* Cold Spring Harb Perspect Biol, 2016. **8**(12).
25. Goebel, E.J., et al., *Structural biology of the TGFbeta family.* Exp Biol Med (Maywood), 2019. **244**(17): p. 1530-1546.
26. Radaev, S., et al., *Ternary complex of transforming growth factor-beta1 reveals isoform-specific ligand recognition and receptor recruitment in the superfamily.* J Biol Chem, 2010. **285**(19): p. 14806-14.
27. Shi, M., et al., *Latent TGF-beta structure and activation.* Nature, 2011. **474**(7351): p. 343-9.
28. Janssens, K., et al., *Camurati-Engelmann disease: review of the clinical, radiological, and molecular data of 24 families and implications for diagnosis and treatment.* J Med Genet, 2006. **43**(1): p. 1-11.
29. Zhao, B., et al., *Prodomain-growth factor swapping in the structure of pro-TGF-beta1.* J Biol Chem, 2018. **293**(5): p. 1579-1589.
30. Rifkin, D., et al., *The role of LTBPs in TGF beta signaling.* Dev Dyn, 2022. **251**(1): p. 95-104.
31. Singh, M., et al., *Structural studies of elastic fibre and microfibrillar proteins.* Matrix Biol Plus, 2021. **12**: p. 100078.
32. Duan, Z., et al., *Specificity of TGF-beta1 signal designated by LRRC33 and integrin alphaVbeta8.* Nat Commun, 2022. **13**(1): p. 4988.
33. Lienart, S., et al., *Structural basis of latent TGF-beta1 presentation and activation by GARP on human regulatory T cells.* Science, 2018. **362**(6417): p. 952-956.
34. Robertson, I.B. and D.B. Rifkin, *Unchaining the beast; insights from structural and evolutionary studies on TGFbeta secretion, sequestration, and activation.* Cytokine Growth Factor Rev, 2013. **24**(4): p. 355-72.
35. Yoshinaga, K., et al., *Perturbation of transforming growth factor (TGF)-beta1 association with latent TGF-beta binding protein yields inflammation and tumors.* Proc Natl Acad Sci U S A, 2008. **105**(48): p. 18758-63.
36. Todorovic, V., et al., *Long form of latent TGF-beta binding protein 1 (Ltbp1L) is essential for cardiac outflow tract septation and remodeling.* Development, 2007. **134**(20): p. 3723-32.
37. Dabovic, B., et al., *Bone abnormalities in latent TGF-[beta] binding protein (Ltbp)-3-null mice indicate a role for Ltbp-3 in modulating TGF-[beta] bioavailability.* J Cell Biol, 2002. **156**(2): p. 227-32.
38. Dabovic, B., et al., *Bone defects in latent TGF-beta binding protein (Ltbp)-3 null mice; a role for Ltbp in TGF-beta presentation.* J Endocrinol, 2002. **175**(1): p. 129-41.
39. Sterner-Kock, A., et al., *Disruption of the gene encoding the latent transforming growth factor-beta binding protein 4 (LTBP-4) causes abnormal lung development, cardiomyopathy, and colorectal cancer.* Genes Dev, 2002. **16**(17): p. 2264-73.

40. Wu, B.X., et al., *Glycoprotein A repetitions predominant (GARP) positively regulates transforming growth factor (TGF) beta3 and is essential for mouse palatogenesis.* J Biol Chem, 2017. **292**(44): p. 18091-18097.
41. Butovsky, O., et al., *Identification of a unique TGF-beta-dependent molecular and functional signature in microglia.* Nat Neurosci, 2014. **17**(1): p. 131-43.
42. Travis, M.A. and D. Sheppard, *TGF-beta activation and function in immunity.* Annu Rev Immunol, 2014. **32**: p. 51-82.
43. Dong, X., et al., *Structural determinants of integrin beta-subunit specificity for latent TGF-beta.* Nat Struct Mol Biol, 2014. **21**(12): p. 1091-6.
44. Yang, Z., et al., *Absence of integrin-mediated TGFbeta1 activation in vivo recapitulates the phenotype of TGFbeta1-null mice.* J Cell Biol, 2007. **176**(6): p. 787-93.
45. Anthis, N.J., et al., *The structure of an integrin/talin complex reveals the basis of inside-out signal transduction.* EMBO J, 2009. **28**(22): p. 3623-32.
46. Zhu, J., J. Zhu, and T.A. Springer, *Complete integrin headpiece opening in eight steps.* J Cell Biol, 2013. **201**(7): p. 1053-68.
47. Reed, N.I., et al., *The alphavbeta1 integrin plays a critical in vivo role in tissue fibrosis.* Sci Transl Med, 2015. **7**(288): p. 288ra79.
48. Huang, X.Z., et al., *Inactivation of the integrin beta 6 subunit gene reveals a role of epithelial integrins in regulating inflammation in the lung and skin.* J Cell Biol, 1996. **133**(4): p. 921-8.
49. Munger, J.S., et al., *The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis.* Cell, 1999. **96**(3): p. 319-28.
50. Wang, R., et al., *GARP regulates the bioavailability and activation of TGFbeta.* Mol Biol Cell, 2012. **23**(6): p. 1129-39.
51. Annes, J.P., et al., *Integrin alphaVbeta6-mediated activation of latent TGF-beta requires the latent TGF-beta binding protein-1.* J Cell Biol, 2004. **165**(5): p. 723-34.
52. Dong, X., et al., *Force interacts with macromolecular structure in activation of TGF-beta.* Nature, 2017. **542**(7639): p. 55-59.
53. Cormier, A., et al., *Cryo-EM structure of the alphavbeta8 integrin reveals a mechanism for stabilizing integrin extension.* Nat Struct Mol Biol, 2018. **25**(8): p. 698-704.
54. Mohammed, J., et al., *Stromal cells control the epithelial residence of DCs and memory T cells by regulated activation of TGF-beta.* Nat Immunol, 2016. **17**(4): p. 414-21.
55. Kitamura, H., et al., *Mouse and human lung fibroblasts regulate dendritic cell trafficking, airway inflammation, and fibrosis through integrin alphavbeta8-mediated activation of TGF-beta.* J Clin Invest, 2011. **121**(7): p. 2863-75.
56. Melton, A.C., et al., *Expression of alphavbeta8 integrin on dendritic cells regulates Th17 cell development and experimental autoimmune encephalomyelitis in mice.* J Clin Invest, 2010. **120**(12): p. 4436-44.
57. Worthington, J.J., et al., *Intestinal dendritic cells specialize to activate transforming growth factor-beta and induce Foxp3+ regulatory T cells via integrin alphavbeta8.* Gastroenterology, 2011. **141**(5): p. 1802-12.
58. Kelly, A., et al., *Human monocytes and macrophages regulate immune tolerance via integrin alphavbeta8-mediated TGFbeta activation.* J Exp Med, 2018. **215**(11): p. 2725-2736.
59. Stockis, J., et al., *Blocking immunosuppression by human Tregs in vivo with antibodies targeting integrin alphaVbeta8.* Proc Natl Acad Sci U S A, 2017. **114**(47): p. E10161-E10168.

60. Worthington, J.J., et al., *Integrin alphavbeta8-Mediated TGF-beta Activation by Effector Regulatory T Cells Is Essential for Suppression of T-Cell-Mediated Inflammation.* Immunity, 2015. **42**(5): p. 903-15.
61. Zhu, J., et al., *beta8 integrins are required for vascular morphogenesis in mouse embryos.* Development, 2002. **129**(12): p. 2891-903.
62. Proctor, J.M., et al., *Vascular development of the brain requires beta8 integrin expression in the neuroepithelium.* J Neurosci, 2005. **25**(43): p. 9940-8.
63. Aluwihare, P., et al., *Mice that lack activity of alphavbeta6- and alphavbeta8-integrins reproduce the abnormalities of Tgfb1- and Tgfb3-null mice.* J Cell Sci, 2009. **122**(Pt 2): p. 227-32.
64. Minagawa, S., et al., *Selective targeting of TGF-beta activation to treat fibroinflammatory airway disease.* Sci Transl Med, 2014. **6**(241): p. 241ra79.
65. Travis, M.A., et al., *Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice.* Nature, 2007. **449**(7160): p. 361-5.
66. Worthington, J.J., et al., *Loss of the TGFbeta-activating integrin alphavbeta8 on dendritic cells protects mice from chronic intestinal parasitic infection via control of type 2 immunity.* PLoS Pathog, 2013. **9**(10): p. e1003675.
67. Mu, D., et al., *The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1.* J Cell Biol, 2002. **157**(3): p. 493-507.
68. Edwards, J.P., A.M. Thornton, and E.M. Shevach, *Release of active TGF-beta1 from the latent TGF-beta1/GARP complex on T regulatory cells is mediated by integrin beta8.* J Immunol, 2014. **193**(6): p. 2843-9.
69. Cheerathodi, M., et al., *The Cytoskeletal Adapter Protein Spinophilin Regulates Invadopodia Dynamics and Tumor Cell Invasion in Glioblastoma.* Mol Cancer Res, 2016. **14**(12): p. 1277-1287.
70. Wang, J., et al., *Atypical interactions of integrin alphaVbeta8 with pro-TGF-beta1.* Proc Natl Acad Sci U S A, 2017. **114**(21): p. E4168-E4174.
71. Wang, J., et al., *General structural features that regulate integrin affinity revealed by atypical alphaVbeta8.* Nat Commun, 2019. **10**(1): p. 5481.
72. Campbell, M.G., et al., *Cryo-EM Reveals Integrin-Mediated TGF-beta Activation without Release from Latent TGF-beta.* Cell, 2020. **180**(3): p. 490-501 e16.
73. Ribeiro, S.M., et al., *The activation sequence of thrombospondin-1 interacts with the latency-associated peptide to regulate activation of latent transforming growth factor-beta.* J Biol Chem, 1999. **274**(19): p. 13586-93.
74. Young, G.D. and J.E. Murphy-Ullrich, *The tryptophan-rich motifs of the thrombospondin type 1 repeats bind VLAL motifs in the latent transforming growth factor-beta complex.* J Biol Chem, 2004. **279**(46): p. 47633-42.
75. Schultz-Cherry, S., et al., *Regulation of transforming growth factor-beta activation by discrete sequences of thrombospondin 1.* J Biol Chem, 1995. **270**(13): p. 7304-10.
76. Schultz-Cherry, S., J. Lawler, and J.E. Murphy-Ullrich, *The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-beta.* J Biol Chem, 1994. **269**(43): p. 26783-8.
77. Murphy-Ullrich, J.E. and M.J. Suto, *Thrombospondin-1 regulation of latent TGF-beta activation: A therapeutic target for fibrotic disease.* Matrix Biol, 2018. **68-69**: p. 28-43.
78. Bailly, S., et al., *Analysis of small latent transforming growth factor-beta complex formation and dissociation by surface plasmon resonance. Absence of direct interaction with thrombospondins.* J Biol Chem, 1997. **272**(26): p. 16329-34.

