Receptors of the TNFSF in the biology and regulation of Tregs

Rezeptoren der TNFSF in der Biologie und Regulation von regulatorischen T-Zellen

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submitted by

Juan Fernando Gamboa Vargas

From

Bogotá, Colombia

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Submitted on: ……..……..
Office stamp

Members of the Thesis Committee:

Chairperson: ……....

Primary Supervisor: Prof. Dr. Dr. Andreas Beilhack

Supervisor (Second): Priv.-Doz. Dr. med. Niklas Beyersdorf

Supervisor (Third): Prof. Dr. Manfred Lutz

Supervisor (Fourth): Prof. Dr. rer. nat. Harald Wajant

Date of Public Defence: …….……...

Date of Receipt of Certificates: ……...

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II. Acknowledgements

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This is not the end of it, but it is impossible to go on and mention everyone who made this time of my life not a complete waste.

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VI. List of Abbreviations

TNF	Tumor necrosis factor
mTNF-α	membrane TNF-α
CRD	Cysteine rich domain
THD	TNF homology domain
TACE	TNA alpha converting enzyme
sTNF-α	soluble TNF-α
TNFR	Tumor necrosis factor
TNFRSF	TNF receptor superfamily
TRADD	TNFR associated death domain
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
TRAF	TNF receptor associated factor
cIAP	Cellular inhibitor of apoptosis
LUBAC	Linear ubiquitin chain assembly complex
IKK	Inhibitor of kappa-B kinases complex
IKK2	Phosphorylation of IKK subunit 2
IκBα	Inhibitor of kappa B-alpha
PI3K	Phosphoinositide 3-kinases
NK	Natural killer cells
DAMP	Damage associated molecular patterns
Treg	Regulatory T cell
MSC	Mesenchymal stem cells
EPC	Endothelial progenitor cells
VEGF	Vascular endothelial growth factor
Breg	Regulatory B cell
MDSC	Myeloid-derived suppressive cell
APC	Antigen presenting cell
DR3	Death receptor 3
TL1A	TNF-like ligand 1A
GITR	Glucocorticoid-Induced TNFR-Related protein

NKT	Natural killer T cell
Th	T helper cell
RA	Rheumatoid arthritis
MS	Multiple sclerosis
BBB	Blood brain barrier
IBD	Inflammatory bowel disease
UC	Ulcerative colitis
CD	Chron's disease
SLE	Systemic lupus erythematosus
PA	Psoriatic arthritis
DCs	Dendritic cell
NIU	Non-infectious uveitis
IFN	Interferon
pDC	Plasmacytoid dendritic cells
CGD	Chronic granulomatous disease
TME	Tumor microenvironment
ALCL	Anaplastic large cell lymphoma
MM	Multiple myeloma
PLAD	Pre-ligand assembly domain
ADCC	Antibody-dependent cellular cytotoxicity
TNC	Tenascin-C derived trimerization domain
HLA	Human leukocyte antigen
miHA	Minor-histocompatibility antigens
HCT	Hematopoietic cell transplantation
GvHD	Graft vs. Host disease
Allo-HCT	Allogenic hematopoietic cell transplantation
aGvHD	Acute GvHD
cGvHD	Chronic GvHD
PAMPs	Pathogen associated molecular patterns
Tc	Cytotoxic T cell
CLP	Common lymphoid progenitor
MAIT	Mucosal associated invariant T cell

CNI	Calcineurin inhibitors
iNKT	Innate NK T cells
DSC	Decidua stromal cells
DPP-4	Dipeptidyl peptidase-4
BCR	B-cell receptor
JAK	Janus kinase
CAR	Chimeric antigen receptor
BiTE	Bi-specific T cell engager
i.v.	Intravenously
GMP	Good manufacturing practices
IDO	Indoleamine 2,3-dioxygenase
PGE2	Prostaglanding E2
GvL	Graft vs. Leukemia
ICANS	Immune effector cell-associated neurotoxicity syndrome
mTEC	Medullary thymic epithelial cells
tTregs	Thymus Tregs
eTregs	Effector Tregs
pTregs	Peripheral Tregs
Nrp-1	Neuropilin-1
iTregs	Induced Tregs
nTregs	Natural Tregs
TSDR	Treg-specific demethylation region
Tfh	T follicular helper cells
A2AR	Adenosine receptor 2A
UCB	Umbilical cord blood
AIR	Artificial immune receptor
LTβR	Lymphotoxin-β receptor
l.d. IL-2	Low-dose IL-2
RBC	Red blood cell
RPMI	Roswell Park memorial institute 1640 medium
FACS	Fluorescence-Activated Cell Sorting
DMEM	Dulbecco's Modified Eagle Medium

HBSS	Hank's balanced salt solution
cRPMI	Complete RPMI
FCS	Fetal calf serum
PBS	Phosphate buffered saline
hPBMCs	Human peripheral blood mononuclear cells
IU	International units
i.p.	Intraperitoneally
eGFP	enhanced green fluorescence protein
CTV	Cell trace violet
ROI	Region of interest
TBI	Total body irradiation
NRS	Normal rat serum
SD	Standard deviation
MFI	Mean fluorescence intensity
TIGIT	T cell immunoreceptor with Ig and ITIM domains
ICAM1	Intercellular adhesion molecule 1
cDC2	Conventional DCs 2
miRNA	Micro RNA
shRNA	Short hairpin RNA

VII. Summary

In this work we expanded upon a study from our group where a ligand-based TNF-α mutein was developed to engage specifically TNFR2 and not TNFR1 activating Tregs and expanding them, which in an allo-HCT context conferred protection from GvHD. Fusing TNF trimers to the heavy chain of an Fc-dead and mouse irrelevant antibody, a new generation of this agonist was developed called NewSTAR2. It is believed that other members of the TNFSF can also target Tregs, therefore additional agonists against DR3 and GITR were developed under the same principles as for NewSTAR2. Phenotyping analysis of the expression of these three receptors were done to confirm their specificity for Tregs before in vitro and in vivo testings with mice or murine splenic cells. A potent expansion of Tregs was seen with NewSTAR2 and the other agonists as well as upregulation of activation markers on Tregs. Thorough analyses with NewSTAR2-treated mice showed how Tregs in several immune and non-immune organs were expanded and upregulated immunomodulatory receptors. A miniature suppressive assay and other cocultures with responder cells confirmed their enhanced suppression over unstimulated Tregs through contact dependent and independent mechanisms. Despite other myeloid cells also being increased after treatment, no undesired effects were observed under steady-state and prophylactic administration of a single dose of NewSTAR2 improved survival frequencies and lessened development of clinical symptoms. Prophylactic treatment with the other TNFRSF agonists showed similar protection yet Fc(DANA)-muTL1A was superior in in terms of less death events and lower clinical score. It was found that not all the three TNFSF members have redundant functions as development of skin lesions was observed with GITRL-based agonist Fc(DANA)-muGITRL, although its expansion of Tregs in steady-state was remarkable with no apparent adverse effects. Neither agonist had an impact on donor cell engraftment or allorective T cell response, however NewSTAR2-treatmend proved to reduce inflammation in small intestine and liver. This work is proof of concept of the effectivity of selectively engaging TNFSF to activate Tregs and expand them systemically allowing them to control strong and complex immune interactions like those governing GvHD.

VIII. Zusammenfassung

In dieser Arbeit erweiterten wir eine Studie unserer Gruppe, in der ein ligandenbasiertes TNF-α-Mutein entwickelt wurde, um spezifisch TNFR2 und nicht TNFR1 zu aktivieren und Tregs zu erweitern, was in einem allo-HCT-Kontext Schutz vor GvHD verlieh. Durch die Fusion von TNF-Trimeren mit der schweren Kette eines Fc-toten und Maus-irrelevanten Antikörpers wurde eine neue Generation dieses Agonisten namens NewSTAR2 entwickelt. Es wird angenommen, dass andere Mitglieder des TNFSF ebenfalls auf Tregs abzielen können. Daher wurden zusätzliche Agonisten gegen DR3 und GITR nach denselben Prinzipien wie für NewSTAR2 entwickelt. Eine phänotypische Analyse der Expression dieser drei Rezeptoren wurde durchgeführt, um ihre Spezifität für Tregs vor In-vitro- und In-vivo-Tests mit Mäusen oder murinen Milzzellen zu bestätigen. Bei NewSTAR2 und den anderen Agonisten wurde eine starke Expansion der Tregs sowie eine Hochregulierung der Aktivierungsmarker auf Tregs beobachtet. Gründliche Analysen mit NewSTAR2-behandelten Mäusen zeigten, wie Tregs in mehreren Immun- und Nichtimmunorganen erweitert und immunmodulatorische Rezeptoren hochreguliert wurden. Ein Miniatur-Suppressionstest und andere Kokulturen mit Responderzellen bestätigten deren verstärkte Unterdrückung nicht stimulierter Tregs durch kontaktabhängige und unabhängige Mechanismen. Obwohl auch andere myeloische Zellen nach der Behandlung zunahmen, wurden unter der Steady-State- und prophylaktischen Verabreichung einer Einzeldosis NewSTAR2 keine unerwünschten Wirkungen beobachtet, was die Überlebenshäufigkeit verbesserte und die Entwicklung klinischer Symptome verringerte. Die prophylaktische Behandlung mit den anderen TNFRSF-Agonisten zeigte einen ähnlichen Schutz, Fc(DANA)-muTL1A war jedoch in Bezug auf weniger Todesereignisse und einen niedrigeren klinischen Score überlegen. Es wurde festgestellt, dass nicht alle drei TNFSF-Mitglieder über redundante Funktionen verfügen, da die Entwicklung von Hautläsionen mit dem GITRL-basierten Agonisten Fc(DANA)-muGITRL beobachtet wurde, obwohl die Expansion der Tregs im Steady-State bemerkenswert war und keine offensichtlichen nachteiligen Auswirkungen auftrat. Keiner der beiden Agonisten hatte einen Einfluss auf die Transplantation von Spenderzellen oder die allorektive T-Zell-Reaktion, allerdings reduzierte die NewSTAR2-Behandlung nachweislich Entzündungen im Dünndarm und in der Leber. Diese Arbeit ist ein Beweis für die Wirksamkeit der selektiven Aktivierung von TNFSF, um Tregs zu aktivieren und sie systemisch zu erweitern, sodass sie starke und komplexe Immuninteraktionen steuern können, wie sie GvHD steuern.

1. Introduction

1.1. TNF axis and its modulation as a proposed strategy to treat inflammatory diseases

1.1.1. TNF axis biology

Cellular and biological systems' communication employ different mechanisms to translate, coordinate and regulate interactions and processes. Among these, the release of cytokines constitutes a key method to transmit information between cells and particularly immune cells. As small pleiotropic proteins and glycoproteins that can target specific receptors or cells, trigger cascades by inducing robust changes within the cell and possess a certain degree of redundancy in some cases, they can elicit a fast response against a stimulus and amplify this initial event to integrate different pathways and engage different cells and organs. Initially, cytokines were classified based on the sharing of subunits in their receptors, later by their cellular source, now a subgrouping is also distinguished depending if they elicit an anti- or proinflammatory effect, however despite this categorizing cytokines defined under this frame overlap greatly (Liu et al., 2021; Nicola, 1994; Ozaki & Leonard, 2002). A prominent example is tumor necrosis factor (TNF), which was firstly identified as a cytokine released by activated immune cells that caused cell death (necrosis) in tumor cells. The findings of the scientific community 40 years since its discovery show clearly that the nature of TNF is much more complex than what was thought at first (Holbrook et al., 2019).

The basic structure of the TNF unit is a 17 kDa protein of 157 amino acids folded into two opposing antiparallel β-sheets (Jones et al., 1990). A homologous protein sharing some of the amino acids and structure is lymphotoxin (TNF-β) (Idriss & Naismith, 2000). When produced in the cell, three units of TNF associate to generate a transmembrane homotrimer (mTNF-α) with its C-terminal domain in the extracellular side and an intracellular N-terminus. A conserved C-terminus with a sequence identity of around 25% (TNF homology domain, THD) composed partly of cysteine rich domains (CRD) involved in a trimeric self-assembly is found in 19 other proteins that together define the TNF superfamily (TNFSF). Like some of the 19 members of the TNFSF, mTNF-α can be cleaved by the metalloprotease TNF alpha converting enzyme (TACE) and released as a soluble form of the cytokine (sTNF-α) (Bodmer et al., 2002; Idriss & Naismith, 2000). The membrane bound form is encoded by four exons found in chromosome

6 and is a precursor of the soluble form that is cleave after cell activation (Leone et al., 2023). mTNF-α activate the two known receptors of TNF, TNFR1 and TNFR2, which as members of the TNF receptor superfamily (TNFRSF), are transmembrane proteins with extracellular CRDs, however sTNF-a can only efficiently activate TNFR1. Although certain homology is seen in the extracellular domain of the members of the TNFRSF, their intracellular domains are variable, allowing activation of different pathways. Such is the case of TNFR1 an TNFR2, having the former a death domain (TNFR associated death domain or TRADD) that activates receptor-interacting serine/threonine-protein kinase 1 (RIPK1) inducing apoptosis or necroptosis in the cell (depending on the involvement or inhibition of caspase 8 respectively and of the availability of other cytosolic proteins), and the latter a TNF receptor associated factor (TRAF) that recruits TRAF1/2 and cellular inhibition of apoptosis protein 1 and 2 (cIAP1/2) to trigger classic NFκB activation (Park et al., 2014; Wajant & Siegmund, 2019).

A closer examination of the molecular biology of these receptors reveals the intricate nature of the TNF axis: TNFR1 efficiently recruits TRAF2 homotrimers, interacting with TRAD, thereby inducing classic NFκB signaling and other pathways, including kinase activations (Wajant et al., 2003)). TRAF2 and RIPK1 facilitate the binding of cellular inhibitor of apoptosis protein 1 and 2 (cIAP1/2). This interaction allows the E3 ligase linear ubiquitin chain assembly complex (LUBAC) to join the signaling complex. Mutual ubiquitin modifications attract various kinases and the inhibitor of kappa-B kinases complex (IKK). Phosphorylation of IKK subunit 2 (IKK2) initiates the proteolysis of inhibitor of kappa B-alpha (IκBα), releasing NFκB transcription factors. TNFR2 also drives classic NFκB activation by recruiting LUBAC and IKK, even in the absence of TRADD or RIPK1 in the receptor signalling complex. This partly explains the lower efficiency of classic NFκB activation via TNFR2. Specifacylly, clusters of TNFR2 can recruit TRAF2, which associates with TRAF1-cIAP1/2 complexes. Simultaneously, TRAF3, normally bound to NFκB associated kinase NIK, undergoes degradation through ubiquitinylation). This induction not only activates classic NFκB but also depletes TRAF1-cIAP1/2 complexes in the cytoplasm, adversely affecting the pool of signaling molecules necessary for TNFR1 classical NFκB activation. Upon inhition of NIK ubiquitinylation, it phosphorylates IKK1, which subsequently phosphorylates p100-RelB, thereby releasing the necessary transcription factor for alternative NFκB activation (Medler et al., 2022; Wajant & Scheurich, 2011; Wicovsky et al., 2009).

The TNFSF ligands activates diverse pathways, including NFAT, MAPK (c-Jun N-terminal kinases, extracellular signal-regulated kinases, p38), protein kinase B (Akt) and phosphoinositide 3-kinases (PI3K), illustrating the extensive crosstalk with complex signaling

pathways (Croft et al., 2012; Ward-Kavanagh et al., 2016). The involvement of TNFSF members in cell survival/apoptosis, cell development, activation of stem cells, homeostasis, hematopoiesis, tissue regeneration, and immune regulation is not surprising. It's noteworthy that many TNFRSF members activate NFκB, implying that this pathway serves as a convergence point. By employing various regulatory strategies, diverse outcomes can ensue. The cleavage and shedding of the receptors, their restricted expression on certain cell subsets, and the varying degree of receptor expression on the cell surface are mechanisms contributing to the achievement of these diverse outcomes (Mizrahi & Askenasy, 2014; Wajant & Siegmund, 2019).

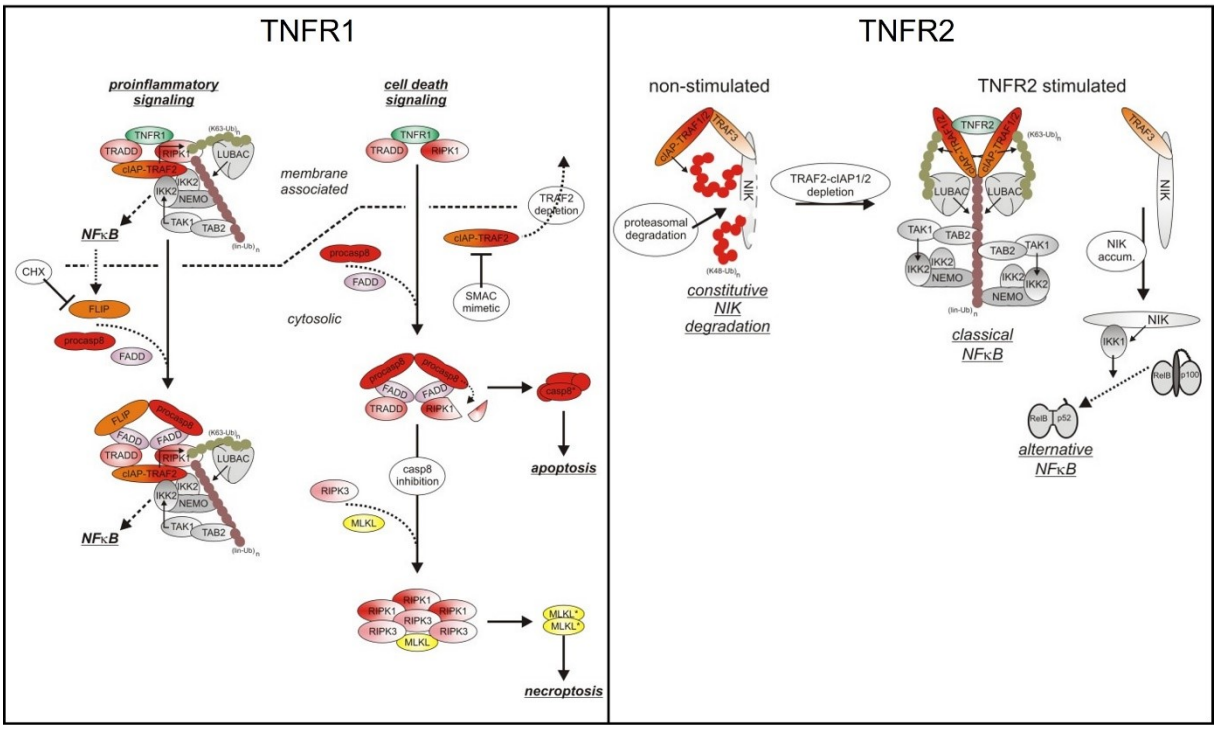

Figure 1. TNFR1 and TNFR2 signaling. Current model of TNFR1 and TNFR2 signaling. Under normal conditions, cIAP-TRAF2 complexes bind to TRADD of TNFR1, facilitating the recruitment of IKK2 and LUBAC, subsequently leading to their ubiquitinylation and classic NFκB activation. In the absence of TRAF2, signaling shifts towards apoptosis or necroptosis. TNFR2 can recruit IKK2 and trigger classic NFκB activation. Alternatively, the recruitment of TRAF2-cIAP1/2 or TRAF1-TRAF2cIAP1/2 complexes by TNFRS prevents their binding to NIK, avoiding its subsequent degradation and resulting in alternative NFκB activation. Figure is adapted from (Wajant & Siegmund, 2019).

1.1.2. Distribution of TNFRs and TNFRSF

Expression of TNFR1 is found to be constitutive across a diverse range of tissues whereas TNFR2 is predominantly expressed on endothelial, immune (largely immunosuppressive), and neural cells (Naserian et al., 2020). Macrophages and monocytes predominantly secrete TNF-

α, with additional production by lymphocytes, mast cells, natural killer (NK) cells, neutrophils, and fibroblasts (Holbrook et al., 2019; Kany et al., 2019). The acute release of TNF-α by macrophages and neutrophils elicits robust tissue inflammation, inducing secretion of TNF-α, IL-6, and IL-1β secretion, along with apoptosis. This sets off a feedback loop through the release of inflammation-inducing damage-associated molecular patterns (DAMPs). Stimulation of TNFR1 on dendritic cells and macrophages activates them, leading to the secretion of cytokines crucial for T cell differentiation. However, prolonged exposure can desensitize macrophages, impairing their pro-inflammatory activities. TNFR2 activation on T cells serves as a mild costimulatory molecule, promoting proliferation and survival. In contrast, TNFR2 on regulatory T cells (Tregs) has a profound net effect, encompasing increased survival by fostering expansion, stabilizing the regulatory phenotype through heightened FoxP3 expression, inducing histone methylation-mediated epigenetic changes, and enhancing regulatory function through increased secretion of anti-inflammatory cytokines. Additionally, Tregs upregulate TNFRSF that act as “Treg activation markers” ((Chen et al., 2007; Lubrano di Ricco et al., 2020; Salomon, 2021; Urbano et al., 2018; Vargas et al., 2022).

Mesenchymal stem cells (MSCs), characterized as multipotent fibroblast-like cells with the ability to differentiate into cells from any germ layer, play crucial roles in tissue regeneration, with their expression of TNFR2 linked to these functions. A recent study revealed the importance of TNF-α and TNFR2 in the immunoregulatory properties of MSCs, including the reduction of T cell proliferation, downregulation of T cell activation markers, and induction of Tregs (Beldi, Khosravi, et al., 2020). Similarly, endothelial progenitor cells (EPCs) exhibit immunomodulatory effects dependent on TFNR2. TNFR2 binding on EPCs promotes the release of IL-10 and TGF-β, suppressing T cell responses, and vascular endothelial growth factor (VEGF), contributing to angiogenesis and neo-angiogenesis (Naserian et al., 2020).

The increasing recognition of TNFR2’s importance in immunoregulation is evident as newly described immunoregulatory cell subsets such as regulatory B cells (Bregs), CD8 Tregs and, myeloid-derived suppressive cells (MDSCs) rely on TNFR2 to execute their immunomodulatory functions (Beldi, Bahiraii, et al., 2020; Beldi, Khosravi, et al., 2020; Leclerc et al., 2016; Polz et al., 2014; Ticha et al., 2018). Despite the presence of TNFR1 on the surface of these cells, they are predominantly described as anti-inflammatory, likely due to differences in receptor density on the surface (Wajant & Siegmund, 2019). Furthermore, other TNFRSF members can be constitutively or inducibly coexpressed on immune cells, adding another layer of complexity to the dual nature of the TNF-axis.

CD27 and its ligand CD70 are expressed in T, B, and NK cells with CD70 also expressed by antigen presenting cells (APCs) and other cells, considered to drive Th1 differentiation. Death receptor 3 (DR3) and its ligand TNF-like ligand 1A (TL1A) share a similar expression pattern as CD27 and C70, and their interaction is associated with Th1/Th17 differentiation. OX40 is induced in activated T cells and its ligand OX40L in neutrophils and activated APCs. The OX40-OX40L receptor-ligand interaction has been implicated in Th2, but also Th17, differentiation and neutrophil activation (Croft et al., 2012; Jin et al., 2019). Th functions of 4-1BB and 4-1BBL are ambiguous, described to increase survival signals and reduce apoptosis, although their expression and response to certain stimuli from the ligand and the receptor are not identical. While 4-1BB is inducible in T cells after activation and in epithelial and endothelial cells, 4-1BBL is inducible in professional antigen-presenting cells. Moreover, the complete knockout (KO) of the ligand appears to negatively affect the accumulation of memory CD8 T cells, while KO of the receptor positively affects the accumulation of activated CD8 T cells (Humphreys et al., 2010). Glucocorticoid-Induced TNFR-Related protein (GITR) is constitutively expressed in lymphocytes and NK T cells (NKTs), with its ligand (GITRL) constitutively expressed in APCs and endothelial cells. The expression in naïve Tcons is considerably lower than in Tregs and recent studies describe diverse effects upon its activation. GITR activation aids in the maturation of thymic Tregs but also abolishes Treg suppressive function. Conversely, GITR blocking improves effector T cell resistance to Treg suppression (Shimizu et al., 2002; Ward-Kavanagh et al., 2016). By acting as costimulatory molecule on effector T cells, GITR can increase T cell-activation, proliferation, and cytokine secretion (Kohm et al., 2004). Other studies also show the resistance of effector T cells to Treg-mediated regulation once GITR has been engaged by its ligand presented on APCs (Stephens et al., 2004). Additional TNFRSF members with known functions include CD30-CD30L, which play a role in induction of cell death, survival, and proliferation of lymphocytes and APCs, and CD40-CD40L crucial in the interaction between helper T cells (Th) and antigen-activated B cells leading to germinal center formation, B cell differentiation, antibody secretion, and isotype switching (Croft et al., 2012; Kawabe et al., 1994; Ward-Kavanagh et al., 2016). Notably, some of these molecules serve as signature markers in various immunological disorders and malignancies, underscoring the relevance of TNFRSF members and their potential as therapeutic targets.

1.1.3. Dysregulation of the TNFRSF in disease

The myriad roles of TNFRSF in the immune response correlate with a range of conditions, including inflammatory diseases, cancers, and autoimmune disorders. The pivotal role of TNF- in initiating systemic proinflammatory cascades and modulating T cell responses is a critical inflection point, where even minor irregularities can have profound repercussions, represending a proverbial double-edged sword (Aggarwal, 2003). Genetic predisposition to these diseases involves polymorphisms in the TNF-α gene, mutations in the downstream signaling molecules like RIPK1 or CASP8, and mutations in the first CRD of TNFR1 (Galon et al., 2000; Ghorbaninezhad et al., 2022).

In inflammatory diseases such as rheumatoid arthritis (RA), uncontrolled TNF-α release by macrophages and Th1 cells sustains synovial inflammation, inducing other proinflammatory cytokines including IL-1β, IL-6 and IL-8, and causing damage to soft tissues and, over time, spreading to bones and destroying the joints (Jang et al., 2021; Salomon, 2021; Zamri & de Vries, 2020). In multiple sclerosis (MS), TNF-α identified in brain lesions and spinal fluid, when overproduced, alters glutamate levels, demyelinates, and increases neuroinflammation impacting the blood-brain barrier (BBB) (Fresegna et al., 2020). TNF-α production by Th1 cells in the intestine recruits and accumulates fibroblasts, neutrophils, and macrophages leading to fibrosis and epithelial damage in inflammatory bowel diseases (IBD) ulcerative colitis (UC) and Chron's disease (CD) (Jang et al., 2021).

Elevated TNF-α serum levels observed in systemic lupus erythematosus (SLE) and psoriatic arthritis (PA) patients contribute to a Th17-skewed T cell response by stimulating APCs to release increased TNF-α and IL-23. Consequently, the heightened IL-17 levels activate keratinocytes and recruit DCs triggering local hyperplasia and microabscesses formation (Ghorbaninezhad et al., 2022; Jang et al., 2021). TNF-α not only initiates these pathologies but exacerbates them by activating B cells and maintaining self-antigens released from apoptotic cells, enabling autoantibody generation. This insight forms the base for anti-TNF therapies, such as etanercept, adalimumab and infliximab, which inhibit soluble and transmembrane TNF-α blocking TNFR1/2 interactions, offering treatment for various conditions. Approved TNF blockers have been in the market since the late 1990s – early 2000s and examples like infliximab, etanercept and adalimumab are prescribed for psoriasis, RA, CD, UC, non-infectious uveitis (NIU), ankylosing spondylitis, chronic obstructive pulmonary disease among others (Jang et al., 2021; Leone et al., 2023; S. J. Li et al., 2019; Salomon, 2021). New clinical trials continue to explore novel applications for TNF blockers, although controversies exist, as adverse outcomes have been observed in several inflammatory diseases.

TNF-α inhibitor-induced psoriasis may correlate with increased release of type 1 interferon (IFN) release by plasmacytoid dendritic cells (pDCs), which are downregulated by TNF-α. Additionally, blocking TNF-α may elevate Th17 cytokines and reduce FoxP3 expression in some patients, enhancing psoriasis pathology. A general abrogation of the TNF axis could increase infection risk and permit invasion by agents that resemble psoriasis lesions (S. J. Li et al., 2019; Ma et al., 2010). In a mouse model of SLE, TNF inhibition triggered autoantibody production against nuclear contents, similar to observations in patients with RA or CD (Ghorbaninezhad et al., 2022). Overall, two main reasons could explain why TNF blocker therapy fails: On one hand is the underlying circumstances of the patient and the pathology that could require alternative or multiple therapeutic targets, and on the other hand, the systemic effect that a whole blockade of TNF can have in inhibiting TNFR1 proninflammatory signals but also TNFR2 immunomodulatory and tissue recovery signals (Siegmund & Wajant, 2023). Clinically approved TNF blockers will block all binding of TNF receptors by TNF-α but not of other ligands of TNFR1 like LTα, allowing still some TNFR1-mediated proinflammatory signaling. The fact that some TNF blockers were antibodies with capable Fc binding which could bind with higher affinity once TNF has also been bound, also proved to trigger ADCC through FcγR (Mitoma et al., 2008). Moreover, common reported side effects of TNF blockade attributed to TNFR2 are neurodegenerative demyelination and reduced production of TGF-β, IL-10 and HLA-G by endothelial progenitor cells crucial players in the remodeling and repair of vasculature (Naserian et al., 2020; Zhang et al., 2020). Thus, preclinical novel TNF biologics distinguish clearly between TNFR1/2 antagonists and agonists.

Conversely, in infectious diseases, TNF dysregulation can manifest as either excessive or insufficient production, depending on the pathogen involved. For instance, in sepsis, an overwhelming immune response to infection can lead to an uncontrolled release of TNF, causing systemic inflammation and organ dysfunction (Jacobs et al., 2007). On the contrary, in chronic infections like tuberculosis, insufficient TNF production can impair the immune system's ability to control the pathogen, resulting in persistent infection. Patients under TNF blockers are in general susceptible to infections, for instance, Herpes zoster reactivation was observed in patients with hepatitis B, and pulmonary and gastrointestinal infections in patients with chronic granulomatous disease (CGD) (Conrad et al., 2021; Shale et al., 2010).

While TNF was first thought to eliminate tumors, early studies in mouse models reveiled the induction of necrosis in tumors, yet, regrowth in the adjacent tissue and systemic vascular damage was also a consequence limiting the application of recombinant TNF. Clinical trials with recombinant TNF exhibited strong toxicity resembling endotoxin shock. Localized

administration of TNF was implemented to mitigate off-target effects, but this posed limitations in treating metastatic carcinomas (Balkwill, 2009). Subsequent research demonstrated TNF-α's ability to induce angiogenesis in tumors, increase metastasis, suppress immune surveillance, and its production by tumors in the tumor microenvironment (TME). Consequently, TNF antagonists emerged as a promising strategy in cancer research, with some of the previously mentioned TNF blockers currently administered to immunotherapy-resistant cancer patients with advanced solid tumors managing to achieve disease stabilization and partial responses (Brown et al., 2008; Harrison et al., 2007). Other studies showed how adding TNF blockers to PD-L1 checkpoint blockade or chemotherapy can increase efficacy of the given therapy by preventing anti-PD-1tumor-infiltrating T cell death and decreasing inflammation in tissues like liver or intestine, contributing to toxicity and disrupting paracrine inflammatory loops that the cancer hijacks to recruit MDSCs and promote its survival (Bertrand et al., 2015, 2017; Montfort et al., 2021; Paik et al., 2022). Newer alternatives of these TNF antagonists focuse specifically on TNFR2 blockade eliciting Treg depletion, CD8 stimulation, inhibition of MDSCs and abrogation of factors promoting angiogenesis (Bai et al., 2022; M. Li et al., 2022).

Certain TNFSF members associated with disease include increased DR3-TL1A expression in patients with IBD and RA, believed to aid T cell differentiation into Th1 and Th9 (Richard et al., 2015; Twohig et al., 2012). Elevated serum levels of 4-1BB in MS and RA patients are assumed to stimulate T cell responses and T cell cytokine secretion (Martínez Gómez et al., 2012). Increased amounts of OX40-expressing T cells are found in the lungs of asthma patients and the joints in those with RA. CD30-CD30L interactions, initially characterized in blood cancers like Hodgkin's lymphoma, anaplastic large cell lymphoma (ALCL) and multiple myeloma (MM), serve as tumor markers with the potential to induce apoptosis (Croft et al., 2013). Further studies have identified high levels of CD30 in patients with chronic infection, which normalize upon effective infection therapy.

Taken together, TNF- and TNFSF-based therapeutics have the dual capacity to both promote and resolve inflammation depending, depending heavily on the context. Therefore, harnessing the potential of the TNF superfamily requires a detailed understanding of their functions in various cell populations and circumstances in which they play crucial roles. Importantly, research should focus on targeted engagement of these pathways to reduce toxicity and unwanted reactions.

1.1.4. Engineered antibody-based TNFRSF agonists

In recent decades, the development and approval of antibody-based therapies for cancer and other diseases have surged. Advocates for antibodies highlight their increased stability, precise specificity, versatile design capabilities, and streamlined production processes (Goulet & Atkins, 2020; Goydel & Rader, 2021; Kaplon & Reichert, 2019). As proposed by other authors, maximizing the benefit of engineered antibodies necessitates meticulous consideration of the specific target's requirements and desired effect, influencing structural aspects like the binding domai size, antibody or nanobody format, valency, multimerization potential for complement activation, modifications to increase solubility or stability and conjugation granting multispecificity, and selection of a second or third antigen for dual or triple targeting constructs (Goulet & Atkins, 2020).

Engineered antibodies targeting the TNFSF have distinct requirements, depending on whether they function as blockers of the soluble or membrane-bound ligands, blockers of receptor interactions, exclusive blockers of one or more receptor(s), or agonists of receptor/ligand interactions. Multimerization of TNFRSF members is facilitated by the THD, aiding in multimeric assembly. Clustering of receptors relies on the pre-ligand assembly domain (PLAD), a prerequisite for proper activation, as has been exemplified for TNFR2 (Chan, 2000; Prada et al., 2021). Agonistic antibodies directed at TNFR2 must bring together three or more receptors on the same cell for downstream activation. The unspecific binding of anti-TNFSF antibodies to Fc-γ receptors (FcγRs) was initially considered an anchoring domain, mimicking the effect of membrane-bound forms and through clustering inducing receptor activation (Wajant, 2015). However, FcγR binding can also neutralize the effects of an anti-TNFSF agonistic antibodies by marking the targeted cells for elimination through antibody-dependent cellular cytotoxicity (ADCC). Intriguingly, studies revealed that removing the FcγR-binding domain of TNFSF antibodies while adding a cytokine sequence in the C-terminus achieved a similar anchoring effect, offering an avenue to enhance the availability and delivery of TNFSF agonists (Medler et al., 2019). This modification mitigates the elimination of antibody-marked cells and systemic effects, reducing toxicity.

Moreover, our research group has pioneered cutting-edge strategies to expand antibody valency by multimerizating antibody chains, accomplished through additional Fab fragment binding to the heavy or light chains, incorporation of a tenascin-C derived trimerization domain (TNC) or appending oligomers of any given TNFSF chain to the C-terminus of an antibody

bearing an irrelevant Fab fragment. Depending on the approach, we have successfully developed dodecavalent antibody formats with 12 binding sites capable of engaging with the same cell (cis-binding) or two receptor expressing cells through binding sites in opposite orientation (trans-binding) (Anany et al., 2024).

1.2. Acute Graft-versus-Host Disease

1.2.1. Clinical relevance

The potential restoration of various hematological malignancies and congenital immunodeficiencies through the replacement of a patient's hematopoietic system is a compelling therapeutic strategy. However, the highly polymorphic human leukocyte antigen (HLA) system and the presence of minor-histocompatibility antigens (miHA) pose a formidable challenge in identifying a suitable donor for replacing the host's immune system (DeFilipp et al., 2022; Manettas et al., 2022; Touw, 2022). Despite this challenge, allogeneic hematopoietic cell transplantation (allo-HCT) has shown lower relapse rates compared to autologous hematopoietic cell transplantation in patients suffering from hematologic malignancies (Shumilov et al., 2022). Initial studies demonstrated that patients with compromised immune systems benefitted from a conditioning regimen to enhance engraftment and prevend an immune reaction then termed "runt disease" characterized by skin rash, liver dysfunction, and gastrointestinal problems, now known as graft versus host disease (GvHD) (Billingham et al., 1954; Singh & McGuirk, 2016). Conditioning regimens, designed to overcome GvHD, eliminate residual malignant cells, and create a conductive niche for the incoming hematopoietic cells, have significantly improved the success of allo-HCT (Singh & McGuirk, 2016). Consequently, the incidence of allo-HCT has surged annually, establishing it as a primary therapy for treating and in certain instances even curing many hematological diseases (DeFilipp et al., 2022; Malard et al., 2023; Niederwieser et al., 2022; Passweg et al., 2021).

However, despite the increased application of allo-HCT, the development of acute GvHD (aGvHD) remains as the major complication, impeding the widespread utilization of this therapy. Up to 60% of patients may develop GvHD, and approximately 33% succumb to its effects (Ramachandran et al., 2019; Ramdial et al., 2021). Without prophylaxis, all patients undergoing allo-HCT, including those with mismatched and unrelated matched donors, will experience GvHD, leading to dermatological and intestinal manifestations that significantly

degrade their quality of life (Holler et al., 2019; Manettas et al., 2022). Standard treatment often involves immunossuppressants, increasing the risk of opportunistic infections, which contribute to an additional 37.5% of mortality after transplantation (Justiz Vaillant et al., 2023; Ramdial et al., 2021). Moreover, the overall 1-year rate in patients with low grade GvHD is 70%, dropping to 40% in those with mild or severe-grade GvHD, and the 5-year survival rate around 40% (Jagasia et al., 2012; Malard et al., 2023). For patients who successfully overcome GvHD and its associated treatment, long-term complications such as microbiota dysbiosis, multiorgan dysfunction and chronic GvHD (cGvHD) may persist (DeFilipp et al., 2022; Hino et al., 2023; Malard et al., 2023; Ramachandran et al., 2019). Considering the demographics of GvHD, with a mean of age is 53.7 years and an overall poor quality of life, it becomes evident that GvHD stands as a major contemporary health problem.

1.2.2. aGvHD pathophysiology

The pathophysiology of aGvHD is a multifaceted process impacting various organs that has been delineated in three phases (Figure 2): Initiation, T cell activation or immune priming and effector response. The conditioning regimen causes damage to the patient's tissues and microbiome, releasing DAMPs, pathogen-associated molecular patterns (PAMPs) and other inflammatory signals. These signals activate both innate and adaptive immune cells, priming the immune system and creating conditions for efficient antigen presentation to T cells, which skew the differentiation into Th1 and Th17 phenotypes and cytotoxic T cells (Tc). Subsequently, freshly activated donor T cells infiltrate inflamed tissues, recognize mismatched antigens on APCs (including donor), while the inflammatory cues further activate tissue-infiltrating alloreactive T cells and the specific cytokine profile. Chemoattractants and tissue-specific expression of antigens recruit T cells to skin, gut and liver where they release cytokines (TNF-α, IL-6, IL-1 and IFN-γ), engage in cell-to-cell mediated killing through mechanisms such as perforin/granzyme release or Fas/FasL interaction, and recruit other cells, leading tissue destruction via diverse mechanisms of apoptosis and necroptosis (Ichiki et al., 2006; Malard et al., 2023; Ramachandran et al., 2019).

Recent studies have identified key contributors to the pathophysiology of aGvHD, offering new avenues for clinical intervention. Key players include innate lymphoid cells, myeloid cells, and cells mediating the interaction between intestinal epithelium and the microbiota (Nassereddine et al., 2017). NK cells, a subset of radioresistant cells originating from common lymphoid progenitors (CLP), play a role in recognizing missing self, eliminating donor cells. They also

secrete large quantities of IFN-γ, contributing to the pro-inflammatory response (Hill et al., 2021). Patients with intestinal GvHD exhibit low numbers of mucosal-associated invariant (MAIT) T cells, which are involved in microbiota recognition and control in the intestine, partly through IL-17A secretion. The lack of MAIT cells correlates with compromised intestinal barrier function and increased DC alloantigen presentation (Gao et al., 2021; Varelias et al., 2018). Myeloid cells, such as neutrophils and donor CD103⁺ DCs, are heavily implicated in the initiation of aGvHD and intestinal lesions. Neutrophils act as early responders to intestinal inflammatory signals triggered by conditioning-mediated tissue damage and commensal translocation. Previously our team has demonstrated that administration of neutrophil-depleting antibodies reduced aGvHD and lethality after allo-HCT in mouse models (Hülsdünker et al., 2018; Schwab et al., 2014). CD103⁺ DCs contribute by presenting exogenous alloantigens in draining lymph nodes to donor T cells and guiding their infiltration into the intestine by imprinting the expression of gut-homing integrin $\alpha_4\beta_7$ (Koyama et al., 2015)

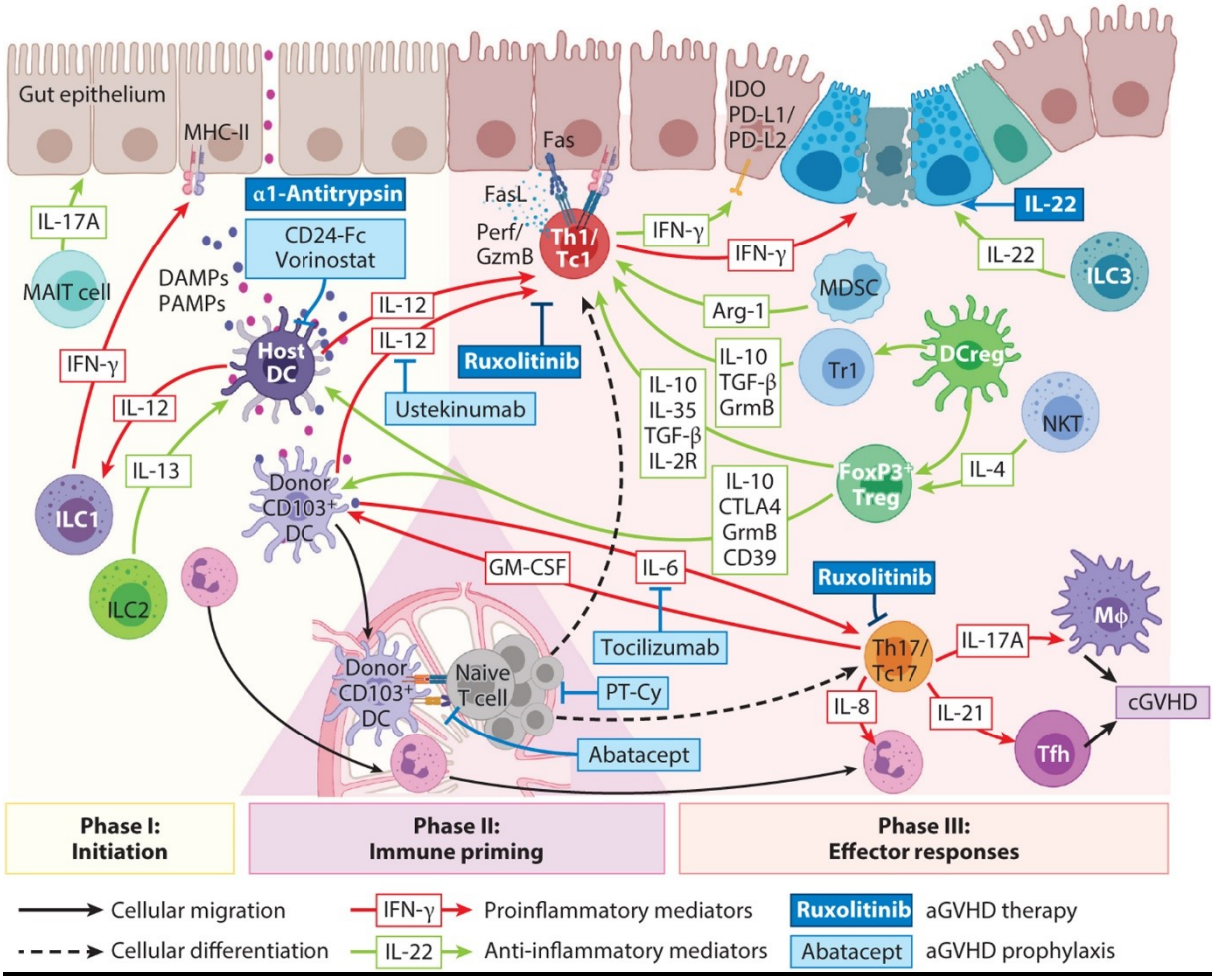

Figure 2. GvHD pathogenesis. Representation of the three pivotal phases of aGvHD pathophysiology. Initiation phase: Conditioning-induced damage in the gastrointestinal tract results in the release PAMPs and DAMPs. This process recruits cells, instigates inflammatory signals, and activatesd DCs. T cell activation/immune priming phase:

Donor CD103+ DCs along with nonhematopoietic APCs activate alloreactive T cells. Effector phase: Activated alloreactive T cells undergo differentiation into Th1 and Th17 subsets. They subsequently migrate to target organs, executing cytotoxic functions that escalate tissue damage and contribute to the perpetuation of the inflammatory environment. Figure adapted from (Hill et al., 2021).

1.2.3. Novel therapies against aGvHD

To prevent aGvHD, the conventional approach involves administering potent calcineurin inhibitors (CNI) during the conditioning regimen, with an alternative being donor T cell depletion in the graft. However, these strategies won't be delved into within this discussion (Flinn & Gennery, 2023; Ho & Soiffer, 2001; Luznik et al., 2008; Simpson, 2003; Zeiser et al., 2023). The primary recourse for GvHD remains classic corticosteroid therapy, effective only in 50% of cases. Consequently, alternative therapies targeting immune response modulators are gaining prominence. Notably among these are Tregs, MSCs, innate NK T cells (iNKTs), Th17 cells, mesenchymal stromal cells, decidua stromal cells (DSCs), Notch signaling, dipeptidyl peptidase-4 (DPP-4) inhibitors, T cell costimulatory molecules (CD28/80/86/40L,OX40), B cell receptor (BCR) signaling, proteasome, Rho associated coiled-coil containing protein kinase 2 (ROCK2), hedgehog signaling pathways and Janus kinase (JAK). Notably, an FDA-approved small-molecule inhibitor against JAK is already available to patients with steroid-refractory aGvHD (Flinn & Gennery, 2023; Inamoto et al., 2021; Martini et al., 2022; Zeiser et al., 2023). Further strategies encompass chimeric antigen receptor (CAR) T cells and bi-specific T cell engager- (BiTEs) T cells (G. Li et al., 2022; Sanber et al., 2021). However, the most promising endeavors center around tissue regeneration and curtailing the unrestrained inflammatory response, either by inducing tolerance or suppressing pro-inflammatory signals, or a synergistic blend of both. Below, a concise overview of these therapeutic targets is provided.

Intravenous (i.v.) administration of infused cells alongside the graft have been tested in pre-clinical models and even in patients to prevent aGvHD. Donor Tregs, whether freshly isolated or ex vivo expanded, or even third-party), have proven protective against aGvHD. Their mechanism involves suppression of alloreactive T cell proliferation, primarily via IL-10 secretion (Cohen et al., 2002; Edinger & Hoffmann, 2011; Hoffmann et al., 2002). However, challenges in achieving high purity Treg populations compliant with good manufacturing practices (GMP) persist, compounded by the diverse effects of various Treg subsets. Hence, defining the distinct populations based on marker expression or origin is crucial for specific isolation (Hefazi et al., 2021). In mixed leukocyte reactions it was observed that placenta-derived DSCs, known for their immunosuppressive functions during pregnancy, need to be in

contact with lymphocytes to suppress their activation via paracrine mechanisms involving Treg expansion. Furthermore, through indoleamine 2,3-dioxygenase (IDO) and prostaglanding E2 (PGE2) they can inhibit DC differentiation and so impair allogenic T cell proliferation (Erkers et al., 2013) (Moll et al., 2015; Zeiser et al., 2023). The application of placenta-derived DSCs poses ethical and GMP compliance hurdles, along with size-related challenges that confine them to the lungs, necessitating heparin supplementation during infusions (Karlsson et al., 2012; Portmann-Lanz et al., 2006; Roelen et al., 2009).

Blockade of OX40L or CD40L+LTβR (and other costimulatory molecules like CD80/86) with hybridoma generated monoclonal antibodies have shown to diminish aGvHD when given to recipient mice by inhibiting the second signal required for T cell activation/cost delivered by activated APCs after TCR and MHC-antigen complex recognition rendering them anergic (Blazar et al., 1996, 2003; Tamada et al., 2002). This importance of such costimulatory pathways, like OX40-OX40L was further supported by the fact that a similar effect was observed when OX40 deficient mice were used as recipients or T cells from OX40L-deficient mice were transplanted. Some of these costimulatory molecules, like OX40L (and in theory other TNFSF members) can also preserve and improve Treg reconstitution (observed in the prevented drop of Treg/Tcon ratio in peripheral blood) after transplantation when given in combination with mammalian target of rapamycin (mTOR). Moreover,this therapy where a monoclonal blocking antibody against OX40L ws given once before transplantation and then weekly for the duration of the experiment accompanied by concomitant sirolimus, showed reduced expansion of CD4 T cells and a lower transcription of gene sets associated to Th/Tc1 and Th/Tc17 (Tkachev et al., 2017). Yet for these OX40, OX40L and CD40L blocking antibodies it cannot be discarded that binding of the antibodies to cells expressing these receptors targeted them for deletion through ADCC having certain confounding effect.

JAK1/2 inhibitors block STAT1/3 phosphorylation affecting directly the transcription pathways involved in secretion of proinflammatory cytokines like IL-6 and IL-12 in T cells plus reducing neutrophil mobilization to intestinal draining lymph nodes, with the downside of indiscriminately blocking this signaling also in other cells impairing for example the maintenance and function of Tregs, Tcs and NKs leading to immunosuppression and increased risk of opportunistic infections (Heine et al., 2013; K. P. A. MacDonald et al., 2018; Zeiser et al., 2020). Notch are a group of conserved receptors and ligands (Delta-like 1/4 and Jagged 1/2 expressed on fibroblastic reticular cells, highlighting the importance of non-hematopoietic APCs in GvHD initiation) proteins that contain a bound transcription factor that is released after receptor/ligand engagement and translocate to the nucleus to activate genes important for T cell differentiation

and T cell function (Th, Tc and Tregs), among others (Radtke et al., 2013). Blocking this interaction has no effect on T cell activation or differentiation in GvHD but impairs their production of IFN- and IL-17, their homing to gut and moreover, favored Treg expansion and homing to gut shifting the ratios of Treg:Tcon in the intestinal tract (Chung et al., 2019; Tkachev et al., 2023).