79. Grainger, D.J. and E.K. Frow, *Thrombospondin 1 does not activate transforming growth factor beta1 in a chemically defined system or in smooth-muscle-cell cultures.* Biochem J, 2000. **350 Pt 1**(Pt 1): p. 291-8.
80. Crawford, S.E., et al., *Thrombospondin-1 is a major activator of TGF-beta1 in vivo.* Cell, 1998. **93**(7): p. 1159-70.
81. Rudensky, A.Y., *Regulatory T cells and Foxp3.* Immunol Rev, 2011. **241**(1): p. 260-8.
82. Sakaguchi, S., et al., *Regulatory T Cells and Human Disease.* Annu Rev Immunol, 2020. **38**: p. 541-566.
83. Polansky, J.K., et al., *DNA methylation controls Foxp3 gene expression.* Eur J Immunol, 2008. **38**(6): p. 1654-63.
84. Sakaguchi, S., et al., *The plasticity and stability of regulatory T cells.* Nat Rev Immunol, 2013. **13**(6): p. 461-7.
85. Zheng, Y., et al., *Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate.* Nature, 2010. **463**(7282): p. 808-12.
86. Francois, V., et al., *The CD4(+) T-cell response of melanoma patients to a MAGE-A3 peptide vaccine involves potential regulatory T cells.* Cancer Res, 2009. **69**(10): p. 4335-45.
87. Stockis, J., et al., *Comparison of stable human Treg and Th clones by transcriptional profiling.* Eur J Immunol, 2009. **39**(3): p. 869-82.
88. Nakamura, K., et al., *TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice.* J Immunol, 2004. **172**(2): p. 834-42.
89. Nakamura, K., A. Kitani, and W. Strober, *Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta.* J Exp Med, 2001. **194**(5): p. 629-44.
90. Bertrand, C., et al., *Combined Blockade of GARP:TGF-beta1 and PD-1 Increases Infiltration of T Cells and Density of Pericyte-Covered GARP(+) Blood Vessels in Mouse MC38 Tumors.* Front Immunol, 2021. **12**: p. 704050.
91. Lecomte, S., et al., *Therapeutic activity of GARP:TGF-beta1 blockade in murine primary myelofibrosis.* Blood, 2022.
92. Salem, M., et al., *GARP Dampens Cancer Immunity by Sustaining Function and Accumulation of Regulatory T Cells in the Colon.* Cancer Res, 2019. **79**(6): p. 1178-1190.
93. Edwards, J.P., et al., *Regulation of the expression of GARP/latent TGF-beta1 complexes on mouse T cells and their role in regulatory T cell and Th17 differentiation.* J Immunol, 2013. **190**(11): p. 5506-15.
94. Nasrallah, R., et al., *A distal enhancer at risk locus 11q13.5 promotes suppression of colitis by Treg cells.* Nature, 2020. **583**(7816): p. 447-452.
95. Miyazono, K., et al., *Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization.* J Biol Chem, 1988. **263**(13): p. 6407-15.
96. Assoian, R.K., et al., *Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization.* J Biol Chem, 1983. **258**(11): p. 7155-60.
97. Rachidi, S., et al., *Platelets subvert T cell immunity against cancer via GARP-TGFbeta axis.* Sci Immunol, 2017. **2**(11).
98. Ahamed, J., et al., *In vitro and in vivo evidence for shear-induced activation of latent transforming growth factor-beta1.* Blood, 2008. **112**(9): p. 3650-60.
99. Abdelouahed, M., et al., *Activation of platelet-transforming growth factor beta-1 in the absence of thrombospondin-1.* J Biol Chem, 2000. **275**(24): p. 17933-6.

100. Blakytny, R., et al., *Latent TGF-beta1 activation by platelets.* J Cell Physiol, 2004. **199**(1): p. 67-76.
101. Metelli, A., et al., *Thrombin contributes to cancer immune evasion via proteolysis of platelet-bound GARP to activate LTGF-beta.* Sci Transl Med, 2020. **12**(525).
102. Gaignage, M., et al., *Blocking GARP-mediated activation of TGF-beta1 did not alter innate or adaptive immune responses to bacterial infection or protein immunization in mice.* Cancer Immunol Immunother, 2022. **71**(8): p. 1851-1862.
103. Wallace, C.H., et al., *B lymphocytes confer immune tolerance via cell surface GARP-TGF-beta complex.* JCI Insight, 2018. **3**(7).
104. Niu, J., et al., *Mesenchymal stem cells inhibit T cell activation by releasing TGF-beta1 from TGF-beta1/GARP complex.* Oncotarget, 2017. **8**(59): p. 99784-99800.
105. Carambia, A., et al., *TGF-beta-dependent induction of CD4(+)CD25(+)Foxp3(+) Tregs by liver sinusoidal endothelial cells.* J Hepatol, 2014. **61**(3): p. 594-9.
106. Jiang, A., Y. Qin, and T.A. Springer, *Loss of LRRC33-Dependent TGFbeta1 Activation Enhances Antitumor Immunity and Checkpoint Blockade Therapy.* Cancer Immunol Res, 2022. **10**(4): p. 453-467.
107. Budi, E.H., D. Duan, and R. Derynck, *Transforming Growth Factor-beta Receptors and Smads: Regulatory Complexity and Functional Versatility.* Trends Cell Biol, 2017. **27**(9): p. 658-672.
108. Marcoe, J.P., et al., *TGF-beta is responsible for NK cell immaturity during ontogeny and increased susceptibility to infection during mouse infancy.* Nat Immunol, 2012. **13**(9): p. 843-50.
109. Flavell, R.A., et al., *The polarization of immune cells in the tumour environment by TGFbeta.* Nat Rev Immunol, 2010. **10**(8): p. 554-67.
110. Sanjabi, S., S.A. Oh, and M.O. Li, *Regulation of the Immune Response by TGF-beta: From Conception to Autoimmunity and Infection.* Cold Spring Harb Perspect Biol, 2017. **9**(6).
111. Smythies, L.E., et al., *Mucosal IL-8 and TGF-beta recruit blood monocytes: evidence for cross-talk between the lamina propria stroma and myeloid cells.* J Leukoc Biol, 2006. **80**(3): p. 492-9.
112. Rani, R., et al., *TGF-beta limits IL-33 production and promotes the resolution of colitis through regulation of macrophage function.* Eur J Immunol, 2011. **41**(7): p. 2000-9.
113. Esebanmen, G.E. and W.H.R. Langridge, *The role of TGF-beta signaling in dendritic cell tolerance.* Immunol Res, 2017. **65**(5): p. 987-994.
114. Ramalingam, R., et al., *Dendritic cell-specific disruption of TGF-beta receptor II leads to altered regulatory T cell phenotype and spontaneous multiorgan autoimmunity.* J Immunol, 2012. **189**(8): p. 3878-93.
115. Laouar, Y., et al., *TGF-beta signaling in dendritic cells is a prerequisite for the control of autoimmune encephalomyelitis.* Proc Natl Acad Sci U S A, 2008. **105**(31): p. 10865-70.
116. Coombes, J.L. and K.J. Maloy, *Control of intestinal homeostasis by regulatory T cells and dendritic cells.* Semin Immunol, 2007. **19**(2): p. 116-26.
117. Sun, C.M., et al., *Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid.* J Exp Med, 2007. **204**(8): p. 1775-85.
118. Paidassi, H., et al., *Preferential expression of integrin alphavbeta8 promotes generation of regulatory T cells by mouse CD103+ dendritic cells.* Gastroenterology, 2011. **141**(5): p. 1813-20.
119. Romani, N., B.E. Clausen, and P. Stoitzner, *Langerhans cells and more: langerin-expressing dendritic cell subsets in the skin.* Immunol Rev, 2010. **234**(1): p. 120-41.

120. Ndaw, V.S., et al., *TGF-beta1 Suppresses IL-33-Induced Mast Cell Function.* J Immunol, 2017. **199**(3): p. 866-873.
121. Haque, T.T. and P.A. Frischmeyer-Guerrerio, *The Role of TGFbeta and Other Cytokines in Regulating Mast Cell Functions in Allergic Inflammation.* Int J Mol Sci, 2022. **23**(18).
122. Lyons, J.J., et al., *ERBIN deficiency links STAT3 and TGF-beta pathway defects with atopy in humans.* J Exp Med, 2017. **214**(3): p. 669-680.
123. Marie, J.C., D. Liggitt, and A.Y. Rudensky, *Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor.* Immunity, 2006. **25**(3): p. 441-54.
124. Letterio, J.J., et al., *Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression.* J Clin Invest, 1996. **98**(9): p. 2109-19.
125. Kobayashi, S., et al., *Beta 2-microglobulin-deficient background ameliorates lethal phenotype of the TGF-beta 1 null mouse.* J Immunol, 1999. **163**(7): p. 4013-9.
126. Hong, C., M.A. Luckey, and J.H. Park, *Intrathymic IL-7: the where, when, and why of IL-7 signaling during T cell development.* Semin Immunol, 2012. **24**(3): p. 151-8.
127. Lee, H.M., et al., *A broad range of self-reactivity drives thymic regulatory T cell selection to limit responses to self.* Immunity, 2012. **37**(3): p. 475-86.
128. Konkel, J.E., et al., *Thymocyte apoptosis drives the intrathymic generation of regulatory T cells.* Proc Natl Acad Sci U S A, 2014. **111**(4): p. E465-73.
129. Marie, J.C., et al., *TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells.* J Exp Med, 2005. **201**(7): p. 1061-7.
130. Li, M.O., S. Sanjabi, and R.A. Flavell, *Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms.* Immunity, 2006. **25**(3): p. 455-71.
131. Ouyang, W., et al., *Transforming growth factor-beta signaling curbs thymic negative selection promoting regulatory T cell development.* Immunity, 2010. **32**(5): p. 642-53.
132. Liu, Y., et al., *A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells.* Nat Immunol, 2008. **9**(6): p. 632-40.
133. Lee, P.W., M.E. Severin, and A.E. Lovett-Racke, *TGF-beta regulation of encephalitogenic and regulatory T cells in multiple sclerosis.* Eur J Immunol, 2017. **47**(3): p. 446-453.
134. Cottrez, F. and H. Groux, *Regulation of TGF-beta response during T cell activation is modulated by IL-10.* J Immunol, 2001. **167**(2): p. 773-8.
135. Sanjabi, S., M.M. Mosaheb, and R.A. Flavell, *Opposing effects of TGF-beta and IL-15 cytokines control the number of short-lived effector CD8+ T cells.* Immunity, 2009. **31**(1): p. 131-44.
136. Ouyang, W., et al., *TGF-beta cytokine signaling promotes CD8+ T cell development and low-affinity CD4+ T cell homeostasis by regulation of interleukin-7 receptor alpha expression.* Immunity, 2013. **39**(2): p. 335-46.
137. Park, J.H., et al., *Signaling by intrathymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells.* Nat Immunol, 2010. **11**(3): p. 257-64.
138. Takimoto, T., et al., *Smad2 and Smad3 are redundantly essential for the TGF-beta-mediated regulation of regulatory T plasticity and Th1 development.* J Immunol, 2010. **185**(2): p. 842-55.
139. Hill, J.A., et al., *Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi Cells.* Immunity, 2008. **29**(5): p. 758-70.

140. Josefowicz, S.Z., L.F. Lu, and A.Y. Rudensky, *Regulatory T cells: mechanisms of differentiation and function.* Annu Rev Immunol, 2012. **30**: p. 531-64.
141. Haribhai, D., et al., *A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity.* Immunity, 2011. **35**(1): p. 109-22.
142. Korn, T., et al., *IL-17 and Th17 Cells.* Annu Rev Immunol, 2009. **27**: p. 485-517.
143. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.
144. Veldhoen, M., et al., *Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease.* Nat Immunol, 2006. **7**(11): p. 1151-6.
145. Dardalhon, V., et al., *IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells.* Nat Immunol, 2008. **9**(12): p. 1347-55.
146. Veldhoen, M., et al., *Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset.* Nat Immunol, 2008. **9**(12): p. 1341-6.
147. Mani, V., et al., *Migratory DCs activate TGF-beta to precondition naive CD8(+) T cells for tissue-resident memory fate.* Science, 2019. **366**(6462).
148. Thomas, D.A. and J. Massague, *TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance.* Cancer Cell, 2005. **8**(5): p. 369-80.
149. Sledzinska, A., et al., *TGF-beta signalling is required for CD4(+) T cell homeostasis but dispensable for regulatory T cell function.* PLoS Biol, 2013. **11**(10): p. e1001674.
150. Zhang, N. and M.J. Bevan, *TGF-beta signaling to T cells inhibits autoimmunity during lymphopenia-driven proliferation.* Nat Immunol, 2012. **13**(7): p. 667-73.
151. Cazac, B.B. and J. Roes, *TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo.* Immunity, 2000. **13**(4): p. 443-51.
152. Medzhitov, R., *The spectrum of inflammatory responses.* Science, 2021. **374**(6571): p. 1070-1075.
153. Maizels, R.M., *The multi-faceted roles of TGF-beta in regulation of immunity to infection.* Adv Immunol, 2021. **150**: p. 1-42.
154. Tinoco, R., et al., *Cell-intrinsic transforming growth factor-beta signaling mediates virus-specific CD8+ T cell deletion and viral persistence in vivo.* Immunity, 2009. **31**(1): p. 145-57.
155. Garidou, L., et al., *Therapeutic blockade of transforming growth factor beta fails to promote clearance of a persistent viral infection.* J Virol, 2012. **86**(13): p. 7060-71.
156. Boettler, T., et al., *TGF-beta blockade does not improve control of an established persistent viral infection.* Viral Immunol, 2012. **25**(3): p. 232-8.
157. Schultz-Cherry, S. and V.S. Hinshaw, *Influenza virus neuraminidase activates latent transforming growth factor beta.* J Virol, 1996. **70**(12): p. 8624-9.
158. Carlson, C.M., et al., *Transforming growth factor-beta: activation by neuraminidase and role in highly pathogenic H5N1 influenza pathogenesis.* PLoS Pathog, 2010. **6**(10): p. e1001136.
159. Marshall, H.D., et al., *The transforming growth factor beta signaling pathway is critical for the formation of CD4 T follicular helper cells and isotype-switched antibody responses in the lung mucosa.* Elife, 2015. **4**: p. e04851.

160. Alatrakchi, N., et al., *Hepatitis C virus (HCV)-specific CD8+ cells produce transforming growth factor beta that can suppress HCV-specific T-cell responses.* J Virol, 2007. **81**(11): p. 5882-92.
161. Karimi-Googheri, M., et al., *Important roles played by TGF-beta in hepatitis B infection.* J Med Virol, 2014. **86**(1): p. 102-8.
162. Khorramdelazad, H., et al., *High Serum Levels of TGF-beta in Iranians With Chronic HBV Infection.* Hepat Mon, 2012. **12**(11): p. e7581.
163. Sun, C., et al., *TGF-beta1 down-regulation of NKG2D/DAP10 and 2B4/SAP expression on human NK cells contributes to HBV persistence.* PLoS Pathog, 2012. **8**(3): p. e1002594.
164. Liu, Y., et al., *Neisseria gonorrhoeae selectively suppresses the development of Th1 and Th2 cells, and enhances Th17 cell responses, through TGF-beta-dependent mechanisms.* Mucosal Immunol, 2012. **5**(3): p. 320-31.
165. Beswick, E.J., et al., *Role of gastric epithelial cell-derived transforming growth factor beta in reduced CD4+ T cell proliferation and development of regulatory T cells during Helicobacter pylori infection.* Infect Immun, 2011. **79**(7): p. 2737-45.
166. Liu, Y. and M.W. Russell, *Diversion of the immune response to Neisseria gonorrhoeae from Th17 to Th1/Th2 by treatment with anti-transforming growth factor beta antibody generates immunological memory and protective immunity.* mBio, 2011. **2**(3): p. e00095-11.
167. Torchinsky, M.B., et al., *Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation.* Nature, 2009. **458**(7234): p. 78-82.
168. Brereton, C.F. and J.M. Blander, *Responding to infection and apoptosis--a task for TH17 cells.* Ann N Y Acad Sci, 2010. **1209**: p. 56-67.
169. DePaolo, R.W., et al., *A specific role for TLR1 in protective T(H)17 immunity during mucosal infection.* J Exp Med, 2012. **209**(8): p. 1437-44.
170. Gomez-Escobar, N., W.F. Gregory, and R.M. Maizels, *Identification of tgh-2, a filarial nematode homolog of Caenorhabditis elegans daf-7 and human transforming growth factor beta, expressed in microfilarial and adult stages of Brugia malayi.* Infect Immun, 2000. **68**(11): p. 6402-10.
171. Sulaiman, A.A., et al., *A Trematode Parasite Derived Growth Factor Binds and Exerts Influences on Host Immune Functions via Host Cytokine Receptor Complexes.* PLoS Pathog, 2016. **12**(11): p. e1005991.
172. Grainger, J.R., et al., *Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-beta pathway.* J Exp Med, 2010. **207**(11): p. 2331-41.
173. Smyth, D.J., et al., *TGF-beta mimic proteins form an extended gene family in the murine parasite Heligmosomoides polygyrus.* Int J Parasitol, 2018. **48**(5): p. 379-385.
174. Doetze, A., et al., *Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-beta but not by a T(h)1 to T(h)2 shift.* Int Immunol, 2000. **12**(5): p. 623-30.
175. King, C.L., et al., *Cytokine control of parasite-specific anergy in human lymphatic filariasis. Preferential induction of a regulatory T helper type 2 lymphocyte subset.* J Clin Invest, 1993. **92**(4): p. 1667-73.
176. Barral, A., et al., *Transforming growth factor beta as a virulence mechanism for Leishmania braziliensis.* Proc Natl Acad Sci U S A, 1993. **90**(8): p. 3442-6.
177. Barral-Netto, M., et al., *Transforming growth factor-beta in leishmanial infection: a parasite escape mechanism.* Science, 1992. **257**(5069): p. 545-8.