To conclude this short review, last generation CAR T cells are designed with an extracellular anti-tumor domain and an intracellular signaling domain of the CD3ζ of the TCR complex and additional domains from costimulatory molecules such as CD28 or 4-1BB (Depil et al., 2020; G. Li et al., 2022). Only autologous CAR T cells are produced, avoiding any allogenic reaction and targeting exclusively tumor cells preserving the graft versus leukemia effect (GvL) without causing GvHD. Producing autologous CAR T cells does not work with every patient, particularly those with T cell dysfunctions, and is a cost and time demanding procedure with a narrow time window for the CAR T cell infusion to guarantee a positive response (Depil et al., 2020). Alloreactive CAR T cells are therefore ways of solving these limitations and increase the access to this therapy but then the risk of GvHD and graft (T cell infusion) rejection represents a problem. Gene editing of TCR genes through CRISPR/Cas9 have been successful to inactivate normal host MHC recognition by removing the β2 microglobulin domain or other HLA components in the CAR T cells but a total knockout in the whole infused population has not been possible yet leaving a potential risk for GvHD development and NK recognition of missing self (Bonini et al., 2023; Hu et al., 2021; Kagoya et al., 2020; Sanber et al., 2021). To overcome other common CAR-related toxicity side effects like cytokine release syndrome caused by continuous activation and high expansion of the CAR T cells leading to release of proinflammatory cytokines which in turn can recruit and activate bystander myeloid cells, endogenous TCR editing has been performed to increase their specificity. Direct modification of the TCR is preferred over functional deletion of the original TCR and subsequent insertion of a new CAR because it eliminates the possible recombination of both TCR sequence generating an unspecific new antigen recognition domain (Bonini et al., 2023). Additional engineered CAR T cells have been designed to silence the target of the concomitant therapies like CD52-depleting antibody to improve their survival, and the α-chain of the TCR to prevent endogenous TCR expression. This has been achieved through delivery of transcription activator-like effector nuclease (TALEN) which contain mRNA sequences that bind to the gene targets and prevent their expression (Qasim et al., 2017).

Despite these efforts, the appropriate conditions for CAR T cell therapy still need to be carefully defined since other factors have been known to influence the outcome of this therapy. Immune

effector cell-associated neurotoxicity syndrome (ICANS) is not yet completely characterized but is believed to be associated with a high dose infusion of CAR T cells, microglia bystander activation and high MCP-1, IL-6 an IL-8 concentrations in the central nervous system. The direct role of CAR T cell in increased BBB permability is not defined though, however has been the cause of conclusion of acute lymphoblastic leukemia clinical trial ("JCAR015 in ALL," 2018). Furthermore, microbial dysregulation has been associated with ICANS as demonstrated in a study on B-cell lymphoma and leukemia patients receiving CD19 CAR T cell therapy. Exposure to broad spectrum antibiotics that reduce the abundance of species from the Clostridiales order or *Faecalibacterium* s.p., *Ruminococcus* s.p. over the treatment course led to increase ICANS and worse survival (Smith et al., 2022).

On a similar note as CAR T cell therapies, another T cell population for which an artificial receptor can be engineered to induce TCR activation are Tregs, which have demonstrated promising results when transferred to patients suffering from post-transplantat complications (see 1.3.2) and in preclinical models of autoimmune disorders due to their modulatory properties (Kohm et al., 2004; McGeachy et al., 2005; Scalapino et al., 2006). Recognition domains for CAR Tregs include antigens involved in antigen presentation and T cell activation, as such several of the tested CAR T reg studies have developed receptors specific against HLA-A2, a highly mismatched antigen between donor-recipients (Kaljanac & Abken, 2023). These CAR Tregs exhibited higher migration to inflammation sites, increased protection against solid transplant rejection and improved survival in murine xenogeneic GvHD models (Boardman et al., 2017; K. G. MacDonald et al., 2016; Noyan et al., 2017; Sicard et al., 2020). Latest proposed improvements to CAR Treg therapy are the inclusion of a third domain into the CAR with a functional motif either acting on the Treg itself or conferring a receptor/ligand to interact with neighboring cells. This functional domain could improve survival, migration, tropism, allow Tregs to block a given interaction or activate certain cell types (Bittner et al., 2023). Early phase clinical trials on kidney or liver transplantation using CAR Tregs against HLA-A2 are currently ongoing so the evidence supporting its application will come up in the following years.

1.3. Regulatory T cells

1.3.1. Tregs and their function

Tregs are a subset of CD4 T cells that possess regulatory mechanisms to suppress the immune response, maintain tissue homeostasis and induce tolerance (including self-tolerance). During thymic selection, some mild self-reactive T cells that engage weakly through their TCR and hence receive weak signals from medullary thymic epithelial cells (mTEC) for a short time, will avoid elimination and receive additional signals like IL-2 and IL-7 to demethylate gene regions and express transcription factors, being FoxP3 the Treg master regulator, that drive differentiation into thymus-derived Tregs (tTregs) (Josefowicz, Lu, et al., 2012; Shevach & Thornton, 2014). tTregs can then leave the thymus to reside in peripheral lymphoid organs where their TCR can engage a MCH-antigen complex and become highly functional Tregs, termed effector Tregs (eTregs) (Goswami et al., 2022). In the periphery, mature naïve CD4 T cells subjected to specific conditions like chronic suboptimal antigen (from commensal, allergens or food) stimulation, lymphopenia or suppressive cytokines like TGF-β can differentiate into peripheral Tregs (pTregs) and become a tissue-resident population. The ensuing changes resemble those undergone by tTregs but expression of certain transcription factors differs, which led to the proposed definition of tTregs as Helios^{+}Neuropilin-1(Nrp-1)$^{+}$ and pTregs as Helios^{-}Neuropilin-1^{-}, however expression of these transcription factors can be induced after Treg activation limiting their role as tTreg markers in mouse (Feuerer et al., 2010; Yadav et al., 2013). Interestingly, recent studies demonstrated how Treg TCR repertoire in peripheral organs overlap between those found in tTregs and pTregs implying that tissue-resident Tregs also originate from migrated tTregs but in certain organs there is a clear majority of Tregs expressing thymic markers (Hsieh et al., 2006; Weiss et al., 2012). pTregs and tTregs are known as natural Tregs (nTregs), and an additional subset of induced Tregs (iTregs) has been described as also generated from CD4 T cells exposed to TGF-β and IL-2 but with incomplete demethylation of the Treg-specific demethylation region (TSDR) and unstable suppressive phenotype that can revert to Tcons (Shevach & Thornton, 2014; Yadav et al., 2013).

Mouse models have shown how deletion of Tregs results in fatal autoimmunity as demonstrated by findings that show how thymectomy of newborn mice is the cause for developing T cell-mediated inflammation that could be restored by transfer of lymphocytes from non-thymectomized adult mice (Bonomo et al., 1995; Sakaguchi et al., 1982). Furthermore, loss of function of the FoxP3 gene in mice and humans causes a fatal disease characterized by lymphoproliferative autoimmune disorder with lymphadenopathy, diabetes, anemia and dermatitis symptoms (Gambineri et al., 2003). This effect is not limited to the developmental stages, as chronic deletion of FoxP3-expressing cells in adult mice led to their death after two weeks by lympho- and myeloproliferative disease supporting the key role of

Tregs in immunological tolerance during the whole life (J. M. Kim et al., 2007). Immune homeostasis is also tightly regulated by Tregs in a T cell subset-dependent manner, and some research groups suggest that actually pTregs are the dominant Treg population in this regard (Josefowicz, Niec, et al., 2012). In the last decade, research has proved how Tregs display high plasticity in their transcription factor signatures and that by sensing environmental signals they adjust the level of expression of them (Kitz et al., 2018; Komatsu et al., 2009; Vahedi et al., 2013). In this way they can act as negative regulators of the immune response by sensing the same environmental cues that determine Th differentiation and express the respective Th-like master regulator and chemokine receptors to specifically migrate together and suppress that particular Th phenotype. (Chaudhry & Rudensky, 2013; Kitz et al., 2018; Lu et al., 2010). In accordance, Th1-like Tregs, expressing T-bet and FoxP3 were found to effectively control Th1 responses, and similar results have been found with Th2, Th17, Th9 and T follicular helper cells (Tfh) (Goswami et al., 2022; Koch et al., 2009)..

Tregs employ various mechanisms to dampen not only T cells but also the broader immune response. These encompass both contact-dependent and independent pathways, involving direct actions on T cells and indirect regulatory mechanisms. Tregs have been categorized based on the specific effects they exert: metabolic disruption, secretion of anti-inflammatory cytokines, inhibition of DC and B cell function and maturation and inhibition of effector T cells (Goswami et al., 2022; Kempkes et al., 2019). CD39 and CD73 ectoenzyme on the membrane can reduce ATP in the medium into adenosine reducing its availability for other cells and also binding to the adenosine receptor 2A (A2AR) on effector T cells reducing effector cytokine secretion (Maj et al., 2017). Increased Treg expression of the high-affinity IL-2 receptor depletes the IL-2 concentration impairing T cell expansion (Höfer et al., 2012). Another recognized mechanism involves the secretion of IL-10, IL-35 and TGF-β. Upon binding to their corresponding receptors, these cytokines induce transcriptional changes in the cell, leading to diminished glycolysis, oxidative phosphorylation, cell maturation, and inhibition of CD28 phosphorylation, among other effects (Komai et al., 2018; Taylor et al., 2006; Ye et al., 2021). The release of the IDO enzyme limits tryptophan catabolism exerting a negative influence on DC maturation and the expression of chemokine receptors important for their migration (Goswami et al., 2022; Hwang et al., 2005). Furthermore, expression of CTLA-4 and Lag3 can futher suppress DCs by downregulating CD80/86 and competing with CD4 to bind to MHCII (Josefowicz, Lu, et al., 2012). Tregs can induce apoptosis in T and B cells by releasing perforin and granzyme (Grossman et al., 2004). The overview above highlights some of the mechanisms employed by Treg to directly suppress T cells, hindering activation, expansion, and effector functions. Additionally Tregs exert indirect suppression by blocking other cells

required for a comprehensive T cell and response. Nevertheless, it is essential to note that other regulatory lymphocyte populations, such as CD8 Tregs and Bregs, play a supportive role in controlling immune reactions, including GvHD, although their functions and biology will not be discussed in this work (Hill et al., 2021).

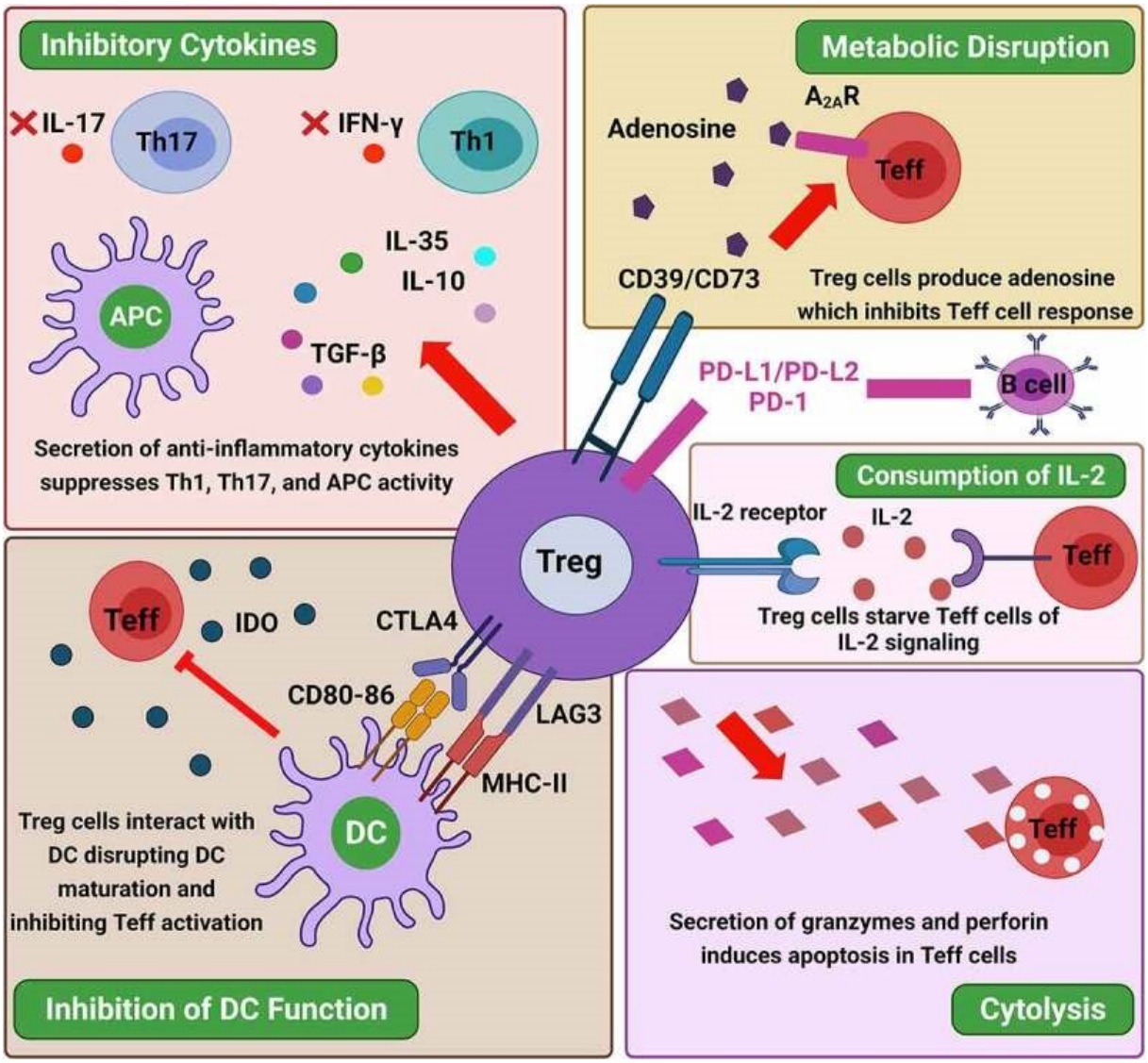

Figure 3. Treg functions. Overview of Treg functions. Metabolic disruption through ATP catalysis into adenosine can inhibit effector T cell function while depletion of IL-2 blocks expansion. Secretion of anti-inflammatory cytokines like IL-10, IL-35 and TGF-β can have negative effects on important pathways like glycolysis and oxidative phosphorylation as well as impair Th effector functions, particularly secretion of cytokines. Binding of surface markers to receptors on DCs can reduce their activation and block binding of T cells avoiding their activation. Finally, secretion of cytotoxic enzymes and cytolytic proteins can eliminate effector T cells. Figure taken from (Goswami et al., 2022).

1.3.2. Treg-based therapies against aGvHD

As discussed in 1.2.3, Tregs have emerged among the many recently investigated targets aiming to devise therapies for preventing GvHD due to their effective regulation of T cell responses and successful preclinical applications (Elias & Rudensky, 2019). However, a straight-forward transfer of Tregs from an unrelated donor's blood to a patient is not a feasible therapeutic approach due to the low number of circulating Tregs. Consequently, in clinical studies exploring Treg transfer, Tregs are first isolated from blood and treated with various agents such as rapamycin, IL-2, or anti-CD3/CD28 antibodies to expand them in vitro before being transferred to the patient (Furlan et al., 2020; Hoffmann et al., 2002, 2002; Riegel et al., 2020; Shin et al., 2011). Other sources, such as umbilical cord blood (UCB) offer higher Treg purity and have demonstrated increased overall survival and lower pathological scores (Elias & Rudensky, 2019; Guo et al., 2021; Parmar et al., 2014). Notwithstanding, this therapeutic approach is not without its limitations, including GMP compliance, low yield, and stability as previously described.

The concept of developing an analogue of CARs for Tregs involves creating a receptor with an extracellular domain capable of binding an inflammatory molecule, accompanied by intracellular CD3 and CD28 costimulatory domains. This engineered immune receptor, termed artificial immune receptor (AIR), is transduced into Tregs and is selectively engaged in inflamed tissues. Unlike CARs, AIR does not target a tissue specific antigen. Proposed domains for guiding AIR Tregs include members of the TNFSF such as lymphotoxin-β receptor (LTβR), DR3 and TNFR2. These domains bind to LTβ, TL1A and mTNF-α, proteins highly expressed in tissues with an ongoing immune response. Transduced Tregs with these AIRs were proved to express latency-associated peptide (required for secretion of TGF-β), transcribe TCR-related genes, proliferate when cocultured with HEK cells expressing the corresponding AIR ligand validating this concept. Furthermore, when tested in a mouse model of GvHD, transplanted AIR Tregs against LTβR improved survival and clinical symptoms. Importantly, these proteins have a short lifespan or are bound to cells, limiting their diffusion to healthy tissues (Bittner et al., 2022). Thus, AIR Tregs are selectively activated in various tissues only in presence of inflammatory signals. This targeted activation provides distinct advantages over CAR Tregs (Salomon, 2022).

In murine models, the in vivo expansion of either donor or recipient Tregs has been investigated. Administering anti-DR3 or anti-TL1A antibodies to donor mice led to Treg expansion, resulting in reduced alloreactive T cell expansion and infiltration into target organs. This translated into lower concentrations of inflammatory cytokines in the serum and improved survival (Copsel et al., 2018; B.-S. Kim et al., 2015; Mavers et al., 2019). Conversely, recipient

Tregs can be activated and expanded with low-dose IL-2 (l.d. IL-2) and in patients with cGvHD, sustained Treg expansion was observed, accompanied by a reduction of symptoms (Guo et al., 2021; Koreth et al., 2011). GITR is elevated in activated alloreactive CD4 and CD8 T cells, and its activation has opposing effects in these populations. A study by Muriglan and colleagues tested an anti-GITR antibody and found that it induces apoptosis of CD4 T cells and reduced proliferation, and enhances CD8 T cell proliferation (Muriglan et al., 2004). This contradictory outcome was hypothesized to protect against GvHD, with alloreactive CD4 T cells being the potent drivers, while preserving GvL effect, which heavily relies on alloreactive CD8 T cell function. Indeed, the GITR agonist DTA-1 protected mice by delaying and decreasing clinical symptoms, and treatment reduced liver and intestinal damage. The authors found that GITR agonism's effect on alloreactive T cell activation was independent on Tregs. However, it's worth noting that, unlike in vitro assays, the transplantation model does not exclude any possible effect on Tregs, and the pre-transplant context, in which high GITR expression is constitutive in Tregs, was not studied. Our group has also demonstrated that a ligand-based TNFR2 agonist given to recipient mice before transplantation can increase Tregs, reduce alloreactive T cell proliferation, and limit infiltration into target organs. Consequently this approach protects against GvHD without compromising GvL or normal immune function after challenging with murine cytomegalovirus (Chopra et al., 2016).

In summary, numerous possibilities exist for designing Treg-based therapies, offering a wide array of choices regarding their isolation, origin (recipient or donor), and timing of administration (before/after transplantation). However, the ultimate success of a Treg therapy will hinge on its swift translation into clinical settings, ensuring broad applicability, simplicity in production, and ease in administration. Moreover, for maximum effectiveness, the therapy should not impede T cell activation and expansion, safeguarding the GvL effect. Taking these considerations into account, this study aims to investigate the effect of DR3-, TNFR2- and GITR-agonists on Tregs through the administration of antibodies designed with the principles outlined in section 1.1.4. This serves as the foundation to specifically target Tregs proposing a straightforward therapy for expanding recipient Tregs while providing protection against GvHD.

1.4. Knowledge gap

TNFSF members play a pivotal role in modulating immune responses, and their expression on Tregs presents a viable target for specific intervention. The engagement of TNFR2 on Tregs is established to expand this cell subset, providing protection against GvHD by mitigating

alloreactive T cell infiltration into target organs. Considering that other TNFRSF members share signaling pathways with TNFR2 and are also expressed on Tregs, underscores the significance of these receptors and ligands as potential targets to prevent or mitigate aGvHD. However, scant evidence is available to demonstrate whether similar protective effects can be achieved with other TNFRSF members and what additional consequences their agonism might entail, both in steady state and during allo-HCT.

2. Specific Aims

1) Determine whether novel antibody-based TNFRSF agonists are superior to ligand-based TNFR2 agonist.
2) Examine the effects of TNFR2 engagement on Tregs and elucidate its potential beneficial role in the context of aGvHD.
3) Investigate the off-target effects of TNFRSF agonism on various cell subsets beyond Tregs.
4) Explore the potential protective effect against aGvHD achieved by engaging TNFR2, DR3 or GITR before transplantation.

3. Materials and Methods

3.1. Materials

3.1.1. Antibodies

Table 1. Mouse-specific antibody list.

Reactivity	Clone	Catalogue number	Manufacturer
CD3	17A2	100241	Biolegend
CD4	RM4-5, GK1.5	100540/100531, 100407	Biolegend
CD8	53-6.7	100725/100722/100712/100752, A15386	Biolegend, Lifetech
FoxP3	FJK-16s	11-5773-82	Invitrogen
TNFR2	TR75-89	113406	Biolegend
GITR	DTA-1, YGL386	126312/ 12-5874-82, 102305	Biolegend, Invitrogen
DR3	4C12	144410	Biolegend
TIGIT	GIGD7	25-9501-82	Invitrogen
CD39	24DMS1	12-0391-82	Invitrogen
CD73	TY/11.8	25-0731-82	Invitrogen
Lag3	C9B7W	12-2231-82	Invitrogen
PD-1	29F.1A12, RMP1-30	135210, 109110	Biolegend
CD25	PC61	102012/102016	Biolegend
Helios	22F6	137216	Biolegend
Ki67	16A8	652408	Biolegend
4-1BB	17B5	106106	Biolegend
OX40	OX-86	17-1341-822	Invitrogen
VISTA	MIH64	46-1083-82	Invitrogen
ICAM1	YN1/1.7.4	116108	Biolegend
ST2	DIH4	146608	Biolegend
KLRG1	2F1	51-5893-82	Invitrogen

PD-L1	10F.9G2	124312	Biolegend
B220	RA3-6B2	103226	Biolegend
Ly6C	AL-21	553104	BD
CD64	X54-5/7.1	139314/139311/139304	Biolegend
CD11b	M1/70	101226	Biolegend
XCR1	ZET	148204	Biolegend
Ly6G	1A8	127624	Biolegend
F4/80	BM8	123110/123122	Biolegend
MHCII	AF6-120.1	116416	Biolegend
SiglecF	E50-2440	562680	BD
CD44	IM7	103032/103008	Biolegend
CD45	30-F11	MCD4528	Invitrogen
CD45.1	A20	110708	Biolegend
CD45.2	104	109820/109816	Biolegend
CD90.1	KW322, HIS51	205903, 47-0900-82	Biolegend
CD90.2	30-H12	105349/105349, 12-0903-82	Biolegend, Invitrogen
CD86	GL-1	105012	Biolegend

Table 2. Human-specific antibody list.