178. Silva, J.S., D.R. Twardzik, and S.G. Reed, *Regulation of Trypanosoma cruzi infections in vitro and in vivo by transforming growth factor beta (TGF-beta).* J Exp Med, 1991. **174**(3): p. 539-45.
179. Laouar, Y., et al., *Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma.* Nat Immunol, 2005. **6**(6): p. 600-7.
180. Drewry, L.L. and J.T. Harty, *Balancing in a black box: Potential immunomodulatory roles for TGF-beta signaling during blood-stage malaria.* Virulence, 2020. **11**(1): p. 159-169.
181. Omer, F.M., J.B. de Souza, and E.M. Riley, *Differential induction of TGF-beta regulates proinflammatory cytokine production and determines the outcome of lethal and nonlethal Plasmodium yoelii infections.* J Immunol, 2003. **171**(10): p. 5430-6.
182. Omer, F.M. and E.M. Riley, *Transforming growth factor beta production is inversely correlated with severity of murine malaria infection.* J Exp Med, 1998. **188**(1): p. 39-48.
183. Tsutsui, N. and T. Kamiyama, *Transforming growth factor beta-induced failure of resistance to infection with blood-stage Plasmodium chabaudi in mice.* Infect Immun, 1999. **67**(5): p. 2306-11.
184. Ocana-Morgner, C., et al., *Role of TGF-beta and PGE2 in T cell responses during Plasmodium yoelii infection.* Eur J Immunol, 2007. **37**(6): p. 1562-74.
185. Frangogiannis, N., *Transforming growth factor-beta in tissue fibrosis.* J Exp Med, 2020. **217**(3): p. e20190103.
186. Lodyga, M., et al., *Cadherin-11-mediated adhesion of macrophages to myofibroblasts establishes a profibrotic niche of active TGF-beta.* Sci Signal, 2019. **12**(564).
187. Wynn, T.A., *Cellular and molecular mechanisms of fibrosis.* J Pathol, 2008. **214**(2): p. 199-210.
188. Rice, L.M., et al., *FresolimumAb treatment decreases biomarkers and improves clinical symptoms in systemic sclerosis patients.* J Clin Invest, 2015. **125**(7): p. 2795-807.
189. Sun, T., et al., *TGFbeta2 and TGFbeta3 isoforms drive fibrotic disease pathogenesis.* Sci Transl Med, 2021. **13**(605).
190. Johnston, C.J., et al., *TGF-beta in tolerance, development and regulation of immunity.* Cell Immunol, 2016. **299**: p. 14-22.
191. Rubtsov, Y.P. and A.Y. Rudensky, *TGFbeta signalling in control of T-cell-mediated self-reactivity.* Nat Rev Immunol, 2007. **7**(6): p. 443-53.
192. Di Sabatino, A., et al., *Blockade of transforming growth factor beta upregulates T-box transcription factor T-bet, and increases T helper cell type 1 cytokine and matrix metalloproteinase-3 production in the human gut mucosa.* Gut, 2008. **57**(5): p. 605-12.
193. Ihara, S., et al., *TGF-beta Signaling in Dendritic Cells Governs Colonic Homeostasis by Controlling Epithelial Differentiation and the Luminal Microbiota.* J Immunol, 2016. **196**(11): p. 4603-13.
194. Li, J., et al., *Myeloid TGF-beta signaling contributes to colitis-associated tumorigenesis in mice.* Carcinogenesis, 2013. **34**(9): p. 2099-108.
195. Oshima, H., et al., *Suppressing TGFbeta signaling in regenerating epithelia in an inflammatory microenvironment is sufficient to cause invasive intestinal cancer.* Cancer Res, 2015. **75**(4): p. 766-76.
196. Ihara, S., Y. Hirata, and K. Koike, *TGF-beta in inflammatory bowel disease: a key regulator of immune cells, epithelium, and the intestinal microbiota.* J Gastroenterol, 2017. **52**(7): p. 777-787.

197. Loeys, B.L., et al., *Aneurysm syndromes caused by mutations in the TGF-beta receptor.* N Engl J Med, 2006. **355**(8): p. 788-98.
198. Naviglio, S., et al., *Severe inflammatory bowel disease associated with congenital alteration of transforming growth factor beta signaling.* J Crohns Colitis, 2014. **8**(8): p. 770-4.
199. Monteleone, G., et al., *Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease.* J Clin Invest, 2001. **108**(4): p. 601-9.
200. Lees, C.W., et al., *New IBD genetics: common pathways with other diseases.* Gut, 2011. **60**(12): p. 1739-53.
201. Sands, B.E., et al., *Mongersen (GED-0301) for Active Crohn's Disease: Results of a Phase 3 Study.* Am J Gastroenterol, 2020. **115**(5): p. 738-745.
202. Marafini, I., et al., *A Pharmacological Batch of Mongersen that Downregulates Smad7 is Effective as Induction Therapy in Active Crohn's Disease: A Phase II, Open-Label Study.* BioDrugs, 2021. **35**(3): p. 325-336.
203. Crooks, B., T. Barnes, and J.K. Limdi, *Vedolizumab in the treatment of inflammatory bowel disease: evolving paradigms.* Drugs Context, 2020. **9**.
204. Yang, Y.C., et al., *Transforming growth factor-beta1 in inflammatory airway disease: a key for understanding inflammation and remodeling.* Allergy, 2012. **67**(10): p. 1193-202.
205. Tirado-Rodriguez, B., et al., *TGF- beta: an important mediator of allergic disease and a molecule with dual activity in cancer development.* J Immunol Res, 2014. **2014**: p. 318481.
206. Hammad, H. and B.N. Lambrecht, *The basic immunology of asthma.* Cell, 2021. **184**(9): p. 2521-2522.
207. Moffatt, M.F., et al., *A large-scale, consortium-based genomewide association study of asthma.* N Engl J Med, 2010. **363**(13): p. 1211-1221.
208. Van Hove, C.L., et al., *Chronic inflammation in asthma: a contest of persistence vs resolution.* Allergy, 2008. **63**(9): p. 1095-109.
209. Silverman, E.S., et al., *Transforming growth factor-beta1 promoter polymorphism C-509T is associated with asthma.* Am J Respir Crit Care Med, 2004. **169**(2): p. 214-9.
210. Hobbs, K., et al., *Interleukin-10 and transforming growth factor-beta promoter polymorphisms in allergies and asthma.* Am J Respir Crit Care Med, 1998. **158**(6): p. 1958-62.
211. Yao, Y.S., et al., *An updated meta-analysis of transforming growth factor-beta1 gene: Three well-characterized polymorphisms with asthma.* Hum Immunol, 2016. **77**(12): p. 1291-1299.
212. Zhang, Y., et al., *Polymorphisms in the transforming growth factor-beta1 gene and the risk of asthma: A meta-analysis.* Respirology, 2010. **15**(4): p. 643-50.
213. Chiang, C.H., et al., *Genetic polymorphism of transforming growth factor beta1 and tumor necrosis factor alpha is associated with asthma and modulates the severity of asthma.* Respir Care, 2013. **58**(8): p. 1343-50.
214. Frischmeyer-Guerrerio, P.A., et al., *TGFbeta receptor mutations impose a strong predisposition for human allergic disease.* Sci Transl Med, 2013. **5**(195): p. 195ra94.
215. Vignola, A.M., et al., *Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis.* Am J Respir Crit Care Med, 1997. **156**(2 Pt 1): p. 591-9.
216. Redington, A.E., et al., *Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid.* Am J Respir Crit Care Med, 1997. **156**(2 Pt 1): p. 642-7.
217. Batra, V., et al., *Bronchoalveolar lavage fluid concentrations of transforming growth factor (TGF)-beta1, TGF-beta2, interleukin (IL)-4 and IL-13 after segmental allergen challenge and their effects on alpha-smooth muscle actin and collagen III synthesis by primary human lung fibroblasts.* Clin Exp Allergy, 2004. **34**(3): p. 437-44.