Reactivity	Clone	Catalogue number	Manufacturer
CD3	OKT3	317314	Biolegend
CD4	OKT4	317428	Biolegend
CD8	RFT-8	A15448	Lifetech
CD25	M-A251	356108	Biolegend
FoxP3	FJK-16s	11-5773-82	Invitrogen
TNFR2	3G7A02	358406	Biolegend
GITR	108-17	371212	Biolegend
DR3	JD3	307106	Biolegend

3.1.2. Buffers, media and solutions

Solution	Composition/preparation
Red blood cell (RBC) lysis buffer (10x)	NH_4Cl (89.9 g), $KHCO_3$ (10 g), EDTA (0.37 g) in 1000 mL distilled water, sterile filtered.
Anesthetic	2 mL of Ursotamin® and 2 ml of Xylavet® in 21 mL PBS
Fluorescence-Activated Cell Sorting (FACS) buffer	2% FCS, EDTA 2mM in (1x) PBS (Ca-, Mg-)
Lymph node enzyme mix	5.76 mL Roswell park memorial institute 1640 medium (RPMI), 60 µL collagenase A (10 mg/mL), 60 µL collagenase D (10 mg/mL), 120 µL DNase I (5 mg/mL) per sample/mouse
Epithelium dissociation buffer	57 mL calcium- and magnesium-free HBSS, 3 mL FCS, 44.6 mg EDTA per sample/mouse
Intestine enzyme mix	7.65 mL HBSS, 850 µL FCS, 750 µL collagenase A (10 mg/mL), 750 µL collagenase D (10 mg/mL), 80 µL DNase I (5mg/mL) per sample/mouse
Intestine washing buffer	54 mL calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS), 6 mL FCS per sample/mouse
Complete RPMI (cRPMI)	10% FCS, 1% Penicillin-Streptomycin (Pen/Strep), 1% L-Glutamine
Lung dissociation buffer 1x	1 mL of 20x Buffer S and 19 mL of ddH_2O per lung
Lung enzyme mix	2.4 mL of lung dissociation buffer 1x, 100 µL of solution D (Reconstituted with 3 mL of lung dissociation buffer 1x) and 15 µL of solution A (Reconstituted with 1 mL of lung dissociation buffer 1x)
Skin digestion mix	5.78 mL Dulbecco's Modified Eagle Medium (DMEM), 2 mL collagenase A (10 mg/mL), 2 mL collagenase D (10 mg/mL), 20 µl DNase I (5mg/mL), 2% v/v FCS

3.1.3. Consumables and chemical reagents

Material	Catalogue Number	Company
Chemical reagents		

2-Mercaptoethanol	M3148	Sigma-Aldrich
6x DNA Loading Dye	R0611	Thermo Fisher Scientific
Agarose NEEO Ultra-Quality	2267.1	Roth
Benzyl alcohol	4478.2	Roth
Benzyl benzoate	B6630	Sigma-Aldrich
Brefeldin A (1000x)	420601	Biolegend
Cell Activation Cocktail (without Brefeldin A)	423301	Biolegend
Collagenase A	10103586001	Roche
Collagenase D	11088866001	Roche
Collagenase P	11213857001	Sigma-Aldrich
Collagenase type VIII	2139-500MG	Sigma-Aldrich
DEPC-Treated Water	AM9906	Invitrogen
DMEM	12491023	Thermo Fisher Scientific
Diphteria toxin	D0564	Sigma-Aldrich
Deoxyribonuclease (DNase) I from bovine pancreas	D5025-15KU	Sigma-Aldrich
eBioscience™ Permeabilization Buffer (10X)	00-8333-56	Thermo Fisher Scientific
Entelan®	1.07961	Merck
Ethanol absolute	2246	TH Geyer
Orniflox(Baytril®)	2130202	Dechra
Fetal Calf Serum (FCS)		Invitrogen
FITC-dextran 4kDa	46944-500MG-F	Sigma-Aldrich
Fix/Perm Concentrate	00-5123-43	Thermo Fisher Scientific
Fix/Perm Diluent	00-5223-56	Thermo Fisher Scientific
Hanks Balanced Salt Solution (HBSS) without Ca^{2+}/Mg^{2+}	14170112	Thermo Fisher Scientific
HBSS with Ca^{2+}/Mg^{2+}	14025092	Thermo Fisher Scientific
HD Green Plus DNA Stain		iNTAS
Histopaque® -1077	10771-500ML	Sigma-Aldrich
Hydrogen Chloride (37%)	4625.1	Roth
Lymphoprep	07851	Stemcell technologies
Liquid blocker super PAP pen	N71310	Science Services

L-Glutamine	3772.1	Roth
n-Hexane	1.04367	Merc
Normal Rat Serum	10710C	Invitrogen
O' Range Ruler 100 bp ladder	SM0623	Thermo Fisher Scientific
Paraformaldehyde	P6148	Sigma-Aldrich
Penicillin-Streptomycin	11074440001	Roche
Phosphate buffered saline (PBS) without Ca^{2+}/Mg^{2+}	P04-36500	Pan Biotech
RPMI1640 medium	12633012	Thermo Fisher Scientific
Tissue-Tek ® Optimal Cutting Temperature ™ (OCT) Compound	4583	Sakura Finetek
TritonX-100	2051.3	Roth
Trypan blue	A0668,0025	Applichem
Ursotamin (Ketamine®)		Serumwerk
Vectashield® antifade mounting medium with DAPI	H-1200	Vector Laboratories
Xylavet (Xylazine®)		CP-Pharma
Consumables		
6-well flat base culture plates	83.3920.005	Sarstedt
96-well flat base culture plates	83.3924.500	Sarstedt
96-well round base culture plates	83.3925.500	Sarstedt
96-well conical base culture plates	83.3926.500	Sarstedt
1, 5, 10, 25, 50 ml single use pipettes		Greiner Bio-one
10, 200, 1000 µL pipette tips	70.3010.100, 70.3030.100, 70.3050.100	Sarstedt
15 ml and 50 ml centrifuge tube	188261, 210261	Greiner Bio-One
Animal feeding needle, sterile, disposable, malleable 304 SS (20G. L x diam. 1.5 in. X 1.9 mm, ball)	CAD9921-100EA	Sigma-Aldrich
UltraComp eBeads™	01-2222-42	Thermo Fischer Scientific

Cell counting chamber (Neubauer)	ZK03	Hartenstein
Cell strainer 70 µm, EASYstrainer™	542070	Greiner Bio-one
Corning® HTS Transwell®-96 Permeable Support with 1.0 µm Pore Polyester Membrane	3380	Corning
Combitips advanced 2.5 ml	0030089650	Eppendorf
GentleMACS C tubes	130-093-237	Miltenyi Biotec
Introcan®-W 0,90 x 25 mm G 22 blau	4254090B	B. Braun
MACS® SmartStrainers (100 µm)	130-098-463	Miltenyi Biotec
Micro tube 0.5 ml	72. 698	Sarstedt
Microvette® 200 Serum Gel	20.1291	Sarstedt
Pipette controller (Accu-jet® Pro)	26300	Brand
Safe seal micro tube 2 ml	72.695.500	Sarstedt
Safe seal tube 1.5 ml	72.706	Sarstedt
Scalpel blades, feather #10, sterile	BB510	B. Braun
SepMate™ 50 (IVD)	85460	Stemcell technologies
Syringe, 1 ml insulin (30Gx×1/2″ (0,3mm × 12mm), Omnican® 100	9151141	B. Braun
Syringe, 5 ml BD Discardit™ II	300850	Becton Dickinson
T75 Greiner culture flasks, tissue culture treated	C7231	Sigma-Aldrich
Tissue embedding cassette	09-0303	R. Langenbrinck GmbH
TT Cryomold® Standard, eckig (25x20x5mm)	4557	Sakura Finetek

3.1.4. Devices and equipment

Device	**Company**
Laminar flow hood Hera safe	Thermo Fisher Scientific
Megafude ST4 Plus Series	Thermo Fisher Scientific

Eppendorf® Repeater™ E3/E3x	Eppendorf
CFX Connect™ Real-Time PCR Detection System	Bio-Rad
Cryostat (CM1950)	Leica Biosystems
Infinite® 200 PRO Plate reader	Tecan group LTD
Nanodrop ™ 2000 Spectrophotometer	Thermo Fisher Scientific
Precellys® 24 Homogenizator	Bertin GmbH
GentleMACS™ octo dissociator with heaters	Miltenyi Biotec
Attune NxT Flow cytometer equipped with 405 nm, 488 nm, 561 nm and 633 nm lasers and a high-throughput sampler	Thermo Fisher Scientific
IVIS Spectrum in vivo imaging system	Perkin Elmer
Faxitron CP-160 X-ray system	Faxitron X-ray

3.1.5. Kits

Name	**Catalogue number**	**Company**
Dynabeads™ Untouched™ Mouse T Cells	11413D	Invitrogen
Dynabeads™ Untouched™ Mouse CD4 Cells	11415D	Invitrogen
Mouse lung dissociation kit	130-095-927	Miltenyi Biotec
LEGENDplex™ Mouse Inflammation Panel (13-plex) with V-bottom Plate	740446	Biolegend
BD OptEIA™ Human IL-8 ELISA Set	555244	BD Bioscience
IL-10 Mouse Instant ELISA™ Kit	BMS614INST	Invitrogen
TGF beta-1 Mouse ELISA Kit	BMS608-4	Invitrogen
CD4+CD25+ Regulatory T Cell Isolation Kit, mouse	130-091-041	Miltenyi Biotec
CellTrace Violet Cell Proliferation Kit Protocol	C34571	Invitrogen
Dynabeads™ Mouse T-Activator CD3/CD28 for T-	11456D	Invitrogen

Cell Expansion and Activation

3.1.6. Mouse lines

Mouse line	Abbreviation	Origin
C57BL/6JTyrc−2J/Foxp3.Luci.DTR-4 (Suffner et al., 2010)	B6a.FoxP3.Luci.DTR	In house breeding, Center for Experimental Molecular Medicine (ZEMM) animal facility, Würzburg University animal facility
C57BL/6-Tyrc^{2J}/J	B6a	In house breeding, ZEMM animal facility
C57BL/6	B6	Charles River
C57BL/6-Tyrc^{2J}/129S2-Tnfrsf1b^{tm1Mwm}/J	B6a.TNFR2ko	In house breeding, ZEMM animal facility
FVB/NCrl	FVB	Charles River
FVB-Tg(CAG-luc,-GFP)L2G85Chco/J	FVB.L2G85	In house breeding, ZEMM animal facility

3.1.7. Softwares

Software (version)	Company
Living Image (4.5.5)	Perkin Elmer
FlowJo (10.8.1)	FlowJo LLC
GraphPad Prism (9)	GraphPad software

3.2. Methods

3.2.1. In vitro stimulation assays

3.2.1.1. Human peripheral blood mononuclear cells (hPBMCs) cultures

Approximately 7 mL of leukapheresis products from healthy patients provided by the department of transfusion medicine of the university clinic of Würzburg, were diluted 1:3 with PBS and transferred to a Sepmate 50 mL falcon tube previously filled with 15 mL of

Histopaque®-1077 or Lymphoprep solution for density gradient centrifugation. 10 mL of the diluted leukapheresis product were gently pipetted on top of the Histopaque®-1077/Lymphoprep. Tubes were centrifuged at 1200 RPM and room temperature for 10 min. Lymphocytes were carefully removed by taking out the buffy coat of the centrifuged Sepmate tubes and transferred to a 50 mL falcon tube. Two washing steps were done by adding PBS to fill the tube completely and then centrifuged at 1200 RPM and room temperature for 5 min. The supernatant was then resuspended in 40 mL of pre-warmed cRPMI medium for determining viable cell count by trypan blue exclusion and adjusting the concentration to $1x10^7$ cells/mL cRPMI. A high density culture was prepared by seeding 20 mL of the concentrated cell suspension in a T75 cell culture flask and placing it upright in an incubator at 37°C for 2 days (Römer et al., 2011).

hPBMCs concentration was determined again by trypan blue exclusion and adjusted to $2x10^6$ cells/mL cRPMI. Solutions of TNFRSF agonists to be tested were prepared in cRPMI at double the intended concentration and plated together at 1:1 v/v with 100 µL of hPBMCs in round bottom 96-well plates for a total of $2x10^5$ cells in 200 µL per well. For unstimulated controls, PBS was added instead of the TNFRSF agonist preparation. A single concentration of huNewSTAR2 (E09-N297A-sc(hu)TNF80), Fc-huTL1A (TL1A(72-251)-Flag-TNC-Fc) or Fc-huGITRL (GITRL-2xFlag-TNC-Fc(DANA)) of 1000 ng/mL was prepared in the culture medium. Plates were incubated at 37°C for 4 days before flow cytometric analysis.

3.2.1.2. Murine primary cells cultures

B6 wild type mice of 8-14 weeks of age were euthanized with CO_2 and by cervical dislocation before opening the abdominal cavity, dissecting the spleen and collecting it in PBS. A 70 µm cell strainer was placed on a 50 mL falcon and wet with 2 mL of RBC lysis buffer prior to transferring the spleen and cutting it into small pieces with surgical scissors. With the aid of a syringe plunger, the organ was macerated and the pulp on the cell strainer was rinsed with 8 mL of RBC lysis buffer and left at room temperature for 3 min. To stop the lysis, 10 mL of PBS were passed through the strainer. Cell suspensions were centrifuged at 1200 RPM and 4°C for 5 min and supernatant was resuspended in 10 mL PBS to determine viable cell counts.

Different types of murine cell cultures in round bottom 96-well plates were performed to evaluate Treg proliferation under different conditions: Mixed splenocytes cultures (with cells taken directly after RBC lysis and single cell suspension of spleen), enriched total T cells or enriched CD4 T cells isolated with Dynabeads™ Untouched™ Mouse T Cells / CD4 Cells kits

respectively, were plated at a density of $2x10^5$ cells/well in either in 1.67 nM solutions of STAR2, NewSTAR2 or 40 international untis (IU)/mL IL-2 (low dose IL-2) in cRPMI (TNFRSF agonist-containing media were prepared as in 3.2.1.1). Plates were incubated at 37°C for 4 days before flow cytometric analysis.

3.2.2. Four-day in vivo stimulation and cell isolation protocols

Wild type or reporter B6a.FoxP3.Luci.DTR, that express enhanced green fluorescence protein (eGFP) and luciferase under the FoxP3 promoter were injected intraperitoneally (i.p.) with 140 µg of the TNFRSF agonist in PBS. Depending on the stock concentration of the constructs, the injected volumes ranged from 200 – 400 µL. An equal quantity of an isotype control IgG1 antibody was injected into the mice from the control group. Four days after injection, mice were sacrificed and selected organs were extracted for flow cytometric analysis, tissue lysate analysis or serum collection.

Cell isolation from selected organs – Blood: Following euthanasia and the opening of the abdominal cavity in each mouse, the ribcage was delicately opened, and blood from the heart was collected using an insulin syringe. Approximately 400 µL of blood were combined with 10 mL of RBC lysis buffer in a 50 mL falcon tube and incubated for 15 minutes at room temperature. Subsequently, 10 mL of PBS were added to halt the lysis process, and the tubes underwent two washes by centrifugation at 1200 RPM and 4°C for 5 minutes, followed by resuspension in 10 mL PBS.

Bone marrow: Following euthanasia, hind legs were excised from the mice using surgical scissors to extract femurs and fibulae. Any attached muscle tissue was meticulously removed, and both edges were opened with a scalpel. Utilizing insulin syringes filled with PBS, the bone marrow was gently flushed from within the bones and collected in wells of 6-well plates. Once all bone marrow tissue was effectively flushed, the cell solution underwent filtration through a 70 µm cell strainer and was stored at 4°C for subsequent analysis.

Liver: A modified protocol, adapted from Sokol and colleagues was employed for liver isolation (Sokol et al., 2021). Instead of perfusing the mice, each lobe of the dissected liver was injected once with 2 mL of cold PBS. Subsequent to this, the digestion process was carried out as per the protocol. Notably, no CD31 enrichment was performed, and the obtained cell suspensions were subjected to staining after filtration and cell count..

Lungs: Lungs were dissected and cut into small 1 – 2 cm pieces with surgical scissors then rinsed with PBS to remove blood and transferred to gentleMACS C tubes previously filled with

lung enzyme mix (see 3.1.2). GentleMACS C tubes were sealed placed in a GentleMACS octo dissociator. The initial program, m_lung_01, was executed, and samples were incubated at 37°C for 30 minutes. Following this, the program m_lung_02 was initiated, and after a brief spin-down, the cell suspension was filtered through a 70 µm cell strainer positioned on top of a 50 mL Falcon tube. The strainer was washed with 2.5 mL of lung dissociation buffer (1x), and the filtered suspension underwent centrifugation at 1200 RPM and 4°C for 5 minutes. A 5-minute red blood cell lysis was performed, and the remaining volume in the tube was replenished with PBS. After another round of centrifugation, the supernatant was resuspended in 1 mL of PBS and stored at 4°C for subsequent analysis.

Lung lavage: In experiments requiring lung lavage to isolate alveolar macrophages, the initial step involved securing the euthanized mouse in a dorsal position, with the head immobilized using a bent needle placed behind and perpendicular to the upper incisors. A partial transversal incision was made on the ventral side of the trachea. An intravenous Introcan-W catheter, devoid of the metal needle, was attached to a 1 mL syringe filled with 0.6 mM EDTA/PBS. The catheter was inserted into the tracheal incision, not exceeding a depth of 1 cm, and the lungs were washed by injecting and withdrawing the solution iteratively while massaging the thoracic cavity. This lavage procedure was repeated 8 – 10 times using fresh EDTA/PBS for each iteration, with the collected washes accumulating in a 50 mL Falcon tube. After viable cell counting, the cell suspension underwent centrifugation at 1200 RPM and 4°C for 5 minutes to achieve the desired concentration for subsequent analysis.

Lymph nodes: Inguinal, cervical, axillary, and mediastinal lymph nodes were meticulously dissected from each mouse and perforated multiple times with a needle in a small petri dish before preservation in RPMI, placed on ice upon completion in the operating room. The excised lymph nodes were then transferred to a 15 mL Falcon tube filled with 3 mL of lymph node enzyme mix (refer to 3.1.2) and subjected to a 37°C water bath incubation for 20 minutes, with gentle inversion every 5 minutes. Simultaneously, a 70 µm cell strainer positioned atop a 50 mL Falcon tube was moistened with 5 mL of FACS buffer (3.1.2). Following the initial 20-minute incubation, the liquid phase underwent filtration through the cell strainer, and 3 mL of fresh lymph node enzyme mix were introduced to the lymph nodes. Mixing by pipetting with a 1 mL micropipette, equipped with a cut tip, was performed before another 10-minute incubation in the water bath, with additional pipetting halfway through. The liquid phase was once again passed through the strainer, and 4 mL of fresh lymph node enzyme mix were added to the lymph nodes for a final 10-minute incubation as previously described. The entire solution was then pipetted onto the strainer, and any remaining lymph node tissue was macerated using a syringe plunger. To conclude the procedure, 5 mL of FACS buffer was added to the 15 mL Falcons utilized during the digestion steps to wash any remaining cells and was also used to

rinse the cell strainer. The resulting cell suspension underwent centrifugation at 1200 RPM and 4°C for 5 minutes and was subsequently resuspended in 10 mL PBS before determining the viable cell count for further analysis.

Small/Large intestine: Approximately 10 cm of the small intestine or the entire colon was meticulously dissected, ensuring the removal of all fatty tissue, followed by the extraction of Peyer's patches using surgical scissors and forceps. The tissue was longitudinally opened, and PBS was employed to thoroughly cleanse the intestine, removing all contents. Fragments measuring 1.5 cm in length were then cut and placed in 50 mL Falcon tubes containing 30 mL of pre-warmed (to room temperature) epithelium dissociation buffer (3.1.2). The tubes were positioned horizontally on an orbital shaker set at 100 RPM and incubated at 37°C for 20 minutes. Subsequently, the intestine fragments were filtered through a metal mesh and returned to the tubes with 30 mL of fresh epithelium dissociation buffer for a second round of incubation on the orbital shaker. After the second incubation, the fragments underwent another round of filtration before being poured onto a plastic weighing boat, where they were finely minced with surgical scissors to obtain a pulp-like consistency. The minced tissue was then incubated in new 50 mL Falcon tubes with 10 mL of intestine enzyme mix (3.1.2), set on an orbital shaker at 200 rpm for an additional 20 minutes at 37°C. Following incubation, the samples were vortexed for 20 seconds and filtered through a 100 µm MACS smart strainer. The flow-through was collected, and the strainer was washed with 30 mL of intestine washing buffer (3.1.2). The resulting cell suspension was centrifuged at 1200 RPM for 10 minutes at 4°C. After discarding the supernatant, the cells were resuspended in 10 mL of intestine washing buffer and counted using trypan blue exclusion.

Skin: The protocol for skin cell isolation was adapted from (Delacher et al., 2020). One day prior to the ex vivo analysis, mice were anesthetized, and an approximately 2 x 5 cm area was shaved using an animal hair trimmer. Any remaining fur was removed from the shaved area using depilatory cream. Following euthanasia, the shaved skin area was carefully separated from the back, cut into small pieces with surgical scissors, and placed in GentleMACS C tubes filled with 10 mL of skin digestion mix. The tubes were processed in a GentleMACS Octo Dissociator using the program 37_C_Multi_H. After centrifugation, the supernatant was resuspended in 10 mL of PBS. The cell solution was then filtered through a 70 µm cell strainer, and the viable cell count was determined for subsequent analysis.

Spleen: see 3.2.1.2

Serum collection: A volume of 100 – 200 µL of blood was obtained by delicately piercing the vena fascialis with a sterile lancette, and the blood was collected in a Microvette® capillary blood collection tube with clotting activator/gel. To determine serum retention, the constructs were injected intravenously, and blood was collected 2 minutes after injection. Additional blood

collection time points included 6, 12, and 24 hours after injection. For experiments focusing on cytokine concentration in serum, a single blood draw was scheduled on day +6 after transplantation. Serum isolation was achieved by centrifuging the collection tubes at maximum speed for 10 minutes at room temperature. The serum was then carefully transferred into 0.5 mL reaction tubes and stored at -80°C for subsequent analysis.

3.2.3. Cytokine bead array

Approximately 2 cm fragemnts from liver and small intestine were dissected to generate organ lysates. The intestinal contents were thoroughly flushed with PBS until completely cleaned. These fragments were then placed in Precellys® tissue lysis tubes containing 200 µL PBS and homogenized in two rounds at 5,500 rpm for 45 seconds, with a rest time of 120 seconds between runs. Following homogenization, lysates were centrifuged at 20,000 RPM for 10 minutes, and the supernatant was collected and stored at -80°C until assay execution.

Cytokine concentrations were determined using the LEGENDplex™ mouse inflammation panel (13-plex). Standard 1:4 dilutions were prepared with assay buffer, and 25 µL of standards and their serial dilutions were loaded into the first column of the plate. Serum samples were plated separately from liver and intestinal lysates, following the manufacturer's protocol. For serum samples, 25 µL of Matrix C was added to the standard wells, while for organ lysates, the same volume of assay buffer was used. The mixture of fluorescence-encoded beads was thoroughly vortexed, and 25 µL was added to all samples and standards. Plates were covered with an adhesive film, wrapped in aluminum foil, and incubated on an orbital shaker at room temperature (800 RPM) for 2 hours. After incubation, wells were washed with 150 µL of washing buffer, and plates were centrifuged at 1,050 RPM and room temperature for 5 minutes. Pellets were resuspended with 25 µL of biotinylated detection antibodies and incubated on an orbital shaker at room temperature (800 RPM) for 1 hour. PE-labelled streptavidin, in an equal volume to the detection antibodies, was added, and the plates were incubated again at room temperature (800 RPM) for 30 minutes. Subsequently, wells were washed with 150 µL of washing buffer, followed by a final centrifugation step. After discarding the supernatant, 80 µL of washing buffer were added to all wells for acquisition using the flow cytometer. Analysis of the standard curve and cytokine concentrations of all samples was performed as instructed by the manufacturer, utilizing the LEGENDplex™ Data Analysis Software Suite.

3.2.4. IL-8, IL-10 and TGF-β ELISA

3.2.4.1. IL-8

To assess serum retention, collected serum samples were diluted with PBS and introduced to HT1080-Bcl2-TNFR2 cultures, each containing $2x10^4$ cells in 96-well plates filled with fresh cRPMI to minimize background IL-8 in the medium overnight. Subsequently, supernatants were harvested and subjected to analysis using a BD OptEIA™ Human IL-8 ELISA Set, following the manufacturer's protocol.

3.2.1.1. IL-10 and TGF-β

To quantify IL-10 and TGF-β levels, cell culture supernatants obtained from suppression assays (refer to 3.2.2) underwent analysis using the Invitrogen IL-10 Mouse Instant ELISA™ Kit and TGF beta-1 Mouse ELISA Kit, following the manufacturer's protocol.

3.2.2. Miniature suppression assay

Four days post-injection of NewSTAR2 or the isotype control antibody in B6a (with three mice per group), spleens were dissected, and cell suspensions were prepared, as detailed in section 3.2.2. These splenocytes from each group were pooled together, and Tregs were isolated using the Miltenyi Biotec mouse $CD4^+CD25^+$ Regulatory T Cell Isolation Kit. Subsequently, Tregs were maintained in cRPMI until plated for coculture. Concurrently, a spleen from a B6 mouse was dissected, and total T cells (referred to as responder cells or Tcons) were isolated using the Dynabeads™ Untouched™ Mouse T Cells Kit. Responder cells ($2x10^7$) were labeled with the CellTrace Violet (CTV) Cell Proliferation Kit from Invitrogen. CTV-labeled responder T cells were resuspended in cRPMI, with or without CD3/CD28 activator beads (Dynabeads™ Mouse T-Activator CD3/CD28 for T-Cell Expansion and Activation), following the manufacturer's protocol. For plating, $5x10^4$ responder T cells were placed in 96-well round-bottom plates with enriched Tregs from NewSTAR2-stimulated mice or unstimulated Tregs from isotype control-treated mice at 1:1, 2:1, 4:1, or 8:1 Tcon:Treg ratios. The number of responder T cells was kept constant, and Tregs were titrated down to achieve the specified ratios, resulting in 5 x, 2.5 x, 1.25 x, 0.625 x 10^4 Tregs per well, respectively. Each sample was plated in triplicate, and positive and negative controls were included. Positive control wells contained only responder T cells and activator beads, maximizing proliferation in the absence

of Tregs. Negative control wells contained only responder T cells without activator beads and with 10 ng/mL IL-7. After incubation for 3 days at 37°C, the plates were subjected to flow cytometry analysis. A modified version of the suppression assay was also conducted in a 96-well plate with a 1.0 µm transwell insert, ensuring that Tregs and responder cells did not make direct physical contact.

3.2.3. In vivo bioluminescence imaging

B6a.FoxP3.Luci.DTR or B6a mice received an intraperitoneal injection of 300 mg/kg bodyweight of D-Luciferin. Subsequently, they were anesthetized by placing them in the induction chamber of an IVIS Spectrum in vivo imaging system, using a 2% v/v isoflurane level for 10 minutes before transferring them to the imaging chamber for bioluminescence image acquisition. The exposure time for reporter mice was set at 2 minutes, while for B6a-transplanted mice, it was 5 minutes. A binning factor of 2 was applied for image acquisition, and efforts were made to image mice from the same group together. Image analysis was performed using Living Image version 4.5.5. Initially, a global scale was set for all images within the same experiment. Subsequently, the average radiance signals (p/s/cm²/sr) were extracted from a region of interest (ROI) containing the ventral view of the entire mouse. To account for background signals, measurements were taken at baseline in the empty imaging chamber and then subtracted from the respective position for each group and time point independently in each experiment. The initial baseline signals, recorded before the injection of agonists, were considered the steady-state maximum signal. Consequently, any changes over time were calculated as the relative change at each time point over baseline levels.