218. Kariyawasam, H.H., et al., *Activin and transforming growth factor-beta signaling pathways are activated after allergen challenge in mild asthma.* J Allergy Clin Immunol, 2009. **124**(3): p. 454-62.
219. Al-Alawi, M., T. Hassan, and S.H. Chotirmall, *Transforming growth factor beta and severe asthma: a perfect storm.* Respir Med, 2014. **108**(10): p. 1409-23.
220. Saito, A., M. Horie, and T. Nagase, *TGF-beta Signaling in Lung Health and Disease.* Int J Mol Sci, 2018. **19**(8).
221. Branchett, W.J. and C.M. Lloyd, *Regulatory cytokine function in the respiratory tract.* Mucosal Immunol, 2019. **12**(3): p. 589-600.
222. Fattouh, R., et al., *Transforming growth factor-beta regulates house dust mite-induced allergic airway inflammation but not airway remodeling.* Am J Respir Crit Care Med, 2008. **177**(6): p. 593-603.
223. McMillan, S.J., G. Xanthou, and C.M. Lloyd, *Manipulation of allergen-induced airway remodeling by treatment with anti-TGF-beta antibody: effect on the Smad signaling pathway.* J Immunol, 2005. **174**(9): p. 5774-80.
224. Alcorn, J.F., et al., *Transforming growth factor-beta1 suppresses airway hyperresponsiveness in allergic airway disease.* Am J Respir Crit Care Med, 2007. **176**(10): p. 974-82.
225. Wei, Y., et al., *Assessment of tumor growth factor-beta1 neutralizing antibody in the treatment of allergic rhinitis and asthma.* Exp Ther Med, 2018. **15**(1): p. 649-656.
226. Joetham, A., et al., *Naturally occurring lung CD4(+)CD25(+) T cell regulation of airway allergic responses depends on IL-10 induction of TGF-beta.* J Immunol, 2007. **178**(3): p. 1433-42.
227. Scherf, W., S. Burdach, and G. Hansen, *Reduced expression of transforming growth factor beta 1 exacerbates pathology in an experimental asthma model.* Eur J Immunol, 2005. **35**(1): p. 198-206.
228. Nakao, A., et al., *Blockade of transforming growth factor beta/Smad signaling in T cells by overexpression of Smad7 enhances antigen-induced airway inflammation and airway reactivity.* J Exp Med, 2000. **192**(2): p. 151-8.
229. Hansen, G., et al., *CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation.* J Clin Invest, 2000. **105**(1): p. 61-70.
230. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.
231. Kearley, J., D.S. Robinson, and C.M. Lloyd, *CD4+CD25+ regulatory T cells reverse established allergic airway inflammation and prevent airway remodeling.* J Allergy Clin Immunol, 2008. **122**(3): p. 617-24 e6.
232. Ling, E.M., et al., *Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease.* Lancet, 2004. **363**(9409): p. 608-15.
233. Huang, F., et al., *Association of imbalance of effector T cells and regulatory cells with the severity of asthma and allergic rhinitis in children.* Allergy Asthma Proc, 2017. **38**(6): p. 70-77.
234. Provoost, S., et al., *Decreased FOXP3 protein expression in patients with asthma.* Allergy, 2009. **64**(10): p. 1539-1546.

235. Krogulska, A., et al., *Decreased FOXP3 mRNA expression in children with atopic asthma and IgE-mediated food allergy.* Ann Allergy Asthma Immunol, 2015. **115**(5): p. 415-21.
236. Gavin, M.A., et al., *Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development.* Proc Natl Acad Sci U S A, 2006. **103**(17): p. 6659-64.
237. Tran, D.Q., H. Ramsey, and E.M. Shevach, *Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype.* Blood, 2007. **110**(8): p. 2983-90.
238. Walker, M.R., et al., *Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells.* J Clin Invest, 2003. **112**(9): p. 1437-43.
239. Fan, K., L. Jin, and S. Yu, *Roles of regulatory B cells in the pathogenesis of allergic rhinitis.* Allergol Immunopathol (Madr), 2022. **50**(5): p. 7-15.
240. Staudt, V., et al., *Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells.* Immunity, 2010. **33**(2): p. 192-202.
241. Gounni, A.S., et al., *IL-9-mediated induction of eotaxin1/CCL11 in human airway smooth muscle cells.* J Immunol, 2004. **173**(4): p. 2771-9.
242. Townsend, J.M., et al., *IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development.* Immunity, 2000. **13**(4): p. 573-83.
243. Louahed, J., et al., *Interleukin-9 upregulates mucus expression in the airways.* Am J Respir Cell Mol Biol, 2000. **22**(6): p. 649-56.
244. Erpenbeck, V.J., et al., *Increased expression of interleukin-9 messenger RNA after segmental allergen challenge in allergic asthmatics.* Chest, 2003. **123**(3 Suppl): p. 370S.
245. Seumois, G., et al., *Single-cell transcriptomic analysis of allergen-specific T cells in allergy and asthma.* Sci Immunol, 2020. **5**(48).
246. Tsuda, M., et al., *A role for BATF3 in T(H)9 differentiation and T-cell-driven mucosal pathologies.* Mucosal Immunol, 2019. **12**(3): p. 644-655.
247. Xue, G., et al., *IL-4 together with IL-1beta induces antitumor Th9 cell differentiation in the absence of TGF-beta signaling.* Nat Commun, 2019. **10**(1): p. 1376.
248. Denney, L., et al., *Pulmonary Epithelial Cell-Derived Cytokine TGF-beta1 Is a Critical Cofactor for Enhanced Innate Lymphoid Cell Function.* Immunity, 2015. **43**(5): p. 945-58.
249. Wang, L., et al., *TGF-beta induces ST2 and programs ILC2 development.* Nat Commun, 2020. **11**(1): p. 35.
250. Lambrecht, B.N. and H. Hammad, *The immunology of asthma.* Nat Immunol, 2015. **16**(1): p. 45-56.
251. Bellini, A., et al., *Interleukin (IL)-4, IL-13, and IL-17A differentially affect the profibrotic and proinflammatory functions of fibrocytes from asthmatic patients.* Mucosal Immunol, 2012. **5**(2): p. 140-9.
252. Kudo, M., et al., *IL-17A produced by alphabeta T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction.* Nat Med, 2012. **18**(4): p. 547-54.
253. Leung, S.Y., et al., *Effect of transforming growth factor-beta receptor I kinase inhibitor 2,4-disubstituted pteridine (SD-208) in chronic allergic airway inflammation and remodeling.* J Pharmacol Exp Ther, 2006. **319**(2): p. 586-94.
254. Wnuk, D., et al., *Enhanced asthma-related fibroblast to myofibroblast transition is the result of profibrotic TGF-beta/Smad2/3 pathway intensification and antifibrotic TGF-beta/Smad1/5/(8)9 pathway impairment.* Sci Rep, 2020. **10**(1): p. 16492.

255. Xie, C., et al., *TGFbeta1 transduction enhances immunomodulatory capacity of neural stem cells in experimental autoimmune encephalomyelitis.* Brain Behav Immun, 2018. **69**: p. 283-295.
256. El-Behi, M., et al., *The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF.* Nat Immunol, 2011. **12**(6): p. 568-75.
257. Rodgers, J.M. and S.D. Miller, *Cytokine control of inflammation and repair in the pathology of multiple sclerosis.* Yale J Biol Med, 2012. **85**(4): p. 447-68.
258. Johns, L.D., et al., *Successful treatment of experimental allergic encephalomyelitis with transforming growth factor-beta 1.* J Immunol, 1991. **147**(6): p. 1792-6.
259. Kuruvilla, A.P., et al., *Protective effect of transforming growth factor beta 1 on experimental autoimmune diseases in mice.* Proc Natl Acad Sci U S A, 1991. **88**(7): p. 2918-21.
260. Racke, M.K., et al., *Prevention and treatment of chronic relapsing experimental allergic encephalomyelitis by transforming growth factor-beta 1.* J Immunol, 1991. **146**(9): p. 3012-7.
261. McGeachy, M.J., et al., *TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology.* Nat Immunol, 2007. **8**(12): p. 1390-7.
262. Ghoreschi, K., et al., *Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling.* Nature, 2010. **467**(7318): p. 967-71.
263. Annunziato, F., et al., *Phenotypic and functional features of human Th17 cells.* J Exp Med, 2007. **204**(8): p. 1849-61.
264. Nistala, K., et al., *Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment.* Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14751-6.
265. Kato, H., J. Endres, and D.A. Fox, *The roles of IFN-gamma versus IL-17 in pathogenic effects of human Th17 cells on synovial fibroblasts.* Mod Rheumatol, 2013. **23**(6): p. 1140-50.
266. Calahorra, L., et al., *Regulatory Cells in Multiple Sclerosis: From Blood to Brain.* Biomedicines, 2022. **10**(2).
267. Li, Y.F., et al., *The proportion of peripheral regulatory T cells in patients with Multiple Sclerosis: A meta-analysis.* Mult Scler Relat Disord, 2019. **28**: p. 75-80.
268. Danikowski, K.M., S. Jayaraman, and B.S. Prabhakar, *Regulatory T cells in multiple sclerosis and myasthenia gravis.* J Neuroinflammation, 2017. **14**(1): p. 117.
269. Meoli, E.M., et al., *TGF-beta signaling is altered in the peripheral blood of subjects with multiple sclerosis.* J Neuroimmunol, 2011. **230**(1-2): p. 164-8.
270. Jiang, Z., J.X. Jiang, and G.X. Zhang, *Macrophages: a double-edged sword in experimental autoimmune encephalomyelitis.* Immunol Lett, 2014. **160**(1): p. 17-22.
271. Filippi, M., et al., *Multiple sclerosis.* Nat Rev Dis Primers, 2018. **4**(1): p. 43.
272. Miron, V.E., et al., *M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination.* Nat Neurosci, 2013. **16**(9): p. 1211-1218.
273. Palazuelos, J., M. Klingener, and A. Aguirre, *TGFbeta signaling regulates the timing of CNS myelination by modulating oligodendrocyte progenitor cell cycle exit through SMAD3/4/FoxO1/Sp1.* J Neurosci, 2014. **34**(23): p. 7917-30.
274. Hamaguchi, M., et al., *Circulating transforming growth factor-beta1 facilitates remyelination in the adult central nervous system.* Elife, 2019. **8**.
275. Sotiropoulos, M.G. and T. Chitnis, *Opposing and potentially antagonistic effects of BMP and TGF-beta in multiple sclerosis: The "Yin and Yang" of neuro-immune Signaling.* J Neuroimmunol, 2020. **347**: p. 577358.

276. Moore, E. and C. Putterman, *Are lupus animal models useful for understanding and developing new therapies for human SLE?* J Autoimmun, 2020. **112**: p. 102490.
277. Ohtsuka, K., et al., *The relationship between defects in lymphocyte production of transforming growth factor-beta1 in systemic lupus erythematosus and disease activity or severity.* Lupus, 1999. **8**(2): p. 90-4.
278. Becker-Merok, A., G.O. Eilertsen, and J.C. Nossent, *Levels of transforming growth factor-beta are low in systemic lupus erythematosus patients with active disease.* J Rheumatol, 2010. **37**(10): p. 2039-45.
279. Zheng, S.G., *Transforming growth factor-beta level: indicator for severity of disease and organ damage in patients with systemic lupus erythematosus.* J Rheumatol, 2010. **37**(10): p. 1983-5.
280. Jin, T., et al., *Decreased serum levels of TGF-beta1 are associated with renal damage in female patients with systemic lupus erythematosus.* Lupus, 2012. **21**(3): p. 310-8.
281. Linge, P., et al., *The non-haemostatic role of platelets in systemic lupus erythematosus.* Nat Rev Rheumatol, 2018. **14**(4): p. 195-213.
282. Lee, Y.H. and S.C. Bae, *Association between circulating transforming growth factor-beta1 level and polymorphisms in systemic lupus erythematosus and rheumatoid arthritis: A meta-analysis.* Cell Mol Biol (Noisy-le-grand), 2017. **63**(1): p. 53-59.
283. Song, K., et al., *Genome-wide association study of SNP- and gene-based approaches to identify susceptibility candidates for lupus nephritis in the Han Chinese population.* Front Immunol, 2022. **13**: p. 908851.
284. Stadtlober, N.P., et al., *TGFB1 +869 T > C (rs1800470) variant is independently associated with susceptibility, laboratory activity, and TGF-beta1 in patients with systemic lupus erythematosus.* Autoimmunity, 2021. **54**(8): p. 569-575.
285. Kohut, E., et al., *Expression of TGFbeta1 and its signaling components by peripheral lymphocytes in systemic lupus erythematosus.* Pathol Oncol Res, 2009. **15**(2): p. 251-6.
286. Ohtsuka, K., et al., *Decreased production of TGF-beta by lymphocytes from patients with systemic lupus erythematosus.* J Immunol, 1998. **160**(5): p. 2539-45.
287. Xing, Q., et al., *Role of Treg cells and TGF-beta1 in patients with systemic lupus erythematosus: a possible relation with lupus nephritis.* Immunol Invest, 2012. **41**(1): p. 15-27.
288. Stadtlober, N.P., et al., *Haplotypes of FOXP3 genetic variants are associated with susceptibility, autoantibodies, and TGF-beta1 in patients with systemic lupus erythematosus.* Sci Rep, 2021. **11**(1): p. 5406.
289. Lin, Y.C., et al., *Association of single-nucleotide polymorphisms in FOXP3 gene with systemic lupus erythematosus susceptibility: a case-control study.* Lupus, 2011. **20**(2): p. 137-43.
290. Goschl, L., C. Scheinecker, and M. Bonelli, *Treg cells in autoimmunity: from identification to Treg-based therapies.* Semin Immunopathol, 2019. **41**(3): p. 301-314.
291. Dang, H., et al., *SLE-like autoantibodies and Sjogren's syndrome-like lymphoproliferation in TGF-beta knockout mice.* J Immunol, 1995. **155**(6): p. 3205-12.
292. Singh, R.R., et al., *Induction of autoantibody production is limited in nonautoimmune mice.* J Immunol, 2002. **169**(1): p. 587-94.
293. Saxena, V., et al., *Dual roles of immunoregulatory cytokine TGF-beta in the pathogenesis of autoimmunity-mediated organ damage.* J Immunol, 2008. **180**(3): p. 1903-12.
294. Raz, E., et al., *Modulation of disease activity in murine systemic lupus erythematosus by cytokine gene delivery.* Lupus, 1995. **4**(4): p. 286-92.

295. Xu, A., et al., *TGF-beta-Induced Regulatory T Cells Directly Suppress B Cell Responses through a Noncytotoxic Mechanism.* J Immunol, 2016. **196**(9): p. 3631-41.
296. Scalapino, K.J., et al., *Suppression of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive transfer of ex vivo expanded regulatory T cells.* J Immunol, 2006. **177**(3): p. 1451-9.
297. Scalapino, K.J. and D.I. Daikh, *Suppression of glomerulonephritis in NZB/NZW lupus prone mice by adoptive transfer of ex vivo expanded regulatory T cells.* PLoS One, 2009. **4**(6): p. e6031.
298. Chalkia, A., et al., *TGF-beta1/Smad Signalling in Proliferative Glomerulonephritis Associated with Autoimmune Diseases.* Mediterr J Rheumatol, 2022. **33**(2): p. 176-184.
299. Sisto, M., D. Ribatti, and S. Lisi, *Organ Fibrosis and Autoimmunity: The Role of Inflammation in TGFbeta-Dependent EMT.* Biomolecules, 2021. **11**(2).
300. Yamamoto, T., et al., *Expression of transforming growth factor-beta isoforms in human glomerular diseases.* Kidney Int, 1996. **49**(2): p. 461-9.
301. Ito, Y., et al., *Expression patterns of connective tissue growth factor and of TGF-beta isoforms during glomerular injury recapitulate glomerulogenesis.* Am J Physiol Renal Physiol, 2010. **299**(3): p. F545-58.
302. Chen, L., et al., *Central role of dysregulation of TGF-beta/Smad in CKD progression and potential targets of its treatment.* Biomed Pharmacother, 2018. **101**: p. 670-681.
303. Mathian, A., L. Arnaud, and Z. Amoura, *[Physiopathology of systemic lupus erythematosus: a 2014 update]*. Rev Med Interne, 2014. **35**(8): p. 503-11.
304. Blazar, B.R., G.R. Hill, and W.J. Murphy, *Dissecting the biology of allogeneic HSCT to enhance the GvT effect whilst minimizing GvHD.* Nat Rev Clin Oncol, 2020. **17**(8): p. 475-492.
305. Blazar, B.R., W.J. Murphy, and M. Abedi, *Advances in graft-versus-host disease biology and therapy.* Nat Rev Immunol, 2012. **12**(6): p. 443-58.
306. Ferrara, J.L., et al., *Graft-versus-host disease.* Lancet, 2009. **373**(9674): p. 1550-61.
307. Atilla, E., P. Ataca Atilla, and T. Demirer, *A Review of Myeloablative vs Reduced Intensity/Non-Myeloablative Regimens in Allogeneic Hematopoietic Stem Cell Transplantations.* Balkan Med J, 2017. **34**(1): p. 1-9.
308. Zeiser, R. and B.R. Blazar, *Acute Graft-versus-Host Disease - Biologic Process, Prevention, and Therapy.* N Engl J Med, 2017. **377**(22): p. 2167-2179.
309. Ho, V.T. and R.J. Soiffer, *The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation.* Blood, 2001. **98**(12): p. 3192-204.
310. Kolb, H.J., *Hematopoietic stem cell transplantation and cellular therapy.* HLA, 2017. **89**(5): p. 267-277.
311. Shlomchik, W.D., *Graft-versus-host disease.* Nat Rev Immunol, 2007. **7**(5): p. 340-52.
312. Gallardo, D., et al., *Low-dose donor CD8+ cells in the CD4-depleted graft prevent allogeneic marrow graft rejection and severe graft-versus-host disease for chronic myeloid leukemia patients in first chronic phase.* Bone Marrow Transplant, 1997. **20**(11): p. 945-52.
313. Champlin, R., et al., *Selective depletion of CD8+ T lymphocytes for prevention of graft-versus-host disease after allogeneic bone marrow transplantation.* Blood, 1990. **76**(2): p. 418-23.
314. Yu, Y., et al., *Prevention of GVHD while sparing GVL effect by targeting Th1 and Th17 transcription factor T-bet and RORgammat in mice.* Blood, 2011. **118**(18): p. 5011-20.