3.2.4. MHC major-mismatch allo-HCT GvHD model

Female B6, B6a, or B6a.FoxP3.Luci.DTR mice aged approximately 8-12 weeks were administered 140 µg of TNFRSF agonists or an isotype control antibody either 4 or 5 days before transplantation. From this juncture, mice underwent daily weight assessments and clinical symptom evaluations. On day 0, host mice underwent myeloablative total body irradiation (TBI) with an effective dose of 9 Gy using a Faxitron CP-160 X-ray system. Within the subsequent 4 hours, $6x10^5$ total T cells from the spleen and $5x10^6$ bone marrow cells (refer to 3.2.1.2 and 3.2.2) from FVB or FVB.L2G85 mice were i.v. injected into the anesthetized host mice. A 1:1 mixture of T cells and bone marrow cells was prepared in PBS and administered

in a total volume of 200 µL per mouse. Bone marrow control mice received bone marrow cells in a total volume of 100 µL PBS per mouse. Irradiation controls did not receive any cell injections after irradiation. Mice were closely monitored for 40 days post-transplantation and euthanized if the humane endpoint was reached as described in Table 3.

Table 3. Clinical score for GvHD scoring

	Score					
	0	**1**	**5**	**10**	**15**	**20**
Weight change	Increase	0-4.9%	5-9.9%	10-19.9%		≥20%
General Condition	Smooth/glossy fur, Clean mouth and nose, Clear eyes	Poor or excessive hygiene (Pathologic grooming)	Scruffy, disordered fur, dirty mouth/nose, sunken eyes, Increased muscle tone	Dirty fur AND sticky/wet mouth/nose, abnormal posture, sunken eyes, higher muscle tone		Cramps, paralysis, abnormal breathing sounds, cold animal
Behavior	Normal	Low deviation from normal	Uncommon behavior, limited movement, involuntary movements (hyperkinetic)	Isolation, lethargie, pronounced hyperkinetic, coordination problems		Repetitive painful sounds when handling, self-amputations
GvHD specific symptoms			Licking or scratching skin areas 1x/min or less, Conjunctivitis in one eye or slight in both eyes, poorly formed stool, anal mucosa swollen	Licking or scratching skin areas >1x/min, strong conjuctivitis in both eyes, blisters in bare places (nose, paws)	Strong diarrhea with black and slimy faeces (tarry or bloody), blisters in the body, paralysis	

3.2.5. Immunofluorescence microscopy of histological sections

Secondary lymphoid organs and segments of the small intestine were carefully dissected and embedded in Tissue-Tek cryomolds filled with Tissue-Tek OCT compound, followed by rapid freezing on dry ice. Subsequently, 5 µm-thick fine sections were sliced using a cryostat and transferred onto glass slides. Slides were allowed to air-dry at room temperature, and the tissue perimeter was delineated with a hydrophobic PAP-pen. The samples were fixed using 200 µl of Fix/Perm solution (1:4 concentrate:diluent ratio) and incubated at 4°C for one hour. Afterward, slides underwent three PBS washes in a glass cuvette every 2 minutes at room temperature, followed by blocking with 150 µl of 2% normal rat serum (NRS) in PBS for 15 minutes at 4°C. Subsequent to three additional PBS washes, 200 µl of primary antibodies in blocking solution were applied and incubated overnight at 4°C. Before introducing the secondary antibodies and undergoing a 2-hour incubation at 4°C, the slides underwent the same washing regimen. A final series of five washes were performed to eliminate excess antibodies, followed by the addition of one drop of Vectashield® antifade mounting medium with DAPI. Coverslips were placed over the samples and sealed with Entellan®.

Observations were conducted using either a Zeiss Imager fluorescent microscope or a Zeiss 780 laser scanning confocal microscope. Images were captured with an AxioCam MRm camera and analyzed using Axio Vision 4.8 or ImageJ.

3.2.6. H&E staining of histological samples

The dissected organs underwent fixation in 4% paraformaldehyde (PFA) for a minimum of 24 hours before being embedded in paraffin and sectioned onto microscopy slides. The embedding and staining processes were executed by an experienced pathologist, Prof. Dr. Maike Büttner-Herold, from the University Hospital Erlangen, who was blinded to the experimental groups. A clinical score, determined by factors such as crypt apoptotic body counts, inflammation intensity, structural alterations of crypts, loss of Paneth cells, and the presence of giant cells, was assigned to each small intestine sample.

3.2.7. Flow cytometry

For flow cytometry analysis, cell suspensions (approximately $1x10^6$, or at least $1x10^5$ for in vitro assays) were resuspended in 100 µl FACS buffer in 96-well V-bottom plates. The plates

were then centrifuged at 1200 rpm and 4°C for 5 minutes, and the supernatant was carefully discarded. Following this, cells were blocked with 100 µl of normal rat serum (NRS) (1:20) in PBS for 5 minutes at 4°C. Next, 100 µl of the antibody mix in PBS was added to the corresponding wells and incubated for 30 minutes at 4°C. Afterward, plates were centrifuged as before, and cells were resuspended in 200 µl of FACS buffer. OneComp eBeads™ were stained with antibody mixes for compensation, and fluorescence minus one (FMO) controls were utilized to correct for spillovers between the spectra of the fluorophores. For intracellular stainings, cells were resuspended in 100 µl of Fix/Perm (1:4) and incubated for 30 minutes at 4°C before adding 150 µl of 1:10 eBioscience Permeabilization Buffer (10x) in double-distilled water. Intracellular antibody mixes were added as stated above but were prepared in diluted Permeabilization Buffer. Finally, plates were sampled using an Attune NxT Flow Cytometer, and the data were analyzed with FlowJo version 10.8.1.

3.2.8. Statistical analysis

The data are represented as means ± standard deviation (SD). Statistical differences between treatment groups were analyzed using unpaired t-tests or (either one or two way) ANOVA, with a statistical significance level of $\alpha=0.05$, unless otherwise specified. Data visualization and statistical analysis were conducted using GraphPad Prism version 9.

4. Results

4.1. TNFR2, GITR and DR3 phenotyping

4.1.1. Coexpression of TNFR2 and GITR is shared between T cell subsets but higher frequencies were seen in Tregs as well as higher expression levels

TNFR2 and GITR are well documented as high-expressing markers on Tregs while DR3 is expressed on activated T cells but has also role in Treg differentiation (Chopra et al., 2016; Ephrem et al., 2013; B.-S. Kim et al., 2015; Mavers et al., 2019; Nishikii et al., 2016; Salomon et al., 2018). However, the expression of other TNFSF receptors is less delineated, particularly when compared to other immune cell populations, such as conventional CD4 and CD8 T cells. Given the relevance of a direct comparison for developing strategies to selectively target Tregs with TNFSF agonists while limiting off-target effects on other immune cells, we conducted a phenotypic analysis using flow cytometry to assess the expression of TNFR2, GITR, and DR3 on various T cell populations in the spleens of B6 wild-type mice. The flow cytometric gating strategy is illustrated in **Figure 4A**. As anticipated, TNFR2 was prominently expressed in approximately 80% of Tregs, contrasting with around 25% positivity in CD4 and CD8 T cells. GITR demonstrated widespread expression across all T cell populations, with the highest frequency observed in Tregs (over 90%), followed by CD4 conventional T cells (Tcons) and CD8 T cells (approximately 80% and 60%, respectively). In comparison, DR3 exhibited the lowest expression among these markers, with approximately 10-15% positivity in each T cell subset. However, this analysis did not encompass double or triple expressers.

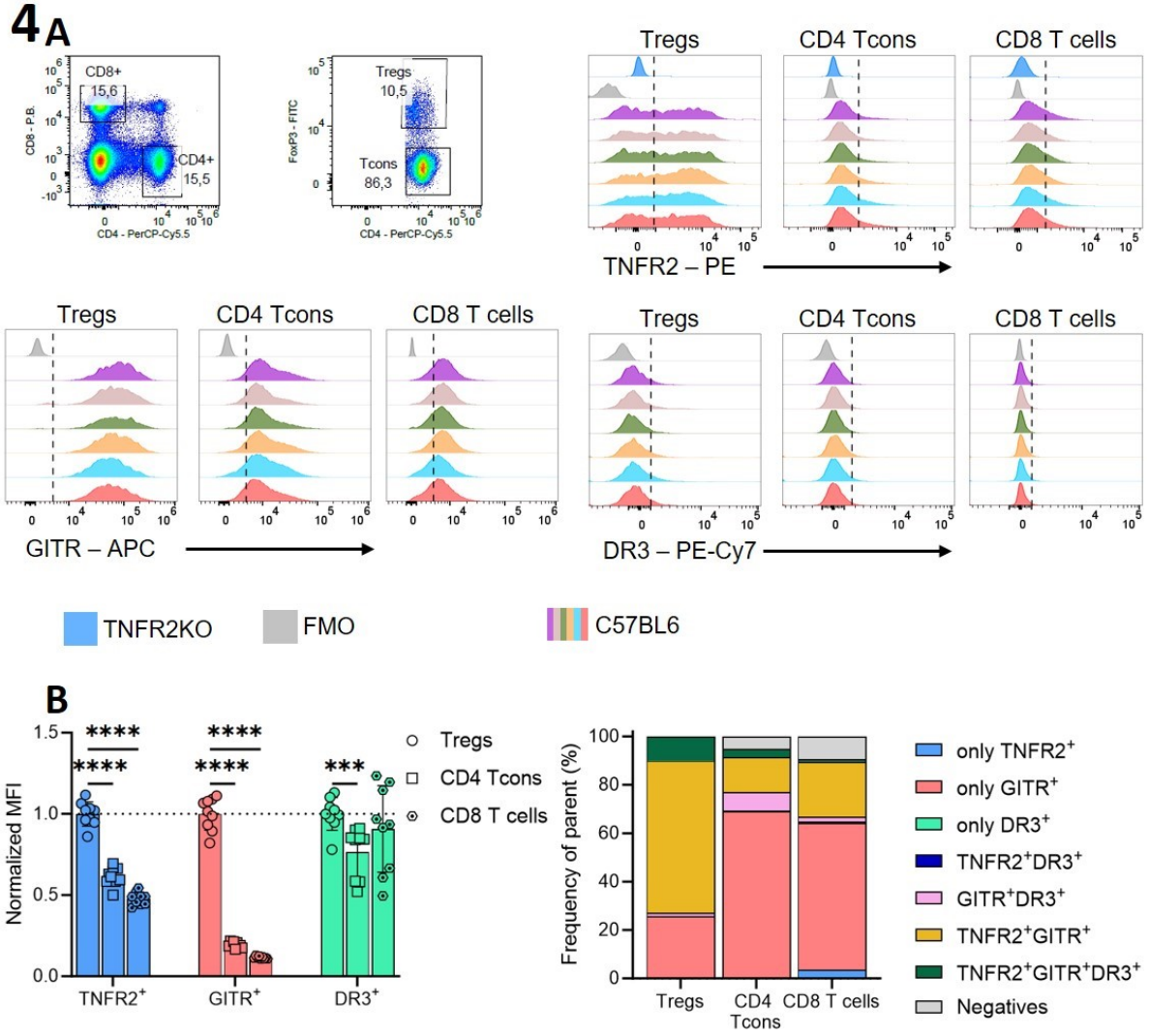

Figure 4. Higher number of cells coexpressing GITR and TNFR2 are found in Tregs than in Tcons subset in B6. A: Representative dot plots of flow cytometry analysis from splenocytes to identify CD4 Tcons, CD8 T cells and Tregs. Pregated from singlets and viable cells. Histograms from the indicated populations on top gated as described before. **B** Left: Frequency of TNFRSF single and coexpressing-cells within each T cell subset. Right: MFI from the depicted markers on each given T cell subset. Values normalized against expression on Tregs. Each datapoint represents one biological replicate. Data shown as mean +/- SD.Two-way ANOVA, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; not significant comparisons not shown.

The distribution of single, double, and triple expressers (**Figure 4B, right panel**) illustrated that the majority of Tregs coexpressed TNFR2 and GITR or TNFR2, GITR, and DR3, with only about 20% being GITR$^+$. In CD4 Tcons and CD8 T cells, frequencies of TNFR2$^+$GITR$^+$ were half of those within the Treg subset, and GITR+ appeared to be the most prominent subpopulation, followed by GITR$^+$DR3$^+$ cells. DR3 alone was not observed in any T cell subset, nor was it coexpressed with TNFR2 in Tregs. For CD4 Tcons and CD8 T cells, a subpopulation

of GITR^{+}DR3^{+} was identified, as well as triple expressers with TNFR2, while the former was absent in Tregs.

To assess the intensity of receptor expression across T cell subsets, we compared the mean fluorescence intensity (MFI) within the single positive subpopulations for each marker, normalizing it to that within the Treg population (**Figure 4B, left**). Tregs consistently exhibited higher expression levels of TNFR2 and GITR compared to CD4 Tcons and CD8 T cells. TNFR2 expression in CD4 Tcons and CD8 T cells was approximately half as strong as in Tregs. Strikingly, GITR intensity was roughly one-tenth in CD4 Tcons and CD8 T cells compared to Tregs. DR3 intensity in CD8 T cells was very similar to that in Tregs, while CD4 Tcons displayed the lowest intensity among the three subsets. The results underscored the consistently high frequencies and expression levels of TNFR2 and GITR on Tregs relative to CD4 Tcons and CD8 T cells. Although DR3 demonstrated more subtle differences, its expression did not correlate with higher frequencies in CD4 or CD8 T cells. Importantly, none of these receptors emerged as exclusive Treg markers, and their distinct expression patterns could influence their targeting across different T cell populations.

4.1.2. Design of molecules targeting TNFR2, GITR and DR3

Recent literature affirms that although TNFR2 is expressed by various T cell subsets and other myeloid populations, systemic administration of TNFR2 agonists primarily positively impacts regulatory T cell (Treg) numbers and function (Chopra et al., 2016; Vargas et al., 2022). Recent work of our group and others supports that also the TNFRSF DR3 can expand and activate Tregs (Nishikii et al., 2016). Moreover, GITR emerges as a promising candidate for Treg specificity with minimal off-target effects on other T cell compartments (Nocentini & Riccardi, 2009; Shimizu et al., 2002). For this study, ligand-based agonists for TNFR2, GITR, and DR3, incorporating immunoglobulin heavy chain domains, were designed, synthesized, and evaluated in the lab of our collaboration partner Prof. Dr. Harald Wajant. In this work we compared the prototypic TNFR2 agonist, termed STAR2, that our group had previously reported (Chopra et al., 2016), with a next-generation construct, termed NewSTAR2. Point mutations to avoid TNFR1 binding by TNFR2 agonists STAR2 and NewSTAR2 were introduced by substituting D221N and A223R in the amino acid sequence of trimeric mTNA-α to induce steric clash (Chopra et al., 2016), and additional point mutations to eliminate binding to FcγR were introduced by substituting aspartic acid to alanine in position 265 (D265A) in the IgG sequence of NewSTAR2. Altogether this and the other constructs described in the following paragraph fused to an antibody domain should posses clear advantages over simple

ligand-based recombinant proteins due to their higher stability through their IgG backbone, better biodistribution by recycling through FcRn and lacking FcγR-mediated clearance, which is what we set to test in our work.

As discussed in the earlier sections of this work, TNFSFRs require clustering of the receptors to induce proper signaling in the inside of the cell (**Fehler! Verweisquelle konnte nicht g efunden werden.**). Consequently, the required valences for proper activation of TNFR2, GITR, and DR3 agonists were established: tetrameric GITRL, hexameric TL1a, and hexa- or nonameric TNF80 molecules (considering TNFR2's need for clustering of at least two TNF trimers for effective activation) were affixed to the heavy chain of immunoglobulin domains (**Figure 5A**). To preclude FcγR binding, point mutations eliminating this interaction were introduced, substituting aspartic acid with alanine at position 265 (D265A). Additional point mutations were incorporated to prevent TNFR1 binding by TNFR2 agonists STAR2 and NewSTAR2, achieved by substituting D221N and A223R in the trimeric mTNA-α sequence to induce steric clash (Chopra *et al.*, 2016). The immunoglobulin domain of NewSTAR2 comprised a full anti-human CD95 antibody derived from antibody E09, as this is irrelevant in mice (Chodorge et al., 2012). Meanwhile, GITR and DR3 agonists were attached to the C-terminus of Fc immunoglobulin domains devoid of an antibody-binding domain (Fab). To facilitate capture and purification, FLAG tags were introduced to GITR and DR3 agonists. Notably, Fc(DANA)-muGITRL and Fc(DANA)-muTL1A Fc domains exhibit an inability to bind both mouse (D265A) and human (N297A) FcγR (designated as DANA).

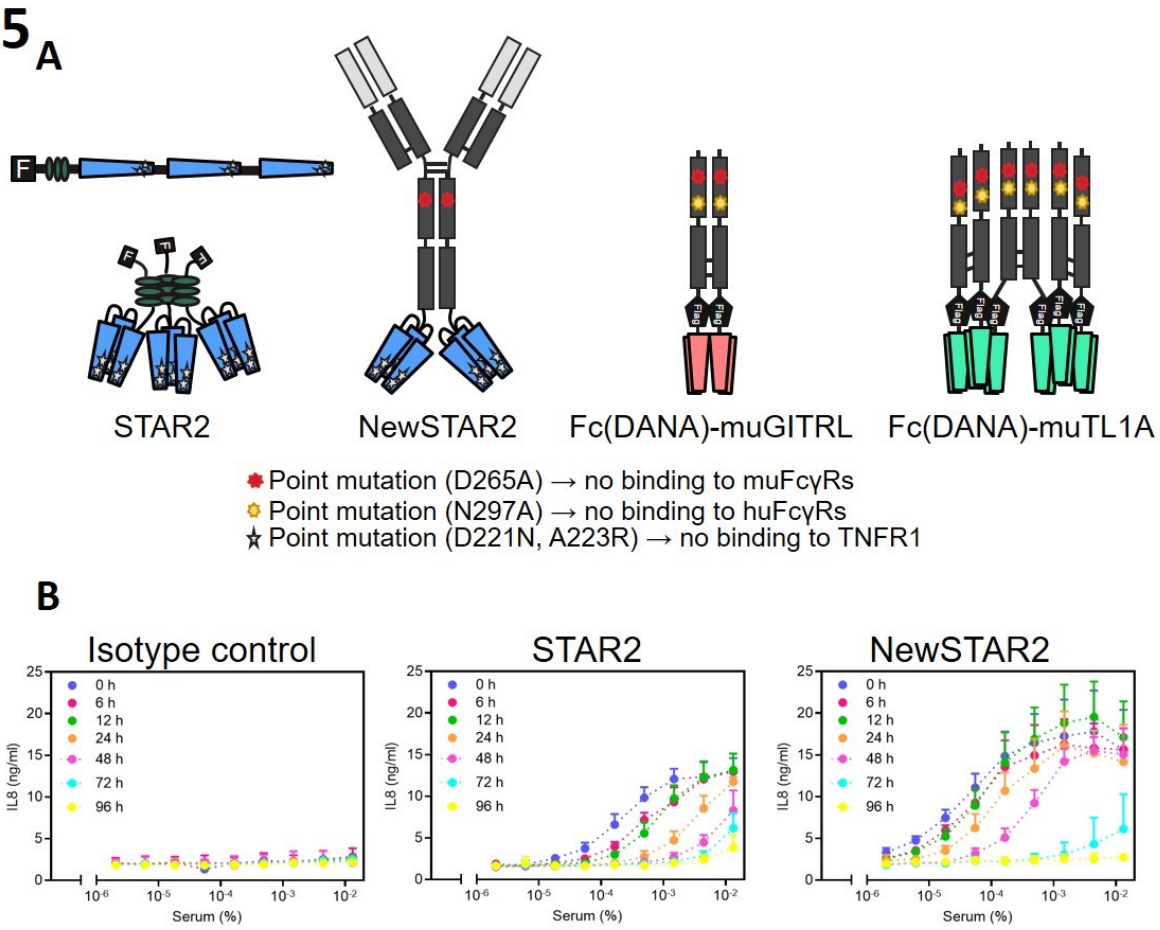

Figure 5. Domain architecture of TNFRSF ligand-based agonists against TNFR2, DR3 and GITR, and enhancement of serum retention with fused antibody backbone of TNFR2 agonist. A: Depictions of the different TNFRSF agonists. **B**: Serum half-life can be extended when an engineered TNFR2 agonist is fused to an IgG heavy chain antibody portion. Concentration of IL-8 in the supernatant of HT1080-Bcl2-TNFR2 cell cultures when stimulated with diluted serum samples from blood taken at the indicated time points after injection in B6a.FoxP3.Luci.DTR mice. Mice were injected with 50 µg of STAR2, 75 µg of NewSTAR2 or PBS. Data pooled from 3 mice per group.

4.2. In-depth analysis of NewSTAR2 in vivo and in vitro

4.2.1. An antibody domain extends TNFR2 agonist half life

To establish whether NewSTAR2 would be a superior TNFR2 agonist, we comprehensively investigated this construct in comparison to the preceding generation agonist, STAR2, elucidating the rationale behind incorporating antibody domains into ligand-based TNFRSF agonists. Subsequently, we conducted selected in vitro and in vivo experiments to compare

the impact of NewSTAR2, Fc(DANA)-muGITRL (GITRL), and Fc(DANA)-muTL1A (TL1A) in mice.

To assess the enhanced stability conferred by an IgG heavy chain antibody domain fused to the TNFR2 agonist, we administered STAR2 or NewSTAR2 intraperitoneally (i.p.) to mice. Blood samples were collected prior to injection and at defined time points specified in **Figure 5B**. Subsequently, we extracted serum from these blood samples, and, as a functional read-out, diluted serum at different concentrations to stimulate HT1080-Bcl2-TNFR2 cells expressing IL-8 after TNFR2 is activated. The measurement of IL-8 in the supernatant served as an indirect indicator of the circulating and active TNFR2 agonist at each time point. IL-8 concentrations not only demonstrated higher levels in the serum from mice administered NewSTAR2 but also revealed sustained detection at elevated concentrations, illustrated by the nearly full Gaussian distribution of the curves, even at 48 hours post-injection. In contrast, STAR2 exhibited a decline after only 12 hours, as indicated by the values in the curve, displaying the initial exponential growth of the distribution. The half-life of NewSTAR2 was consequently higher than that of STAR2, advocating for extended serum retention. This characteristic eliminates the need for repetitive dosing to achieve therapeutic levels, a requirement associated with STAR2 (Chopra *et al*., 2016). Additionally, while a nonameric agonist like STAR2 was known to achieve the required clustering of TNFR2-trimers similar to membrane-bound TNF to activate TNFR2, our findings indicate that a hexameric agonist like NewSTAR2 was equally suitable for the full activation of TNFR2.

4.2.2. NewSTAR2 is a potent TNFR2 agonist targeting Tregs

To test potential differences in Treg activation with a hexameric TNFR2 agonist (NewSTAR2), we stimulated $2x10^5$ murine splenic total T cells in vitro with equimolar concentrations of STAR2 and NewSTAR2. After 3 days of culture, both agonists led to a roughly 2-fold increase inTreg frequency. The Treg expansions induced by STAR2, NewSTAR2 or a low dose (l.d.) IL-2 as positive control were comparable in total T cell cultures derived from WT B6 mice (**Figure 6A**). Stimulation of splenic total T cells from B6 TNFR2KO mice confirmed that the expansion of Tregs with STAR2 or NewSTAR2 is dependent on TNFR2 expression, underscoring the effectiveness and specificity of these agonists. Notably, l.d. IL-2 somehow resulted in a stronger Treg expansion in TNFR2KO cultures than in WT cultures.