315. Iclozan, C., et al., *T helper17 cells are sufficient but not necessary to induce acute graft-versus-host disease.* Biol Blood Marrow Transplant, 2010. **16**(2): p. 170-8.
316. Yi, T., et al., *Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease.* Blood, 2009. **114**(14): p. 3101-12.
317. Koyama, M., et al., *Recipient nonhematopoietic antigen-presenting cells are sufficient to induce lethal acute graft-versus-host disease.* Nat Med, 2011. **18**(1): p. 135-42.
318. Carli, C., M. Giroux, and J.S. Delisle, *Roles of transforming growth factor-beta in graft-versus-host and graft-versus-tumor effects.* Biol Blood Marrow Transplant, 2012. **18**(9): p. 1329-40.
319. Coomes, S.M. and B.B. Moore, *Pleiotropic effects of transforming growth factor-beta in hematopoietic stem-cell transplantation.* Transplantation, 2010. **90**(11): p. 1139-44.
320. Banovic, T., et al., *TGF-beta in allogeneic stem cell transplantation: friend or foe?* Blood, 2005. **106**(6): p. 2206-14.
321. Giroux, M., et al., *SMAD3 prevents graft-versus-host disease by restraining Th1 differentiation and granulocyte-mediated tissue damage.* Blood, 2011. **117**(5): p. 1734-44.
322. Sela, U., et al., *Dendritic cells induce antigen-specific regulatory T cells that prevent graft versus host disease and persist in mice.* J Exp Med, 2011. **208**(12): p. 2489-96.
323. Sato, K., et al., *Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse.* Immunity, 2003. **18**(3): p. 367-79.
324. Malone, F.R., et al., *Prolonged anorexia and elevated plasma cytokine levels following myeloablative allogeneic hematopoietic cell transplant.* Bone Marrow Transplant, 2007. **40**(8): p. 765-72.
325. Visentainer, J.E., et al., *Serum cytokine levels and acute graft-versus-host disease after HLA-identical hematopoietic stem cell transplantation.* Exp Hematol, 2003. **31**(11): p. 1044-50.
326. Li, Q., et al., *Decrease of CD4(+)CD25(+) regulatory T cells and TGF-beta at early immune reconstitution is associated to the onset and severity of graft-versus-host disease following allogeneic haematogenesis stem cell transplantation.* Leuk Res, 2010. **34**(9): p. 1158-68.
327. Wu, J.M., et al., *CD20, AIF-1, and TGF-beta in graft-versus-host disease: a study of mRNA expression in histologically matched skin biopsies.* Mod Pathol, 2010. **23**(5): p. 720-8.
328. Baron, C., et al., *Prediction of graft-versus-host disease in humans by donor gene-expression profiling.* PLoS Med, 2007. **4**(1): p. e23.
329. Hattori, H., et al., *Polymorphisms of transforming growth factor-beta1 and transforming growth factor-beta1 type II receptor genes are associated with acute graft-versus-host disease in children with HLA-matched sibling bone marrow transplantation.* Bone Marrow Transplant, 2002. **30**(10): p. 665-71.
330. Noori-Daloii, M.R., et al., *Transforming growth factor-beta1 codon 10 polymorphism is associated with acute GVHD after allogenic BMT in Iranian population.* Ann Transplant, 2007. **12**(4): p. 5-10.
331. MacDonald, K.P., G.R. Hill, and B.R. Blazar, *Chronic graft-versus-host disease: biological insights from preclinical and clinical studies.* Blood, 2017. **129**(1): p. 13-21.
332. Dertschnig, S., et al., *Impaired thymic expression of tissue-restricted antigens licenses the de novo generation of autoreactive CD4+ T cells in acute GVHD.* Blood, 2015. **125**(17): p. 2720-3.
333. Krenger, W., B.R. Blazar, and G.A. Hollander, *Thymic T-cell development in allogeneic stem cell transplantation.* Blood, 2011. **117**(25): p. 6768-76.
334. Krenger, W. and G.A. Hollander, *The thymus in GVHD pathophysiology.* Best Pract Res Clin Haematol, 2008. **21**(2): p. 119-28.

335. Wu, T., et al., *Thymic damage, impaired negative selection, and development of chronic graft-versus-host disease caused by donor CD4+ and CD8+ T cells.* J Immunol, 2013. **191**(1): p. 488-99.
336. Bleakley, M., et al., *Outcomes of acute leukemia patients transplanted with naive T cell-depleted stem cell grafts.* J Clin Invest, 2015. **125**(7): p. 2677-89.
337. Zhang, Y., et al., *CD4+ T cells generated de novo from donor hemopoietic stem cells mediate the evolution from acute to chronic graft-versus-host disease.* J Immunol, 2007. **179**(5): p. 3305-14.
338. Sarantopoulos, S. and J. Ritz, *Aberrant B-cell homeostasis in chronic GVHD.* Blood, 2015. **125**(11): p. 1703-7.
339. Sarantopoulos, S., et al., *Recovery of B-cell homeostasis after rituximab in chronic graft-versus-host disease.* Blood, 2011. **117**(7): p. 2275-83.
340. Fujii, H., et al., *Biomarkers in newly diagnosed pediatric-extensive chronic graft-versus-host disease: a report from the Children's Oncology Group.* Blood, 2008. **111**(6): p. 3276-85.
341. Sarantopoulos, S., et al., *High levels of B-cell activating factor in patients with active chronic graft-versus-host disease.* Clin Cancer Res, 2007. **13**(20): p. 6107-14.
342. Li, X., et al., *Developing role of B cells in the pathogenesis and treatment of chronic GVHD.* Br J Haematol, 2019. **184**(3): p. 323-336.
343. MacDonald, K.P., B.R. Blazar, and G.R. Hill, *Cytokine mediators of chronic graft-versus-host disease.* J Clin Invest, 2017. **127**(7): p. 2452-2463.
344. Forcade, E., et al., *Circulating T follicular helper cells with increased function during chronic graft-versus-host disease.* Blood, 2016. **127**(20): p. 2489-97.
345. Kier, P., et al., *Autoantibodies in chronic GVHD: high prevalence of antinucleolar antibodies.* Bone Marrow Transplant, 1990. **6**(2): p. 93-6.
346. Patriarca, F., et al., *The development of autoantibodies after allogeneic stem cell transplantation is related with chronic graft-vs-host disease and immune recovery.* Exp Hematol, 2006. **34**(3): p. 389-96.
347. Zorn, E., et al., *Minor histocompatibility antigen DBY elicits a coordinated B and T cell response after allogeneic stem cell transplantation.* J Exp Med, 2004. **199**(8): p. 1133-42.
348. Srinivasan, M., et al., *Donor B-cell alloantibody deposition and germinal center formation are required for the development of murine chronic GVHD and bronchiolitis obliterans.* Blood, 2012. **119**(6): p. 1570-80.
349. Varelias, A., et al., *Lung parenchyma-derived IL-6 promotes IL-17A-dependent acute lung injury after allogeneic stem cell transplantation.* Blood, 2015. **125**(15): p. 2435-44.
350. Distler, J.H.W., et al., *Shared and distinct mechanisms of fibrosis.* Nat Rev Rheumatol, 2019. **15**(12): p. 705-730.
351. Wynn, T.A. and K.M. Vannella, *Macrophages in Tissue Repair, Regeneration, and Fibrosis.* Immunity, 2016. **44**(3): p. 450-462.
352. Erbel, C., et al., *IL-17A influences essential functions of the monocyte/macrophage lineage and is involved in advanced murine and human atherosclerosis.* J Immunol, 2014. **193**(9): p. 4344-55.
353. Ono, R., et al., *Co-activation of macrophages and T cells contribute to chronic GVHD in human IL-6 transgenic humanised mouse model.* EBioMedicine, 2019. **41**: p. 584-596.
354. McCormick, L.L., et al., *Anti-TGF-beta treatment prevents skin and lung fibrosis in murine sclerodermatous graft-versus-host disease: a model for human scleroderma.* J Immunol, 1999. **163**(10): p. 5693-9.

355. Liem, L.M., et al., *Serum transforming growth factor-beta1 levels in bone marrow transplant recipients correlate with blood cell counts and chronic graft-versus-host disease.* Transplantation, 1999. **67**(1): p. 59-65.
356. Anderson, B.E., et al., *Recipient CD4+ T cells that survive irradiation regulate chronic graft-versus-host disease.* Blood, 2004. **104**(5): p. 1565-73.
357. Chen, X., et al., *Absence of regulatory T-cell control of TH1 and TH17 cells is responsible for the autoimmune-mediated pathology in chronic graft-versus-host disease.* Blood, 2007. **110**(10): p. 3804-13.
358. Matsuoka, K., et al., *Altered regulatory T cell homeostasis in patients with CD4+ lymphopenia following allogeneic hematopoietic stem cell transplantation.* J Clin Invest, 2010. **120**(5): p. 1479-93.
359. Matsuoka, K., et al., *Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease.* Sci Transl Med, 2013. **5**(179): p. 179ra43.
360. Fahlen, L., et al., *T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells.* J Exp Med, 2005. **201**(5): p. 737-46.
361. Bleakley, M., et al., *Naive T-Cell Depletion to Prevent Chronic Graft-Versus-Host Disease.* J Clin Oncol, 2022. **40**(11): p. 1174-1185.
362. Dekker, L., et al., *Naive T Cells in Graft Versus Host Disease and Graft Versus Leukemia: Innocent or Guilty?* Front Immunol, 2022. **13**: p. 893545.
363. Chen, X., et al., *Blockade of interleukin-6 signaling augments regulatory T-cell reconstitution and attenuates the severity of graft-versus-host disease.* Blood, 2009. **114**(4): p. 891-900.
364. Belle, L., et al., *Blockade of interleukin-27 signaling reduces GVHD in mice by augmenting Treg reconstitution and stabilizing Foxp3 expression.* Blood, 2016. **128**(16): p. 2068-2082.
365. Kennedy, G.A., et al., *Addition of interleukin-6 inhibition with tocilizumab to standard graft-versus-host disease prophylaxis after allogeneic stem-cell transplantation: a phase 1/2 trial.* Lancet Oncol, 2014. **15**(13): p. 1451-1459.
366. Hannon, M., et al., *Infusion of clinical-grade enriched regulatory T cells delays experimental xenogeneic graft-versus-host disease.* Transfusion, 2014. **54**(2): p. 353-63.
367. Edinger, M., et al., *CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation.* Nat Med, 2003. **9**(9): p. 1144-50.
368. Trenado, A., et al., *Recipient-type specific CD4+CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia.* J Clin Invest, 2003. **112**(11): p. 1688-96.
369. Grossman, W.J., et al., *Human T regulatory cells can use the perforin pathway to cause autologous target cell death.* Immunity, 2004. **21**(4): p. 589-601.
370. Bolivar-Wagers, S., et al., *Cytolytic CD4(+) and CD8(+) Regulatory T-Cells and Implications for Developing Immunotherapies to Combat Graft-Versus-Host Disease.* Front Immunol, 2022. **13**: p. 864748.
371. Di Ianni, M., et al., *Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation.* Blood, 2011. **117**(14): p. 3921-8.
372. Brunstein, C.G., et al., *Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics.* Blood, 2011. **117**(3): p. 1061-70.
373. Brunstein, C.G., et al., *Umbilical cord blood-derived T regulatory cells to prevent GVHD: kinetics, toxicity profile, and clinical effect.* Blood, 2016. **127**(8): p. 1044-51.