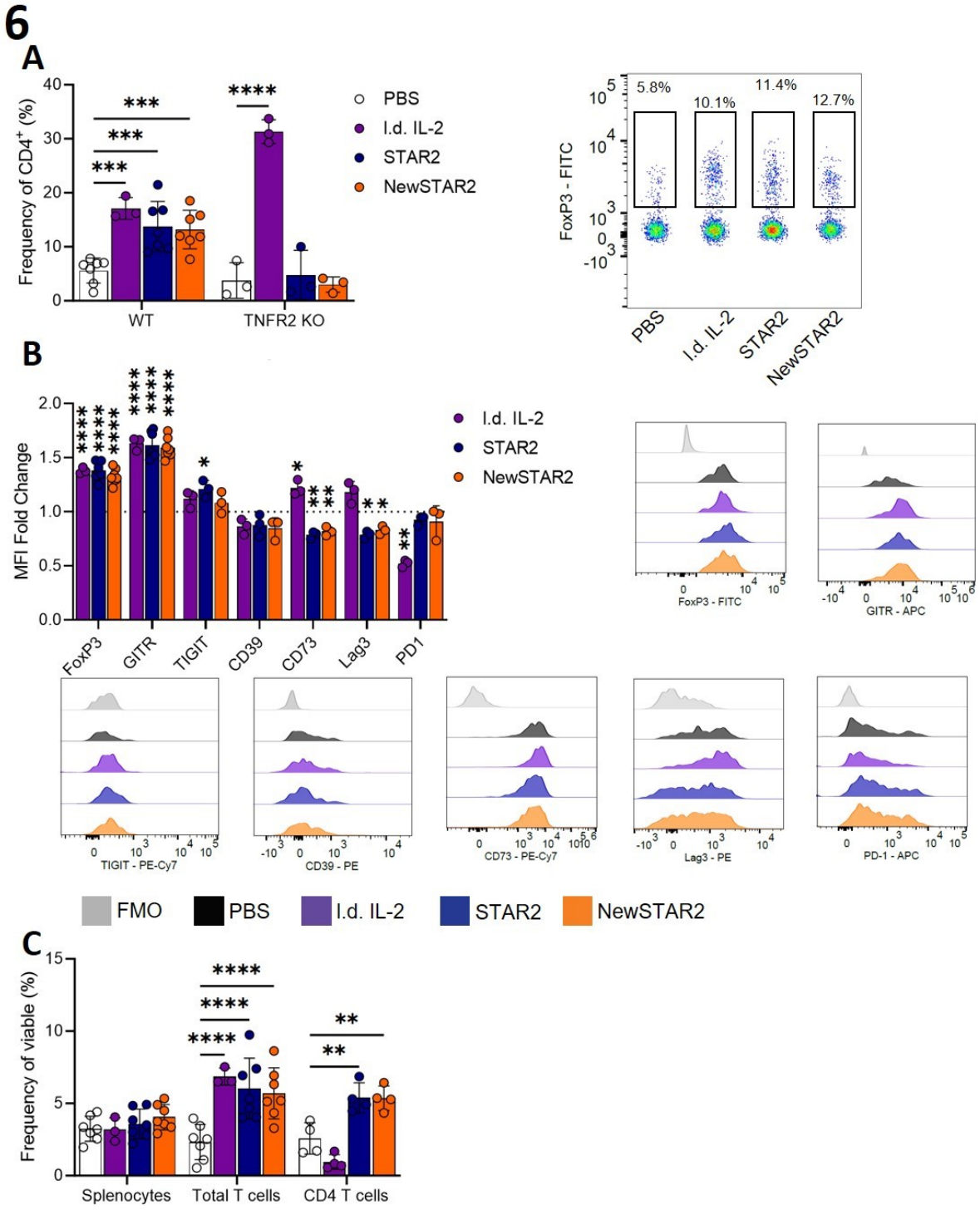

Figure 6. STAR2 and NewSTAR2 induce TNFR2-dependent Treg expansion in T cell cultures comparable to low-dose IL-2. A Left: 2x10^5 enriched total T cells from B6 mice were stimulated with equimolar concentrations (1.67 nM) of STAR2, NewSTAR2, 40 IU/mL (low dose) IL-2 or PBS and cultured for 4 days. Right: representative dot plots showing Treg (FoxP3$^+$) frequency within CD4 population. Pregated from singlets, viable cells, CD4$^+$. **B** Upper left: MFI from the depicted markers on Tregs stimulated with the different TNFR2 agonists or I.d. IL-2. Values normalized against their respective expression in unstimulated cultures (PBS). Upper right and lower: representative histograms of the analyzed markers on Tregs. **C** Comparison of frequency of Tregs within viable cells in mixed splenocytes, enriched total T cells or enriched CD4 T cell cultures stimulated with STAR2, NewSTAR2, I.d. IL-2 or

PBS for 4 days. Each datapoint represents one biological replicate. Data shown as mean +/- SD.Two-way ANOVA (**A,C**), unpaired t-Test (**B**) *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; not significant comparisons not shown.

Subsequently, we examined the expression profile of “Treg activation markers” and observed a similar upregulation of FoxP3, GITR and T cell immunoreceptor with Ig and ITIM domains (TIGIT) upon treatment with STAR2 and NewSTAR2, respectively, (although increased TIGIT upregulation did not reach significance upon NewSTAR2 treatment). Simultaneously, both TNFR2 agonists, but not low dose IL2, induced a downregulation of CD73 and Lymphocyte-activation gene 3 (Lag3) (**Figure 6B**). In the comparison of STAR2 or NewSTAR2 agonist treatments in whole splenocyte in vitro cultures, we observed a significant increase in Tregs when comparing Treg frequencies among all T cells (CD4 and CD8) or in all CD4 T cells. However, this difference was not apparent when comparing Treg numbers with the total numbers of splenocytes (**Figure 6C**). Interestingly, for low dose IL-2 treatment an increase in Treg frequencies became only apparent when compared to total T cells but was not obvious when compared to total splenocyte or total CD4 T cell numbers. These data suggest that STAR2, NewSTAR2 and IL2 also affect other cell populations beside T cells and, by altering the cellular composition in total splenocytes, obscure the agonistic effect on the relatively rare Tregs subpopulation, at least in vitro.

4.2.3. NewSTAR2 outperforms STAR2 in inducing Treg expansion in vivo

To compare the capacity of the novel IgG-based TNFR2 hexavalent agonist NewSTAR2 with the previously published nonavalent STAR2 to expand and activate Tregs in vivo (Chopra et al., 2016), we administered 140 µg i.p. of STAR2 or NewSTAR2 to wild type mice and analyzed spleens of B6 WT mice four days after injection. A single dose of NewSTAR2 induced a robust increase in splenic Tregs, with no effect on CD4 Tcons frequencies and a marginal decrease of CD8 T cells (**Figure 7**). Mice treated with NewSTAR2 but not STAR2 upregulated several “Treg activation markers”, including CD39, TIGIT, GITR, 4-1BB, CD73, Lag3 and PD1. Compared to in vitro assays, more markers were upregulated, and stronger upregulation was evident, as seen in the almost 2-fold increase in TIGIT, Lag3 and PD1.

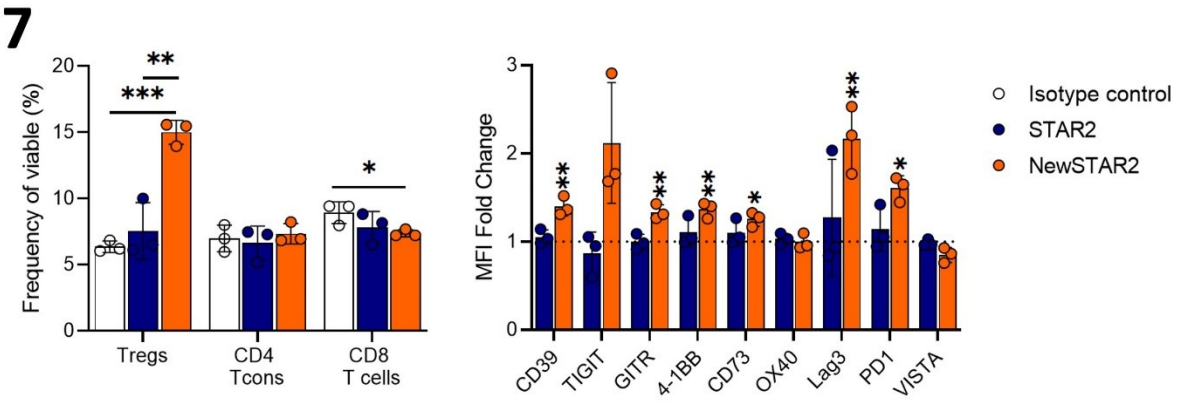

Figure 7. One single dose of NewSTAR2 is enough to expand regulatory T cells and induce upregulation of Treg „activation markers". B6 mice were injected i.p. with 140 µg of STAR2, NewSTAR2 or an isotype control antibody. 4 days later, spleens were dissected and their single cell suspensions were analyzed by flow cytometry. Left: T cell subsets frequencies within viable from each treatment group. Right: MFI from the depicted markers on Tregs stimulated with STAR2 or NewSTAR2. Values normalized against their respective expression on Tregs from mice given isotype control antibody. Each datapoint represents one biological replicate. Data shown as mean +/- SD.Two-way ANOVA, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; not significant comparisons not shown.

Further in vivo assays in B6a.FoxP3.Luci.DTR mice, from which bioluminescence signals were measured in vivo, confirmed that a single dose of NewSTAR2 was able to induce a systemic Treg expansion, reaching an almost 4-fold increase four days after stimulation (**Figure 8A**). In line with the serum retention experiment, it is evident that NewSTAR2 induces more potent TNFR2 activation, as demonstrated in our recent publication (Vargas et al., 2022)) and also achieves Treg expansion that lasts almost two weeks even after concentration in serum has reduced strongly (after 72 hours). STAR2 failed to increase any Treg signals with a single dose, and its relative change throughout the experiment closely resembled that of mice given isotype control antibody.

Flow cytometry analysis of different organs on day 4 after injection (the apparent peak of Treg increase) confirmed systemic Treg expansion in secondary lymphoid organs, as well as, though sometimes less visibly, in non-lymphoid tissues such as the small intestine, liver, colon and lung (**Figure 8B**). Treg frequencies within the CD4 population were not uniformly increased; they were found to be higher in blood, lungs and colon followed by lymph nodes, spleen (as shown later in **Figure 9A**) and bone marrow, challenging the idea that only circulating Tregs are expanded.

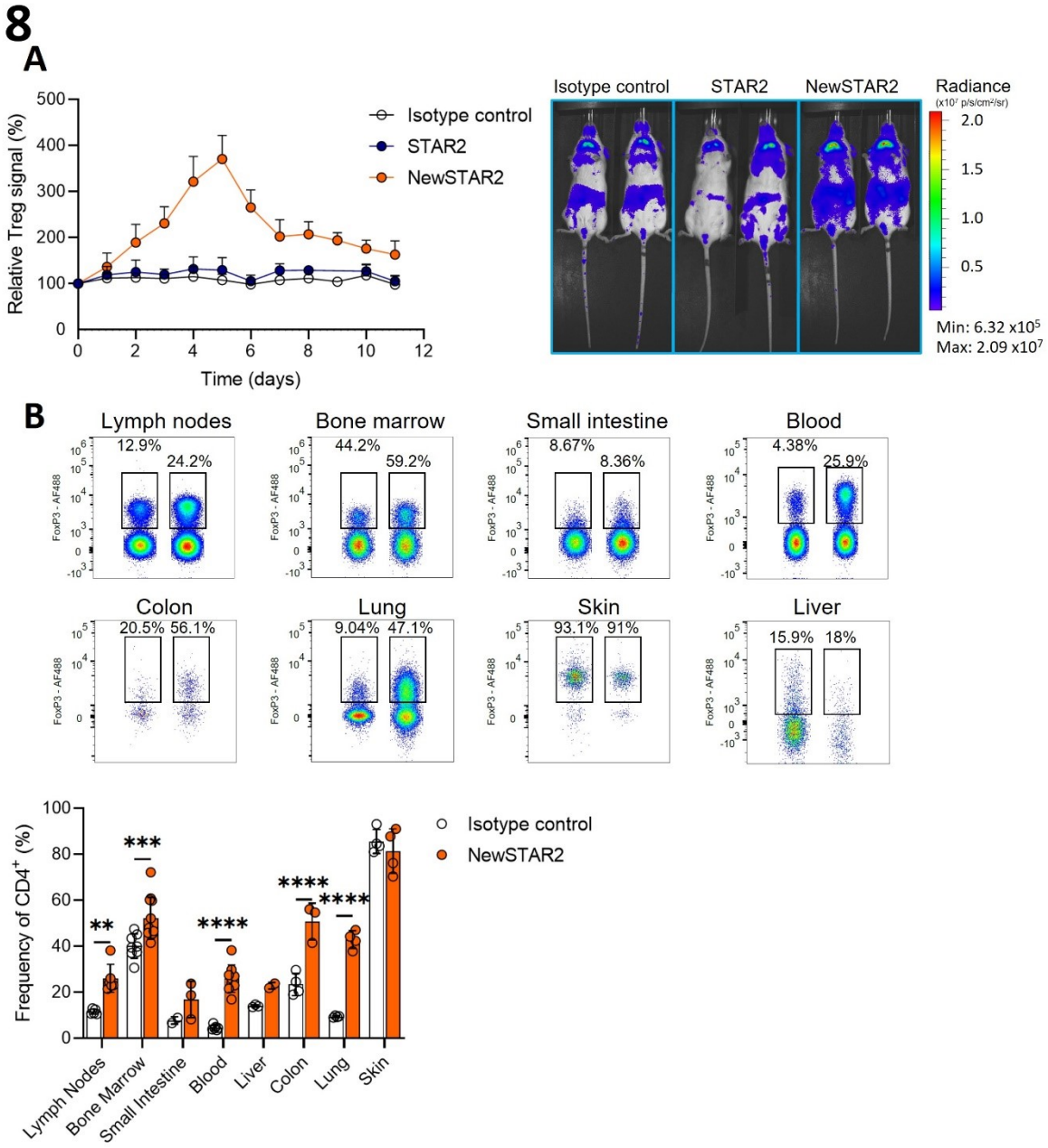

Figure 8. Systemic endogenous Treg expansion by NewSTAR2 peaks 4-5 days after injection and remains higher than baseline for 2 weeks. A Left: Relative average radiance change (day 0 baseline) from Treg in B6a.FoxP3.Luci.DTR injected i.p. on day 0 with 140 µg of STAR2, NewSTAR2 or isotype control antibody. In vivo bioluminescence imaging from ventral view was taken on the shown time points. Right: Representative bioluminescence images from the different treatment groups on day 4 after injection. **B** Upper rows: Representative dot plots depicting the Treg frequencies within $CD4^+$ in different organs on day 4 after injection in B6 mice. Gates and percentages on the left and right represent isotype control and NewSTAR2 respectively. Lower graph: Treg frequency within $CD4^+$ population in the organs shown in the upper panel comparing isotype control and NewSTAR2 on day 4 after injection. Each datapoint represents one biological replicate. Data shown as mean +/- SD.Two-way ANOVA, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; not significant comparisons not shown.

4.2.4. NewSTAR2-treated activates and increases Tregs more than two-fold

Having established the superiority of NewSTAR2 and the ineffectiveness of a single dose of STAR2 on the Treg compartment, subsequent experiments were exclusively performed with NewSTAR2. As described in preceding sections, organs were dissected and analyzed with flow cytometry four days after injection in B6 WT mice. Spleens of treated mice displayed a substantial increase inTreg frequencies within the total CD4 population, rising from the baseline of 15% to 40% (**Figure 9A**). Absolute Treg numbers exhibited a consistent increase of approximately 2.8-fold compared to untreated mice. The CD4 Tcon population, encompassing cells that are not Tregs by definition, showed a clear decrease in frequency within the total CD4 compartment. However, the absolute numbers indicated a minor decrease, yet much smaller than the effect of increased Tregs. In contrast, absolute CD8 T cell numbers remained unaltered.

Given the lack of unanimous accepted markers to define activated Tregs, we considered several markers in this work to demonstrate an activated Treg phenotype. These “Treg activation markers” comprised inhibitory checkpoint molecules like programmed cell death protein-1 (PD-1/CD279), one of its ligands PD-L1, TIGIT and Lag3 which were upregulated in splenic Tregs from treated mice (**Figure 9B**). Other receptors, for instance, ectoenzyme CD39, important for adenosine regulation, GITR and intercellular adhesion molecule 1 (ICAM1), required for Treg-Tcell immunological synapse formation but also a costimulatory marker of

Tregs (Deane et al., 2012; Gottrand et al., 2015), were also upregulated upon NewSTAR2 in vivo treatment.

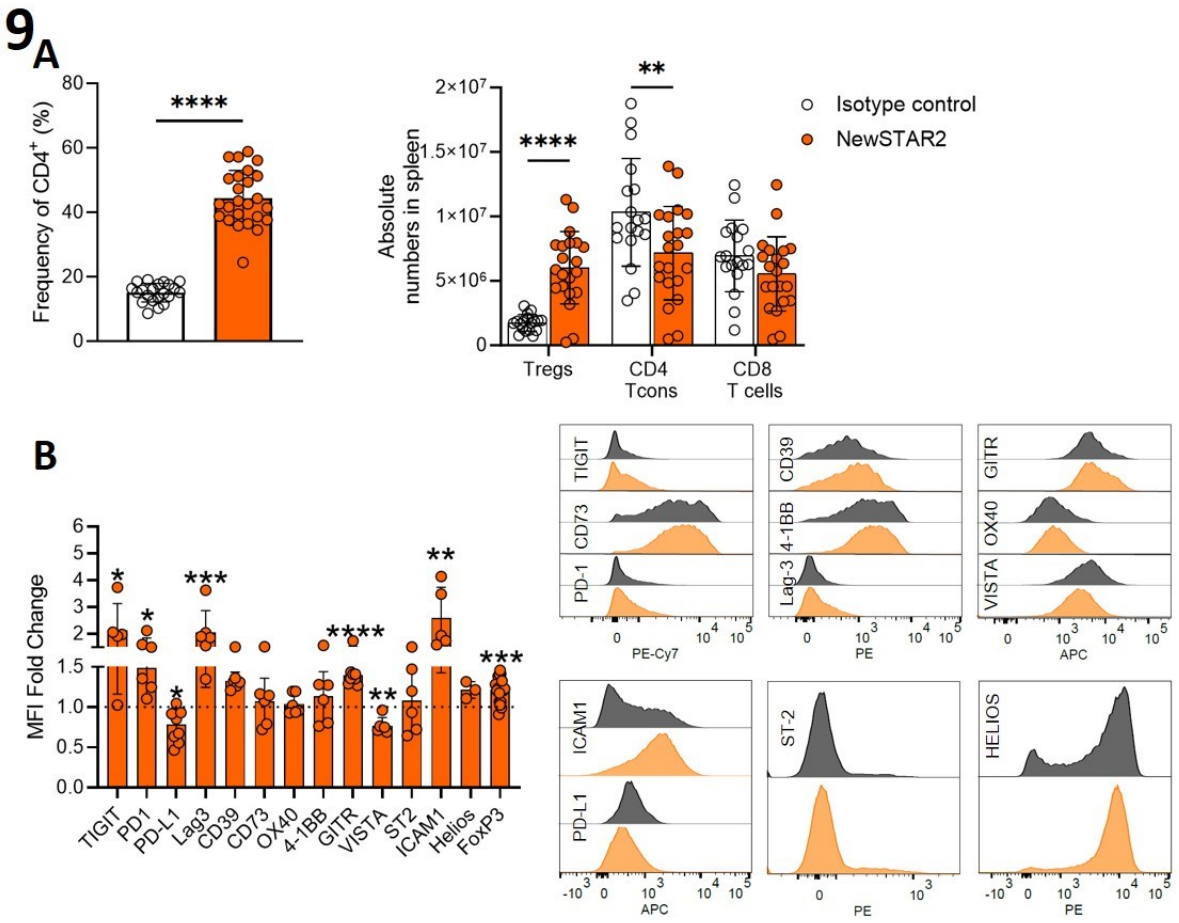

Figure 9. In-depth analysis of NewSTAR2-stimulated splenocytes shows increase of absolute Treg numbers and upregulation of several „activation markers". A Left: Treg frequency within CD4+ population in spleen comparing isotype control and NewSTAR2 on day 4 after injection. Right: Absolute number of T cell subsets in spleen on day 4 after injection. **B** Left: MFI from the depicted markers on splenic Tregs stimulated with NewSTAR2. Values normalized against their respective expression on Tregs from mice given isotype control antibody. Right: Representative offset histograms from the different markers on Tregs from isotype control-stimulated (black) or NewSTAR2-stimulated (orange) mice from panel A. Each datapoint represents one biological replicate. Data shown as mean +/- SD.Two-way ANOVA (**A**), unpaired t-Test (**B**) *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; not significant comparisons not shown.

4.2.5. NewSTAR2 amplifies the myeloid cell frequencies in secondary lymphoid organs

Analyzing mRNA expression data from the ImmGen Gene Skyline database after RNA sequencing of TNFR2 (Supplementary figure S1) revealed that various myeloid and lymphoid cells exhibit high TNFR2 expression levels. In comparison to splenic Tregs, splenic

macrophages, alveolar macrophages, splenic neutrophils, Ly6C^{high} blood monocytes and Ly6C^{low} blood monocytes displayed 3.6-, 3.3-, 6.2-, 13.4- and 19.4-fold increases in TNFR2 expression, respectively. Consequently, we hypothesized that NewSTAR2 might influence some of these populations, although the effects were not robust enough to manifest a phenotype under steady-state conditions during the monitoring period of each experiment.

Differences in the frequencies of myeloid cell populations in bone marrow (BM), blood and spleen were observed when analyzing single-cell suspensions of these organs with flow cytometry. The frequency of living neutrophils, defined as CD45^{+}CD11b^{+}Ly6G^{+}, increased in the BM, blood and spleen of mice treated with 140 µg of NewSTAR2 (**Figure 10A,B**). The frequency of living Ly6C^{high} monocytes (CD45^{+}CD11b^{+}SiglecF^{-}Ly6G^{-}F4/80^{-}) also increased on average, with a statistically significant increase in the spleen. Although the frequencies of other cell subsets, including conventional DCs 2 (cDC2), macrophages, and Ly6C^{low} monocytes, were higher than in untreated mice, the increase was not statistically significant in the BM, blood and spleen. Despite the reported high transcription of TNFR2 in alveolar macrophages, their frequencies remained unaltered in lung lavage, suggesting that their numbers. This could mean in the latter case that the intraperitoneal administration did not lead to an efficient accumulation of NewSTAR2 in the alveolar space or in general. Generally, numbers within the defined populations by the gating strategy may fluctuate after treatment but do not significantly impact the total cell numbers per organ (Supplementary figure S2).

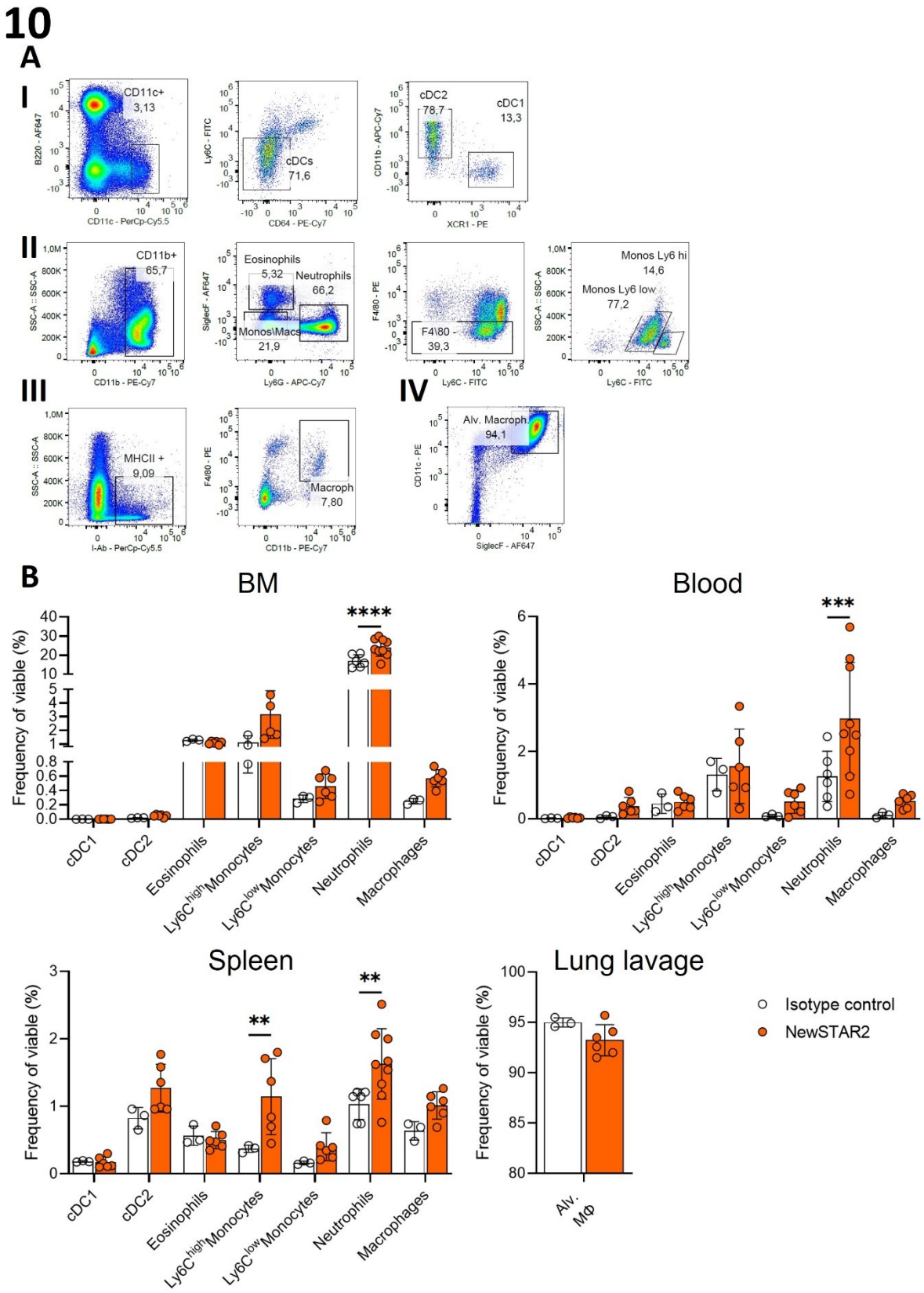

Figure 10. Neutrophils and monocytes in bone marrow, spleen and circulation are also expanded by NewSTAR2. A: Representative gating strategies for the shown myeloid cell populations in panel A. I, II and III pregated from CD45^{+}, singlets and viable cells. IV pregated from singlets and viable cells. **B**: Frequency of different

myeloid cells within viable in bone marrow, blood, spleen and lung lavage on day 4 after injection with 140 µg of isotype control antibody or NewSTAR2. Each datapoint represents one biological replicate. Data shown as mean +/- SD.Two-way ANOVA, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; not significant comparisons not shown.

4.2.6. NewSTAR2 enhances Treg suppressive activity

To assess the impact of NewSTAR2 on Treg function, we devised a miniature suppression assay involving in vivo stimulated splenic Tregs and in vitro activated responder T cells (splenic total T cells). Mice were intraperitoneally administered 140 µg of either NewSTAR2 or an isotype control antibody, and four days later, we dissected and pooled spleens from three mice to isolate Tregs via magnetic sorting (purity shown in **Figure 11B**). $5x10^4$ Tregs were seeded in 96-well plates and titrated down by halves four times by transferring half the volume to wells containing the same volume of only incubation medium. Concurrently, spleens from untreated B6.CD45.1 mice were dissected, and total T cells were isolated through magnetic separation. These cells were labeled with CTV, a compound that covalently binds to cytosolic amines, enabling the determination of cell division generations by the decrease in signal intensity within the cell. The total T cell suspension was prepared in incubation medium with CD3/CD28 activator beads to induce T cell activation. Subsequently, $5x10^4$ total T cells were seeded in the wells with Tregs, resulting in triplicates of different Tcon:Treg ratios spanning from 1:1, 2:1, 4:1 and 8:1 (**Figure 11A**). The number of Tcons remained constant and the Tregs were serially diluted as described earlier so that the highest ratio contained $5x10^4$ of both cell populations and the most diluted (8:1) contained $5x10^4$ total T cells and $0.625x10^4$ Tregs. Plates were incubated for 3 days before flow cytometry analysis.

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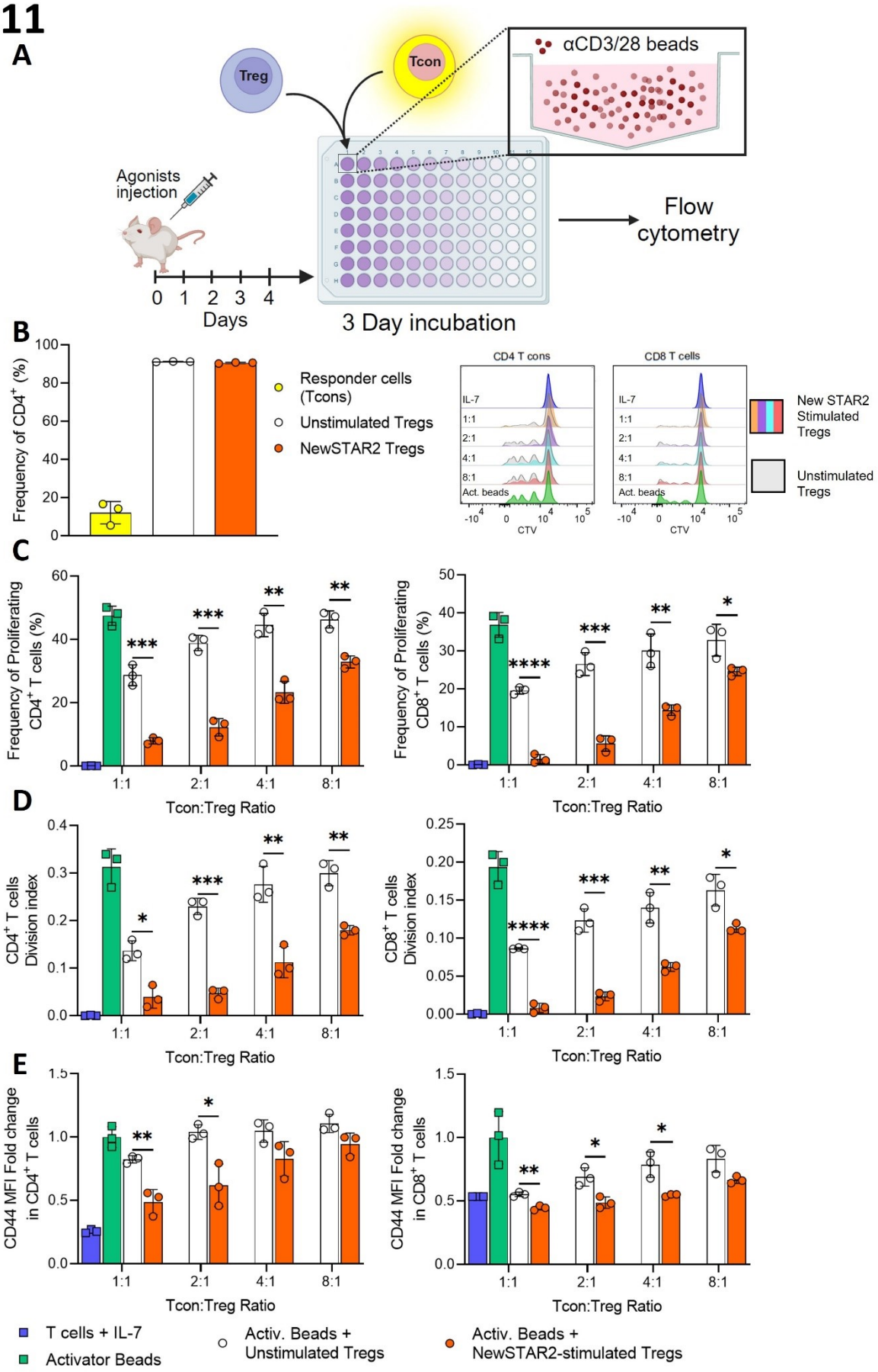
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NewSTAR2 Tregs
CD4 T cons
CD8 T cells
IL-7
1:1
2:1
4:1
8:1
Act. beads
CTV
New STAR2 Stimulated Tregs
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Frequency of Proliferating CD4+ T cells (%)
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CD4+ T cells Division index
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Tcon:Treg Ratio
E
CD44 MFI Fold change in CD4+ T cells
CD44 MFI Fold change in CD8+ T cells
Tcon:Treg Ratio
T cells + IL-7
Activator Beads
Activ. Beads + Unstimulated Tregs
Activ. Beads + NewSTAR2-stimulated Tregs

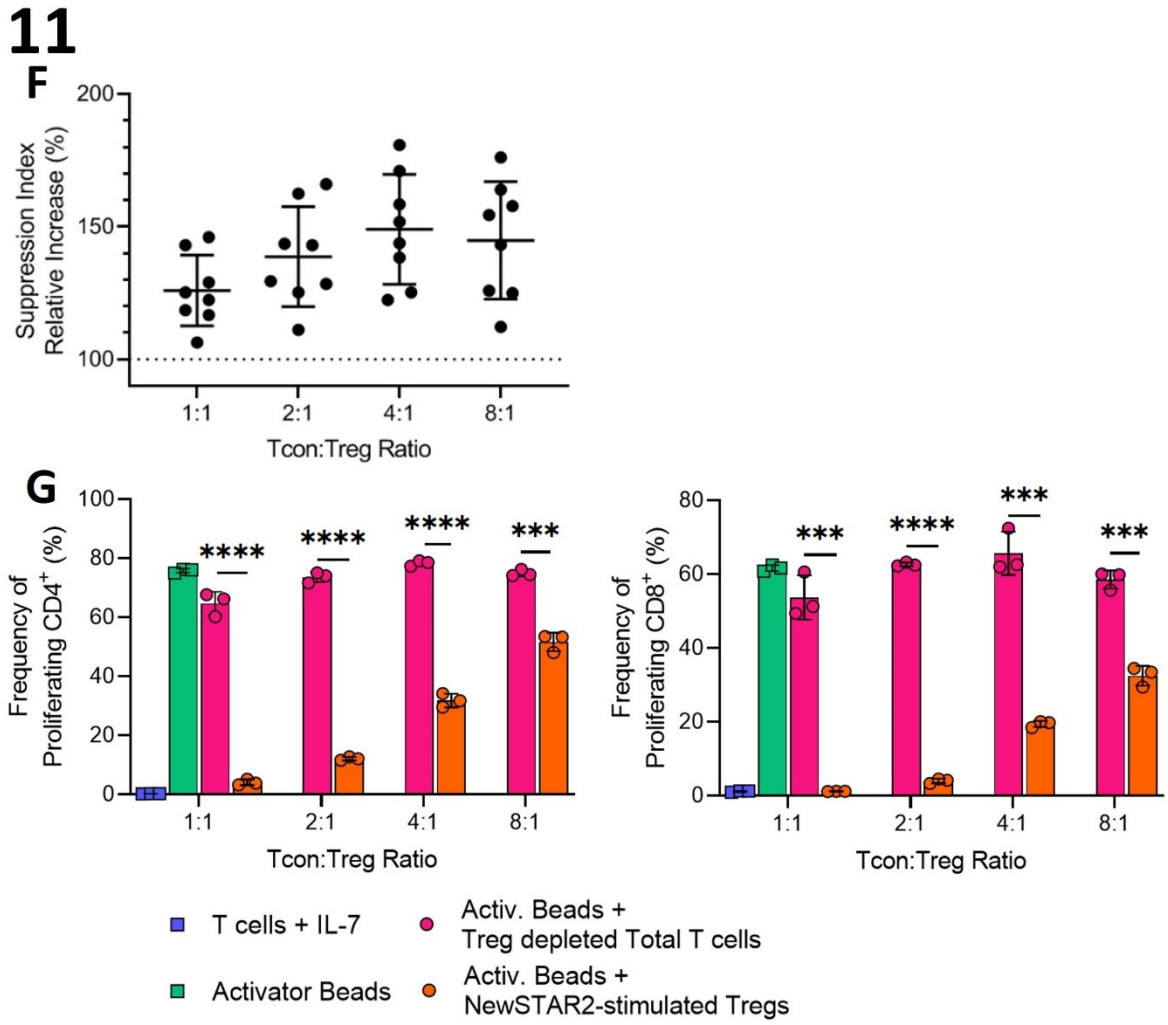

Figure 11. NewSTAR2-stimulated Tregs exhibit higher suppressive activity on conventional T cells. **A**: Experimental setup of Treg suppression assays. B6 mice were injected with 140 µg of isotype control antibody or NewSTAR2. 4 days later single cell suspensions from spleens were prepared and pooled together (3 mice per treatment group) for each group to then enrich Tregs by magnetic cell separation. Simultaneously, total T cells (referred as responder cells or Tcons) from steady-state B6 mice were enriched by magnetic separation and labeled with CTV. $5x10^4$ responder cells were plated together with CD3/CD28 activator beads and NewSTAR2-stimulated or unstimulated Tregs at 1:1, 2:1, 4:1 or 8:1 Tcon:Treg ratios in cRPMI (each ratio by triplicate) in 96-well round bottom plates. Control wells with only Tcons and beads were plated by triplicate (activator beads label) as well as control wells with only Tcons with IL-7 (T cells + IL-7). 3 days after the coculture was seeded, cells were analyzed by flow cytometry. **B** Left: Treg frequencies within CD4+ population after enrichment of the corresponding populations. Right: Representative histograms of responder $CD4^+$ (left) or $CD8^+$ (right) responder T cells 3 days after coculture. Dark blue histograms: undivided cells cultured with IL-7; green: responder cells cultured with CD3/CD28 activator beads only. Orange, magenta, light blue and pink peaks show the generations of divided responder T cells by dilution of the CTV dye when cultured with Tregs from NewSTAR2-stimulated mice at the indicated Tcon:Treg ratios. Overlaid gray histograms represent the generations of divided responder T cells by dilution of the CTV dye when cultured with unstimulated Tregs at the indicated Tcon:Treg ratios. **C**: Frequencies of proliferating $CD4^+$(left) and $CD8^+$(right) responder Tcons defined as the percentage of cells not in generation 0 (peak with highest signal of CTV, hence undivided) at the given Tcon:Treg ratios. **D**: Division index defined as the average number of divisions per cell in $CD4^+$(left) and $CD8^+$(right) responder Tcons at the given Tcon:Treg ratios.

E: MFI of CD44 on $CD4^+$(left) and $CD8^+$(right) responder Tcons normalized against values from activated Tcons alone (activator beads label). **F**: Increase in responder T cell suppression, namely suppression index, defined as the quotient of the frequency of proliferating cells in presence of unstimulated Tregs over the frequency of proliferating cells in presence of NewSTAR2-stimulated Tregs. Data from CD4+ and CD8+ T cells are shown together for each given ratio. Data from 4 different experiments. **G**: Increased suppression observed after stimulation with NewSTAR2 is mostly dependent on Tregs. Frequencies of proliferating $CD4^+$(left) and $CD8^+$(right) responder Tcons defined as the percentage of cells not in generation 0 when responder cells are coculture with Tregs isolated from NewSTAR2-stimulated (as in the previously described experimental setup) B6.FoxP3.Luci.DTR or total T cells from NewSTAR2-stimulated and Treg-depleted B6.FoxP3.Luci.DTR mice. Each datapoint represents one technical replicate from one representative experiment where Tregs were isolated from 3 pooled spleens of treated or untreated mice (**C**, **D**, **E**, **G**). Data shown as mean +/- SD Two-way ANOVA, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; not significant comparisons not shown.

For the identification of Tregs from stimulated/unstimulated mice and responder CTV-labeled T cells, the congenic marker CD45.1 was utilized. Non-proliferating CTV-labeled T cell populations were defined by setting gates on the histograms according to the “generation 0” peak determined in total T cells in wells with only culture medium and IL-7 but without activator beads (**Figure 11B**). Daughter generations, indicated by peaks with lower CTV intensity to the left of the generation 0 peak, exhibited fewer CD4 and CD8 T cells proliferating and generating daughter cells when cultured with NewSTAR2-stimulated Tregs compared to unstimulated Tregs (**Figure 11C**). Although unstimulated Tregs demonstrated some suppressive function, it was weaker at every Tcon:Treg ratio compared to NewSTAR2-stimulated Tregs. The impact on T cell proliferation was more pronounced on the CD8 than CD4 T cells. Additionally, the expression levels of CD44, a marker of T cell activation, were reduced in CD4 and CD8 T cells cocultured with NewSTAR2-stimulated Tregs (**Figure 11E**). Division indexes, indicating the average number of cell divisions that responder T cells of the original population underwent, were significantly reduced in wells with Tregs from treated mice (**Figure 11D**). Overall, our data provide evidence for the reduction in daughter cells generated by an activated T cell and the decrease in activation levels of the expanding T cells. To quantify the treatment effect, we calculated the quotient of the frequency of proliferating cells in presence of unstimulated Tregs over the frequency of proliferating cells in presence of NewSTAR2-stimulated Tregs (**Figure 11F**). In summary, NewSTAR2 enhances suppressive functions of Tregs, even when outnumbered 8:1 by responder T cells.

To confirm if the observed suppression on Tcon proliferation was dependent on Tregs, we repeated the miniature suppression assay with Tregs from B6a.FoxP3.Luci.DTR mice (expressing luciferase and the diphtheria toxin receptor under the FoxP3 promotor) stimulated with NewSTAR2. This time, Tregs were depleted by administering diphtheria toxin (DT) one

and two days before spleen dissection, and they were compared to Tregs from mice that were given NewSTAR2 but not DT (**Figure 11G**). To account for the fact that no Tregs would have been obtained after enrichment of DT-treated mice, we isolated total T cells. When Tregs were depleted, the effect on suppression was completely abrogated. Conversely, when Tregs were isolated from NewSTAR2-treated mice, we observed a significant reduction in the proliferation of responder CD4 and CD8 Tcons. It is important to note that the CTV-labeled total T cells were not depleted of Tregs, and their frequency within the CD4 subset was the same as in untreated mice under steady-state conditions (**Figure 11B**), and thus greater than 0. Assuming that this ratio was constant throughout the five different miniature suppression assays, their effect is negligible.

4.2.7. NewSTAR2 boosts Treg function via contact-dependent and -independent mechanisms

The initial identification of upregulated “Treg activation markers” suggests an augmentation of contact-dependent mechanisms by NewSTAR2. To substantiate this, conducted an additional miniature suppression assay with a 1.0 µm transwell insert to physically separate Tregs from the CTV-labeled responder T cells (**Figure 12A**). Proliferation frequencies of responder T cells were diminished in both treatments—NewSTAR2-stimulated and unstimulated Tregs—but with a more pronounced effect in wells with Tregs from mice given NewSTAR2 (**Figure 12B**). Compared to the normal miniature suppression assays, the reduction of responder cell proliferation was not further impaired, indicating that, for the defined setting of this assay, direct cell-to-cell contact is not indispensable.

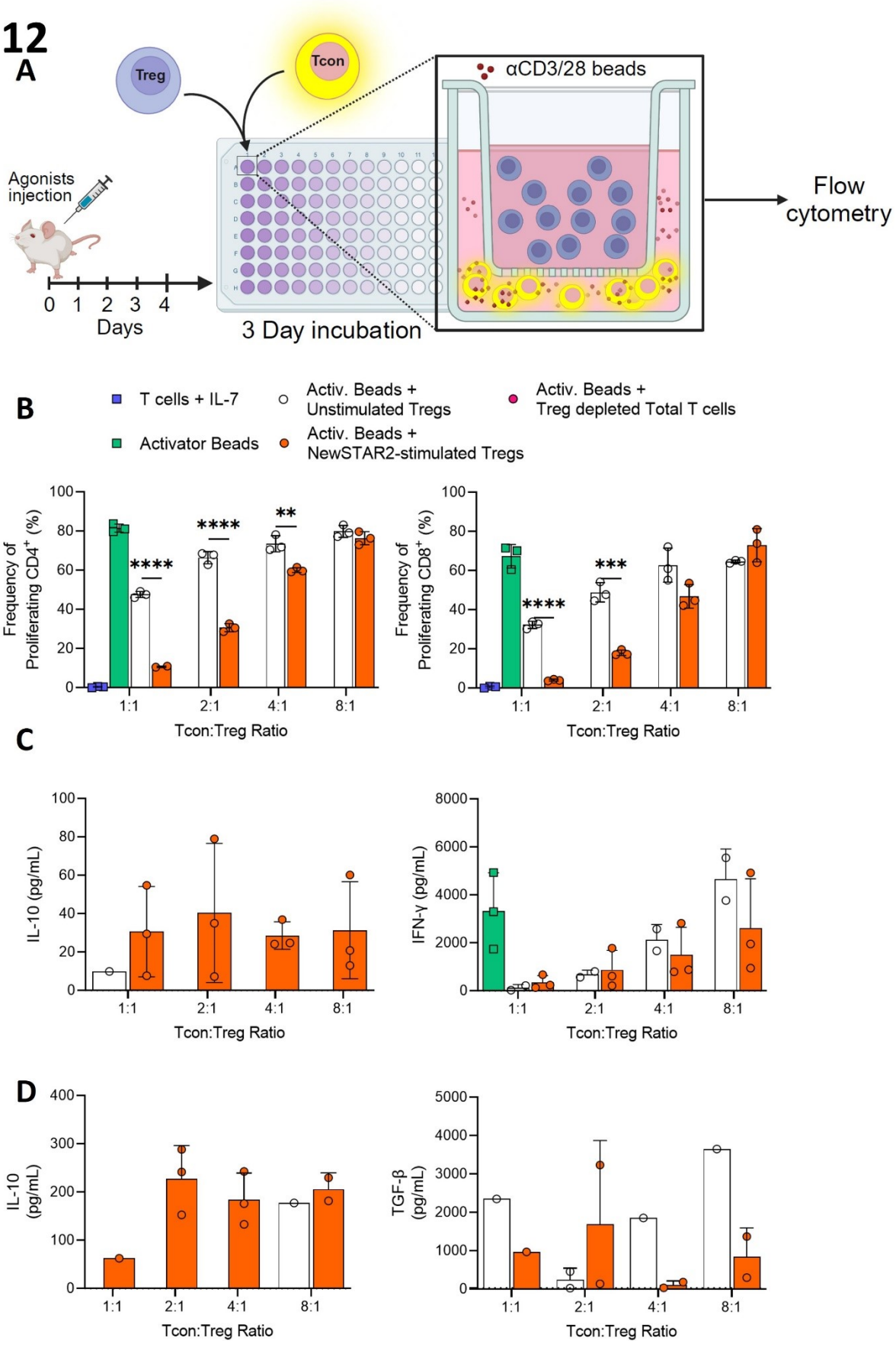

Figure 12. Enhanced suppressive activity after NewSTAR2 stimulation is exerted by contact dependent and independent mechanisms. A: Experimental setup of Treg suppression assay in transwell plate. Suppression

assay was carried out as described for the experiments before but in a 96-well 1 µm transwell plate where Tregs were plated in the insert and responder T cells and activator beads were plated in the well. **B**: Frequencies of proliferating $CD4^+$(left) and $CD8^+$(right) responder Tcons defined as the percentage of cells not in generation 0 (peak with highest signal of CTV, hence undivided) at the given Tcon:Treg ratios. Each datapoint represents one technical replicate from one representative experiment where Tregs were isolated from 3 pooled spleens of treated or untreated mice. **C**: IL-10 (left) and IFN-γ (right) concentrations in suppression assay supernatants (not in transwell plate) at the different Tcon:Treg ratios measured by cytokine bead array. **D**: IL-10 (left) and TGF-β (right) concentrations in suppression assay supernatants at the given Tcon:Treg ratios measure by ELISA. Each datapoint (**C**, **D**) represents the average of two replicates from each of different experiments described in **Figure 11**. Data shown as mean +/- SD.Two-way ANOVA, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; not significant comparisons not shown.

Furthermore, we stored cell culture supernatants from the normal suppression assays, and measured IL-10 and also various proinflammatory cytokine concentrations using cytokine bead array assays. In five different assays, only a few replicates exhibited detectable concentrations (albeit lower than 80 pg/mL), and it was in several wells from cocultures with NewSTAR2-stimulated Tregs where IL-10 was detected. In contrast, only one replicate from cocultures with unstimulated Tregs showed detectable IL-10 (**Figure 12C**). Concentrations of IFN-γ, a signature cytokine of activated T cells, were, on average, lower in supernatants from NewSTAR2-stimulated Tregs but not significantly. Employing more sensitive ELISAs, we conducted IL-10 and TGF-β assays on the culture supernatants. Similarly, IL-10 was predominantly detected in wells containing Tregs from NewSTAR2-stimulated mice, although no correlation between concentration and the amount of Tregs in coculture was observed (**Figure 12D**). TGF-β was found in higher concentrations in supernatants from both treatments, but neither correlation between concentration and Treg ratio, nor differences between stimulated and unstimulated Tregs were discerned. In conclusion, while we cannot exclude the effect of cell-to-cell contact in the enhanced suppression induced by NewSTAR2, we propose that both contact-dependent and -independent mechanisms are heightened, as evidenced by the upregulation of surface markers, the suppression observed in the transwell assay, and the detection of IL-10 in the cell culture supernatants.

4.3. TL1A- and GITRL-based agonists induce comparable Treg expansion as NewSTAR2

4.3.1. Agonistic TL1A (Fc(DANA)-muTL1A) and GITRL (Fc(DANA)-muGITRL)-constructs induce Treg expansion with differential activation profiles in vitro

We assessed the effect of in vitro stimulations with ligand based hexameric Fc(DANA)-muTL1A and tetrameric Fc(DANA)-muGITRL agonists with an Fcγ-deficient IgG backbone on total T cell cultures compared to NewSTAR2. We cultured $2x10^5$ total T cells total T cells with equimolar concentrations of the different agonists or PBS for 4 days before performing flow cytometry analysis. The Treg frequency within the CD4 T cell population increased comparably with all treatments (around a 1.5-fold increase). However, the ligand-based GITR agonist showed an average higher effect, with an almost 2-fold increase (**Figure 13A**). Although the expression of some activation markers like CD25 marginally increased (though not significantly), Helios and Ki67 were upregulated almost 1.5-fold with every agonist. Notably, the DR3 agonist (Fc(DANA)-muTL1A) did not induce upregulation of Helios in Tregs. The impact on other T cell populations was negligible, and the effect on the frequency of Tregs within viable cells was also not observed.