374. Meyer, E.H., et al., *Transplantation of donor grafts with defined ratio of conventional and regulatory T cells in HLA-matched recipients.* JCI Insight, 2019. **4**(10).
375. Martelli, M.F., et al., *HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse.* Blood, 2014. **124**(4): p. 638-44.
376. Trzonkowski, P., et al., *First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells.* Clin Immunol, 2009. **133**(1): p. 22-6.
377. Lu, Y., et al., *Th9 cells promote antitumor immune responses in vivo.* J Clin Invest, 2012. **122**(11): p. 4160-71.
378. Ma, X., et al., *Cholesterol negatively regulates IL-9-producing CD8(+) T cell differentiation and antitumor activity.* J Exp Med, 2018. **215**(6): p. 1555-1569.
379. Purwar, R., et al., *Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells.* Nat Med, 2012. **18**(8): p. 1248-53.
380. Rivera Vargas, T., et al., *T(H)9 cells in anti-tumor immunity.* Semin Immunopathol, 2017. **39**(1): p. 39-46.
381. Reisser, T., et al., *In vitro-generated alloantigen-specific Th9 cells mediate antileukemia cytotoxicity in the absence of graft-versus-host disease.* Leukemia, 2020. **34**(7): p. 1943-1948.
382. Mangus, C.W., et al., *Rapamycin resistant murine th9 cells have a stable in vivo phenotype and inhibit graft-versus-host reactivity.* PLoS One, 2013. **8**(8): p. e72305.
383. Bjorklund, A.T., et al., *Complete Remission with Reduction of High-Risk Clones following Haploidentical NK-Cell Therapy against MDS and AML.* Clin Cancer Res, 2018. **24**(8): p. 1834-1844.
384. Lee, D.A., et al., *Haploidentical Natural Killer Cells Infused before Allogeneic Stem Cell Transplantation for Myeloid Malignancies: A Phase I Trial.* Biol Blood Marrow Transplant, 2016. **22**(7): p. 1290-1298.
385. Shaffer, B.C., et al., *Phase II Study of Haploidentical Natural Killer Cell Infusion for Treatment of Relapsed or Persistent Myeloid Malignancies Following Allogeneic Hematopoietic Cell Transplantation.* Biol Blood Marrow Transplant, 2016. **22**(4): p. 705-709.
386. Simonetta, F., M. Alvarez, and R.S. Negrin, *Natural Killer Cells in Graft-versus-Host-Disease after Allogeneic Hematopoietic Cell Transplantation.* Front Immunol, 2017. **8**: p. 465.
387. de Streel, G. and S. Lucas, *Targeting immunosuppression by TGF-beta1 for cancer immunotherapy.* Biochem Pharmacol, 2021. **192**: p. 114697.
388. Brenet, F., et al., *TGFbeta restores hematopoietic homeostasis after myelosuppressive chemotherapy.* J Exp Med, 2013. **210**(3): p. 623-39.
389. Fortunel, N.O., A. Hatzfeld, and J.A. Hatzfeld, *Transforming growth factor-beta: pleiotropic role in the regulation of hematopoiesis.* Blood, 2000. **96**(6): p. 2022-36.
390. Ottmann, O.G. and L.M. Pelus, *Differential proliferative effects of transforming growth factor-beta on human hematopoietic progenitor cells.* J Immunol, 1988. **140**(8): p. 2661-5.
391. Yamazaki, S., et al., *Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche.* Cell, 2011. **147**(5): p. 1146-58.
392. Isufi, I., et al., *Transforming growth factor-beta signaling in normal and malignant hematopoiesis.* J Interferon Cytokine Res, 2007. **27**(7): p. 543-52.

393. Jakubowiak, A., et al., *Inhibition of the transforming growth factor beta 1 signaling pathway by the AML1/ETO leukemia-associated fusion protein.* J Biol Chem, 2000. **275**(51): p. 40282-7.
394. Letterio, J.J., *TGF-beta signaling in T cells: roles in lymphoid and epithelial neoplasia.* Oncogene, 2005. **24**(37): p. 5701-12.
395. Mori, N., et al., *Human T-cell leukemia virus type I oncoprotein Tax represses Smad-dependent transforming growth factor beta signaling through interaction with CREB-binding protein/p300.* Blood, 2001. **97**(7): p. 2137-44.
396. Timmins, M.A. and I. Ringshausen, *Transforming Growth Factor-Beta Orchestrates Tumour and Bystander Cells in B-Cell Non-Hodgkin Lymphoma.* Cancers (Basel), 2022. **14**(7).
397. Challen, G.A., et al., *Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1.* Cell Stem Cell, 2010. **6**(3): p. 265-78.
398. Aldinucci, D., et al., *Interactions between tissue fibroblasts in lymph nodes and Hodgkin/Reed-Sternberg cells.* Leuk Lymphoma, 2004. **45**(9): p. 1731-9.
399. Chagraoui, H., et al., *Prominent role of TGF-beta 1 in thrombopoietin-induced myelofibrosis in mice.* Blood, 2002. **100**(10): p. 3495-503.
400. Gorelik, L. and R.A. Flavell, *Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells.* Nat Med, 2001. **7**(10): p. 1118-22.
401. Asakura, S., et al., *Alloantigen expression on non-hematopoietic cells reduces graft-versus-leukemia effects in mice.* J Clin Invest, 2010. **120**(7): p. 2370-8.
402. Delisle, J.S., et al., *Graft-versus-host disease causes failure of donor hematopoiesis and lymphopoiesis in interferon-gamma receptor-deficient hosts.* Blood, 2008. **112**(5): p. 2111-9.
403. Fontaine, P., et al., *Adoptive transfer of minor histocompatibility antigen-specific T lymphocytes eradicates leukemia cells without causing graft-versus-host disease.* Nat Med, 2001. **7**(7): p. 789-94.
404. Kummar, S., et al., *Modulation of graft-versus-tumor effects in a murine allogeneic bone marrow transplantation model by tumor-derived transforming growth factor-betaI.* Biol Blood Marrow Transplant, 2001. **7**(1): p. 25-30.
405. Bollard, C.M., et al., *Adapting a transforming growth factor beta-related tumor protection strategy to enhance antitumor immunity.* Blood, 2002. **99**(9): p. 3179-87.
406. Foster, A.E., et al., *Antitumor activity of EBV-specific T lymphocytes transduced with a dominant negative TGF-beta receptor.* J Immunother, 2008. **31**(5): p. 500-5.
407. Trotta, R., et al., *TGF-beta utilizes SMAD3 to inhibit CD16-mediated IFN-gamma production and antibody-dependent cellular cytotoxicity in human NK cells.* J Immunol, 2008. **181**(6): p. 3784-92.
408. Yu, J., et al., *Pro- and antiinflammatory cytokine signaling: reciprocal antagonism regulates interferon-gamma production by human natural killer cells.* Immunity, 2006. **24**(5): p. 575-90.
409. Alvarez, M., et al., *IL-2 and Anti-TGF-beta Promote NK Cell Reconstitution and Anti-tumor Effects after Syngeneic Hematopoietic Stem Cell Transplantation.* Cancers (Basel), 2020. **12**(11).
410. Asai, O., et al., *Suppression of graft-versus-host disease and amplification of graft-versus-tumor effects by activated natural killer cells after allogeneic bone marrow transplantation.* J Clin Invest, 1998. **101**(9): p. 1835-42.

411. Maag, D., et al., *Electrostatic interactions contribute to the control of intramolecular thiol-disulfide isomerization in a protein.* Phys Chem Chem Phys, 2021. **23**(46): p. 26366-26375.
412. Huygens, C., et al., *Lysosomal-associated Transmembrane Protein 4B (LAPTM4B) Decreases Transforming Growth Factor beta1 (TGF-beta1) Production in Human Regulatory T Cells.* J Biol Chem, 2015. **290**(33): p. 20105-16.
413. Razinia, Z., et al., *Filamins in mechanosensing and signaling.* Annu Rev Biophys, 2012. **41**: p. 227-46.
414. Parakh, S., et al., *Radiolabeled Antibodies for Cancer Imaging and Therapy.* Cancers (Basel), 2022. **14**(6).
415. Baron, U., et al., *DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells.* Eur J Immunol, 2007. **37**(9): p. 2378-89.
416. Schroeder, A.M., et al., *Baroreceptor reflex dysfunction in the BACHD mouse model of Huntington's disease.* PLoS Curr, 2011. **3**: p. RRN1266.
417. Reddy, P., R. Negrin, and G.R. Hill, *Mouse models of bone marrow transplantation.* Biol Blood Marrow Transplant, 2008. **14**(1 Suppl 1): p. 129-35.
418. Coomes, S.M., et al., *Induction of TGF-beta 1, not regulatory T cells, impairs antiviral immunity in the lung following bone marrow transplant.* J Immunol, 2010. **184**(9): p. 5130-40.
419. Liossis, S.N. and C. Staveri, *What's New in the Treatment of Systemic Lupus Erythematosus.* Front Med (Lausanne), 2021. **8**: p. 655100.
420. Morel, L., et al., *Functional dissection of systemic lupus erythematosus using congenic mouse strains.* J Immunol, 1997. **158**(12): p. 6019-28.
421. Berthier, C.C., et al., *Cross-species transcriptional network analysis defines shared inflammatory responses in murine and human lupus nephritis.* J Immunol, 2012. **189**(2): p. 988-1001.
422. Bethunaickan, R., et al., *Comparative transcriptional profiling of 3 murine models of SLE nephritis reveals both unique and shared regulatory networks.* PLoS One, 2013. **8**(10): p. e77489.