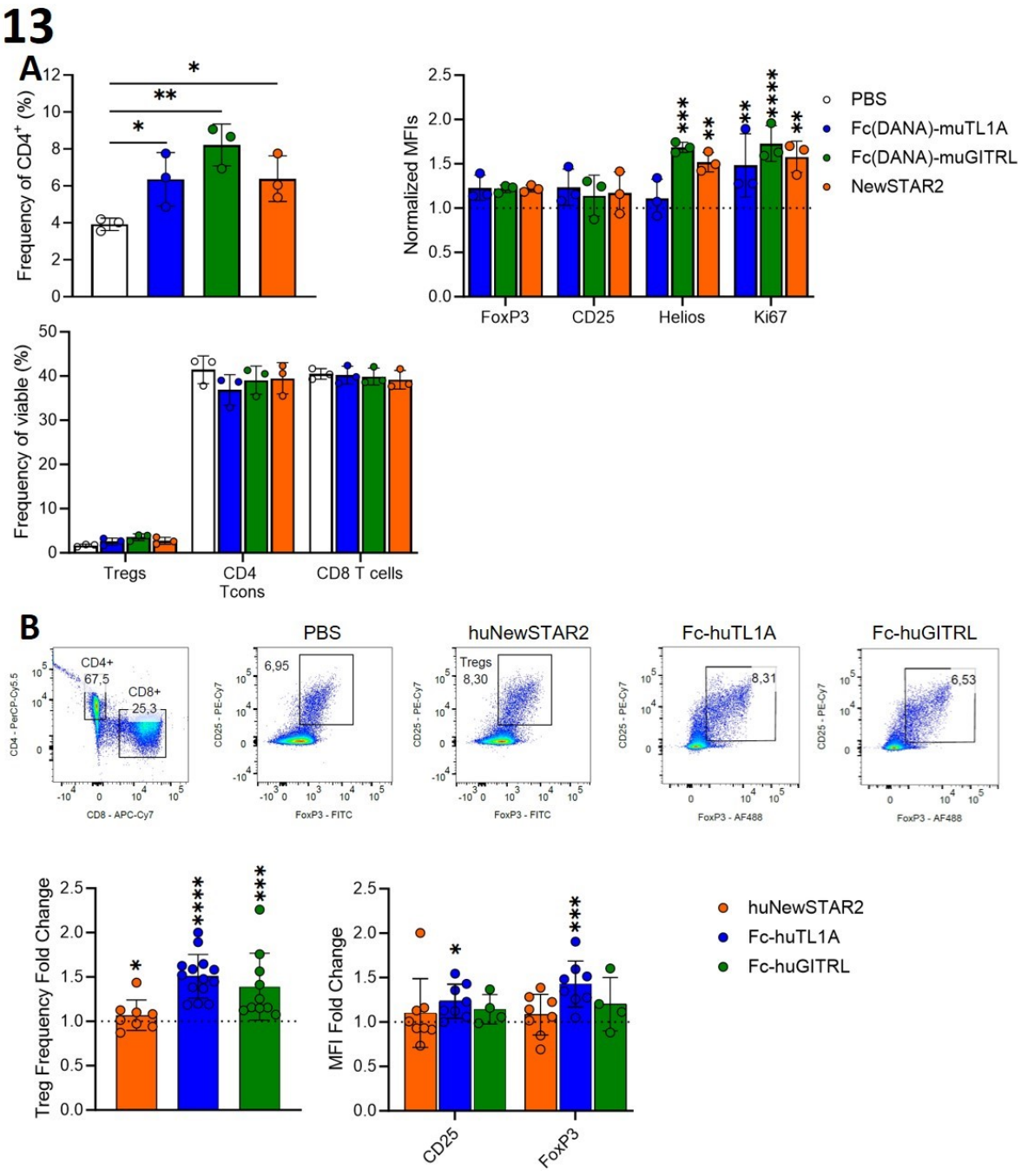

Figure 13. Fc(DANA)-muTL1A and Fc(DANA)-muGITRL induce similar Treg expansion than TNFR2 agonist NewSTAR2 in enriched total T cell cultures but the signature of expression of Treg activation markers may differ. A Treg frequency within $CD4^+$ cells after 4 days of incubation of $2x10^5$ enriched total T cells (B6) per well stimulated with equimolar concentrations (1.67 nM) of NewSTAR2, Fc(DANA)-muTL1A, Fc(DANA)-muGITR or PBS. Upper right: MFI from the depicted markers on Tregs stimulated with the different TNFRSF agonists. Values normalized against their respective expression in unstimulated cultures (PBS). Lower left: Frequency of T cell subsets within viable cells when stimulated with the different TNFRSF agonists. **B** Upper panel: Representative dot plots showing gating strategy for human Tregs. Pregated from singlets, viable, $CD3^+$, $CD4^+$. Lower panel: Fold change of Treg ($CD25^{high}FoxP3^+$) frequency within $CD4^+$ after 4 days of incubation of $2x10^5$ human PBMCs stimulated with 1 µg/mL of huNewSTAR2, Fc-huTL1A or Fc-huGITRL. Each datapoint represents one biological

replicate. Data shown as mean +/- SD.unpaired t-Test (13A upper left panel and 13B lower panel) and Two-way ANOVA (13A upper right panel), *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; not significant comparisons not shown.

Human versions of these agonists have also been developed to engage the human receptors. Fc(DANA)-muGITRL had been designed as a dimeric molecule because natural GITRL forms stable homodimers to bind the receptor, while human GITRL, like other TNFSF members, forms homotrimers, explaining the stronger binding reported for human GITR:GITRL than murine GITR:GITRL (Chattopadhyay et al., 2008). For this reason, the human GITRL-based agonist was designed as a hexameric molecule. In in vitro cultures with human PBMCs isolated from blood after leukapheresis the tested human TL1A-based agonist (Fc-huTL1A), human GITRL-based agonist (Fc-huGITRL), and huNewSTAR2 induced expansion of the Treg population (defined as $CD4^{+}CD25^{high}FoxP3^{+}$ (**Figure 13B**). The effect was lower than in mice, and greater differences between patients were observed. However, an average fold change increase of 1.2, 1.5, and 1.1 was reached with huNewSTAR2, Fc-huTL1A, and Fc-huGITRL, respectively. In murine assays, Fc-huGITRL appeared to induce a stronger increase in Treg frequencies, but in human assays, Fc-huTL1A exhibited the strongest increase, with the lowest increase belonging to Fc-huGITRL treatment, suggesting species-dependent differential effects. Consistently with previous findings, only Fc-huTL1A induced upregulation of CD25 and FoxP3 in human Tregs.

4.3.2. In vivo testing of TL1A-based agonist yields superior Treg expansion than NewSTAR2 and GITRL-based agonist but combination therapy exerts an even higher expansion

In the next stage, we assessed the in vivo efficacy of murine agonists in B6 WT mice. For this purpose, we administered 140 µg of each agonist separately to different mice i.p., and four days later, we analyzed splenocytes with flow cytometry. To explore potential synergistic or inhibiting effects, we opted to test a combination therapy involving all three agonists for the TNFRSF receptors DR3, GITR, and TNFR2. Treg frequencies in the spleen exhibited a mild increase after Fc(DANA)-muGITRL single treatment and a robust increase with Fc(DANA)-muTL1A. Interestingly, the in vivo results were not fully concordant with our previous in vitro findings, as Fc(DANA)-muGITRL now induced the weakest expansion of Tregs, and Fc(DANA)-muTL1A even surpassed the effect observed with NewSTAR2 (**Figure 14A**). The Fc(DANA)-muTL1A construct achieved an almost equivalent frequency of CD4 Tcons and Tregs in spleens, representing an almost 5-fold increase, potentially leading to a higher

suppression of the general T cell response, although no pathological symptoms were observed during the short duration of the experiment. Remarkably, combination therapy proved to be the most effective in expanding Tregs. While no additive effects were observed, a modest complementary effect was evident, emphasizing the potential to enhance Treg frequency beyond what a single therapy can achieve. Frequencies of ICAM1+ and Helios+ within Tregs were also significantly boosted to almost 100%, regardless of the treatment (**Figure 14B**).

14

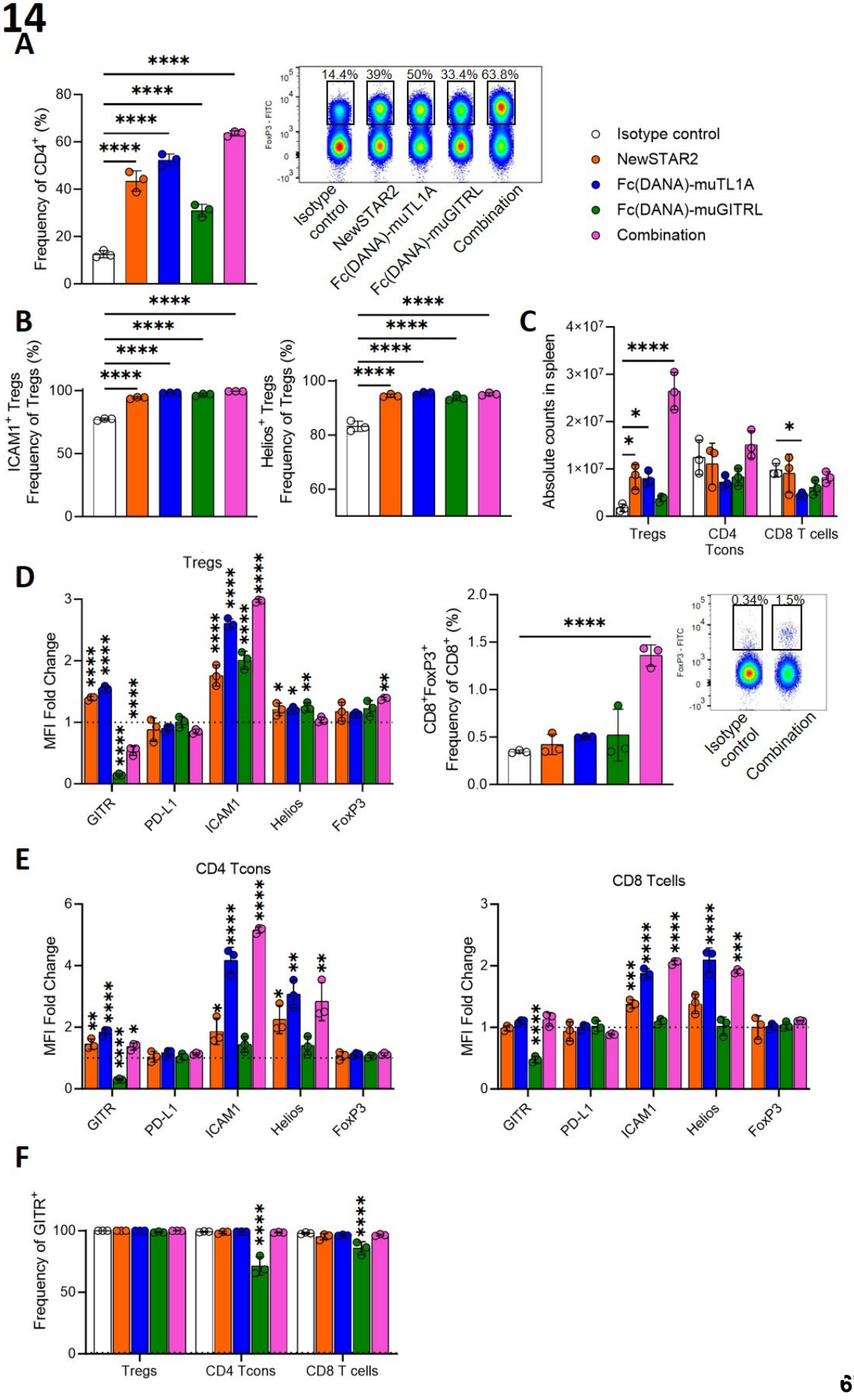
A
Frequency of CD4+ (%)
14.4%
39%
50%
33.4%
63.8%
FoxP3 - FITC
Isotype control
NewSTAR2
Fc(DANA)-muTL1A
Fc(DANA)-muGITRL
Combination
B
ICAM1+ Tregs Frequency of Tregs (%)
Helios+ Tregs Frequency of Tregs (%)
C
Absolute counts in spleen
Tregs
CD4 Tcons
CD8 T cells
D
Tregs
MFI Fold Change
GITR
PD-L1
ICAM1
Helios
FoxP3
CD8+FoxP3+ Frequency of CD8+ (%)
0.34%
1.5%
E
CD4 Tcons
CD8 Tcells
F
Frequency of GITR+

Figure 14. In vivo stimulation with other TNFRSF agonists also increase Tregs in spleen and their expression of „activation markers". A Left: Treg frequencies within CD4+ population in spleen comparing mice given 140 µg of isotype control, NewSTAR2, Fc(DANA)-muTL1A or Fc(DANA)-muGITRL four days before flow cytometry analysis. Right: Representative dot plots depicting the Treg frequencies within $CD4^+$ in spleens on day 4 after injection in B6 mice. **B**: $ICAM1^+$ (left) and $Helios^+$ (right) Treg frequency within total Treg population. **C**: Absolute numbers of T cell subsets in spleen. **D** Left: MFI from the depicted markers on splenic Tregs stimulated with the corresponding TNFRSF agonist. Values normalized against their respective expression on Tregs from mice given isotype control antibody. Right: Frequencies of $CD8^+FoxP3^+$ cells within $CD8^+$ cells in spleen on day 4 after injection and a representative dot plot of this population. Gates and percentages on the left and right represent isotype control and combination therapy respectively. **E**: MFI from the depicted markers on splenic CD4 Tcons (left) and CD8 T cells (right) stimulated with the corresponding TNFRSF agonist. Values normalized against their respective expression on CD4 Tcons or CD8 T cells from mice given isotype control antibody. **F**: $GITR^+$ T cell frequencies within their respective T cell population. Each datapoint represents one biological replicate. Data shown as mean +/- SD.Two-way ANOVA (**D** left panel, **E**, **F**) or one-way ANOVA (**A**, **B**, **D** right panel), *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; not significant comparisons not shown.

The absolute numbers of Tregs in the spleen followed a similar trend, exhibiting a substantial increase in Treg frequencies when treating mice with NewSTAR2 or Fc(DANA)-muTL1A. However, unlike the relative frequency within CD4, no significant increase in absolute numbers was observed with Fc(DANA)-muGITRL. In contrast, mice receiving combination therapy experienced a dramatic increase in absolute Treg numbers (**Figure 14C**). Although splenomegaly is typically associated with NewSTAR2 treatment, larger spleen sizes were observed with Fc(DANA)-muTL1A or combination therapy (not shown), supporting the findings of increased total splenocytes and particularly Tregs. The expression of several "Treg activation markers" was upregulated, including GITR, ICAM1, and Helios (**Figure 14D**). Fc(DANA)-muTL1A induced higher upregulation of GITR and ICAM1 compared to the other agonists, whereas Fc(DANA)-muGITRL induced a similar upregulation of ICAM1 and Helios as NewSTAR2. With combination therapy, an increase in the expression of all markers in Tregs was expected beyond what was seen with single treatments, but only ICAM1 and FoxP3 upregulation were enhanced compared to the other treatments. As a notable exception, Helios upregulation observed with individual treatments was not evident in the context of combination therapy.

The reduced intensity of GITR expression could be caused by partial competitive receptor blocking by the agonist not allowing for proper fluorescent antibody staining for flow cytometry or by receptor internalization. In vitro assays with splenocytes demonstrated that Fc(DANA)-muGITRL could reduce the detected GITR MFI in Tregs, CD4 Tcons, and CD8 T cells when incubated for 10 minutes with the cells before FACS staining, and this effect followed a dose-dependent curve. However, this did not affect the frequencies of $GITR^+$ cells, suggesting that

the amount of conjugated antibodies binding to GITR could be less when Fc(DANA)-muGITRL is given, but this does not block the binding and subsequent detection of GITR-expressing cells by flow cytometry (Supplementary figure S3). Accordingly, the decrease in $GITR^+$ CD4 Tcons and CD8 T cells four days after stimulation with Fc(DANA)-muGITRL could indeed be a consequence of receptor regulation (**Figure 14F**). Moreover, a distinct $CD8^+FoxP3^+$ population had never been observed in spleens of NewSTAR2-treated but combination therapy proved to increase the number of cells in this population, an effect that was not seen with neither Fc(DANA)-muTL1A nor Fc(DANA)-muGITRL (**Figure 14D**). It is also essential to note that considering the serum retention data of NewSTAR2, the circulating amount of the other agonists may approach 0 already four days after injection, supporting the idea of natural downregulation after stimulation. Furthermore, in combination therapy, the same amount of Fc(DANA)-muGITRL was administered, but the baseline GITR expression was not restored in Tregs, while it was completely restored and even increased in CD4 Tcons and CD8 T cells, indicating differential regulations in the T cell populations. Another surprising observation was the stark increase in ICAM1 and Helios in Tcons, mostly seen with Fc(DANA)-muTL1A or combination therapy, highlighting that a negative effect, as hinted by the absolute numbers, is not necessarily the only effect triggered by the different agonists (**Figure 14E**). Once again, these findings provide evidence of varying Treg activation patterns resulting from different TNFSF agonists and further suggest the potential to target other T cell populations when TNFSFRs are simultaneously engaged.

4.4. NewSTAR2, Fc(DANA)-muTL1A and Fc(DANA)-muGITRL as prophylactic therapy for aGvHD

4.4.1. Prophylactic NewSTAR2 protects from aGvHD prolonging mean survival and reducing intestinal damage

Previously, our research group has assessed the effectiveness of STAR2 in preventing GvHD in murine allo-HCT models (Chopra et al., 2016). During those studies, it was demonstrated that repetitive injections enhanced host Tregs before transplantation. Although this did not influence alloreactive T cell activation, it did impede their proliferation and migration into target organs, particularly the intestine, leading to a partial reduction in intestinal lesions and an increase in overall survival. Building on these findings, we conducted similar experiments, wherein recipient B6 mice were injected with 140 µg of NewSTAR2 or isotype control antibody four days before TBI and allo-HCT, involving $5x10^6$ BM cells from femurs and $6x10^5$ total T

cells from spleens of FVB mice. This model, characterized by major MHC mismatch, typically results in acute mortality within the first two weeks after transplantation, accompanied by skin and gastrointestinal symptoms. To examine alloreactive T cell activation we analyzed spleens as T cell priming sites with flow cytometry on Day 3 analysis, while we preserved target organs for subsequent histopathological evaluation and cytokine concentration analysis on Day 6. We continued daily monitoring of the mice until 40 days after transplantation to observe the development of symptoms and weight changes, or reaching the humane endpoint if applicable (**Figure 15A**).

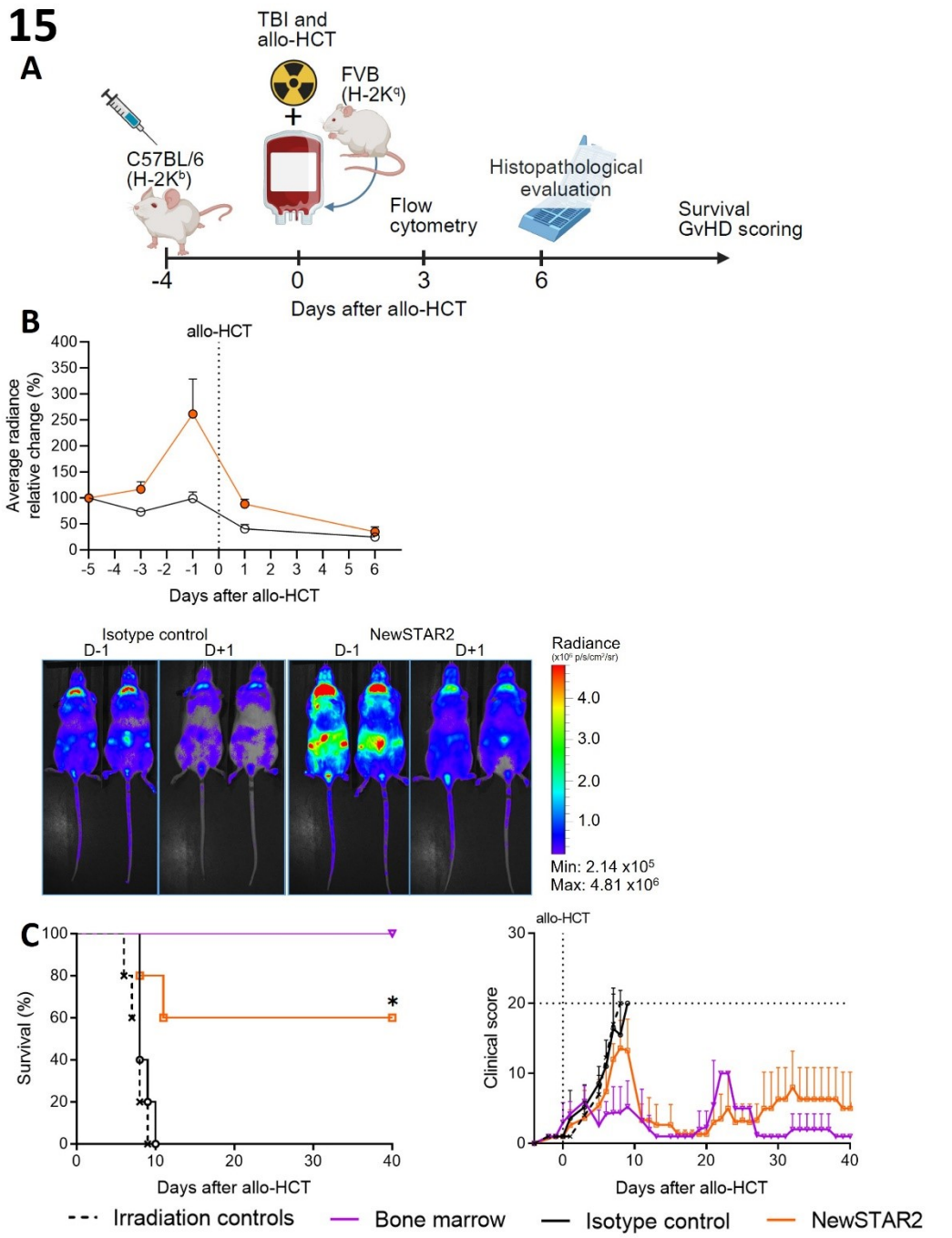
15
A
TBI and allo-HCT
FVB (H-2K^q)
C57BL/6 (H-2K^b)
Flow cytometry
Histopathological evaluation
Survival GvHD scoring
-4
0
3
6
Days after allo-HCT
B
allo-HCT
Average radiance relative change (%)
Days after allo-HCT
Isotype control
NewSTAR2
D-1
D+1
Radiance
Min: 2.14 x10^5
Max: 4.81 x10^6
C
Survival (%)
Days after allo-HCT
Clinical score
Irradiation controls
Bone marrow
Isotype control
NewSTAR2
D
NewSTAR2
Isotype control
Bone marrow
Ileum
100 µm
Pathology score

Figure 15. Prophylactic endogenous Treg expansion protects against GvHD. A: Experimental setup of allogenic hematopoietic cell transplantation major mismatch aGvHD model: B6 or B6a.FoxP3.Luci.DTR mice were injected with 140 µg of isotype control antibody or NewSTAR2 5 days before myeloablative total body irradiation and transplantation with $5x10^6$ bone marrow and $6x10^5$ enriched total T cells from FVB/N mice. Mice were weighed and scored daily for 40 days after transplantation. **B** Upper row: Relative average radiance change (day -5 baseline) from Tregs of recipient B6a.FoxP3.Luci.DTR injected i.p. on day 0 with 140 µg of NewSTAR2 or isotype control antibody. In vivo bioluminescence imaging from ventral view was taken on the shown time points. Dotted line denotes the day of allo-HCT. Lower row: Representative bioluminescence images from the different treatment groups on day -1 and day +1 after allo-HCT. C: Kaplan-Meier survival graphs (left) and clinical score (right). **D**: Representative hematoxylin and eosin stainings of the small bowel at day +6 after allo-HCT (upper) and the respective histopathological score (lower). Each datapoint represents one biological replicate. Data shown as mean +/- SD.Two-way ANOVA, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; not significant comparisons not shown.

Additionally, we transplanted B6a.FoxP3.Luci.DTR mice to assess changes in the in vivo bioluminescence signal emitted by Tregs before and after allo-HCT. As observed in our previous in vivo experiments, NewSTAR2 effectively increased the total signal from Tregs throughout the entire mouse four days after injection. Recognizing that TBI has the potential to eliminate almost all lymphocytes, with Tregs displaying greater resilience and persisting after myeloablation, we utilized in vivo BLI signal as a parameter for Treg numbers in the mouse. The preemptive Treg expansion with NewSTAR2 mitigated the negative impact caused by the irradiation, maintaining the Tregs within the normal ranges. One day after conditioning and transplantation, the treated mice exhibited nearly baseline BLI signals, with around 100% persistence (**Figure 15B**). This suggested that this modest increase in persistent Tregs sufficed to provide protection, as evidenced by the low clinical score throughout the experiment and reduced pathology score in the small intestine. This was characterized by fewer round-cell infiltrates between intestinal crypts and normal spacing between neighboring crypts (**Figure 15D**). Compared to untreated mice, median survival increased from 9 days to 40 days, and disease development was significantly delayed (**Figure 15C**).

4.4.2. Prophylactic TL1A-based agonist enhances survival and alleviates GvHD symptoms

Given that most analyses following in vivo stimulation occurred four days after agonist injection, we conducted new allo-HCT experiments to compare the different TNFRSF agonists administered at this time point as prophylaxis. Transplanting BM cells and T cells from FVB donors into B6a or B6a.FoxP3.Luci.DTR recipient mice, we assessed the in vivo BLI signal of Tregs before and after transplantation. Additionally, we evaluated the activation of the T cell

response and initial tissue damage by analyzing splenocytes on day 3 and target organs on day 6 after transplantation, respectively.

The increase in in vivo BLI signal of FoxP3$^+$ host cells from day -4 to day -1 was similar among treatment groups, approximately doubling (**Figure 16A,B**). On day 0, before irradiation and allo-HCT, NewSTAR2- and Fc(DANA)-muGITRL-treated mice exhibited signal increases similar to those on the previous day, while the Fc(DANA)-muTL1A-treated mice already showed a decline. The observed difference in the kinetics of Treg population maintenance between treatments was also reflected in later time points of in vivo BLI analysis, where the average signal in Fc(DANA)-muTL1A-treated mice was higher than in the other groups. Ventral imaging could not clarify if the Treg signal was preferentially high in a particular organs unique to each treatment. For all groups, most of the signal came from cervical lymph nodes and the intestinal tract (**Figure 16B**). Flow cytometry analysis of spleens on day 3 after allo-HCT showed that the absolute number of host Tregs remained higher in NewSTAR2- and Fc(DANA)-muGITRL-treated mice; however, this increase was not statistically significant in the Fc(DANA)-muTL1A group. Notably, it caught our attention that the Treg signal from BM control mice was still detectable in mice after 2 and 3 weeks, compared to the treatment groups where it had practically disappeared. These results suggested a higher host chimerism after transplantation in the absence of alloreactive T cells.

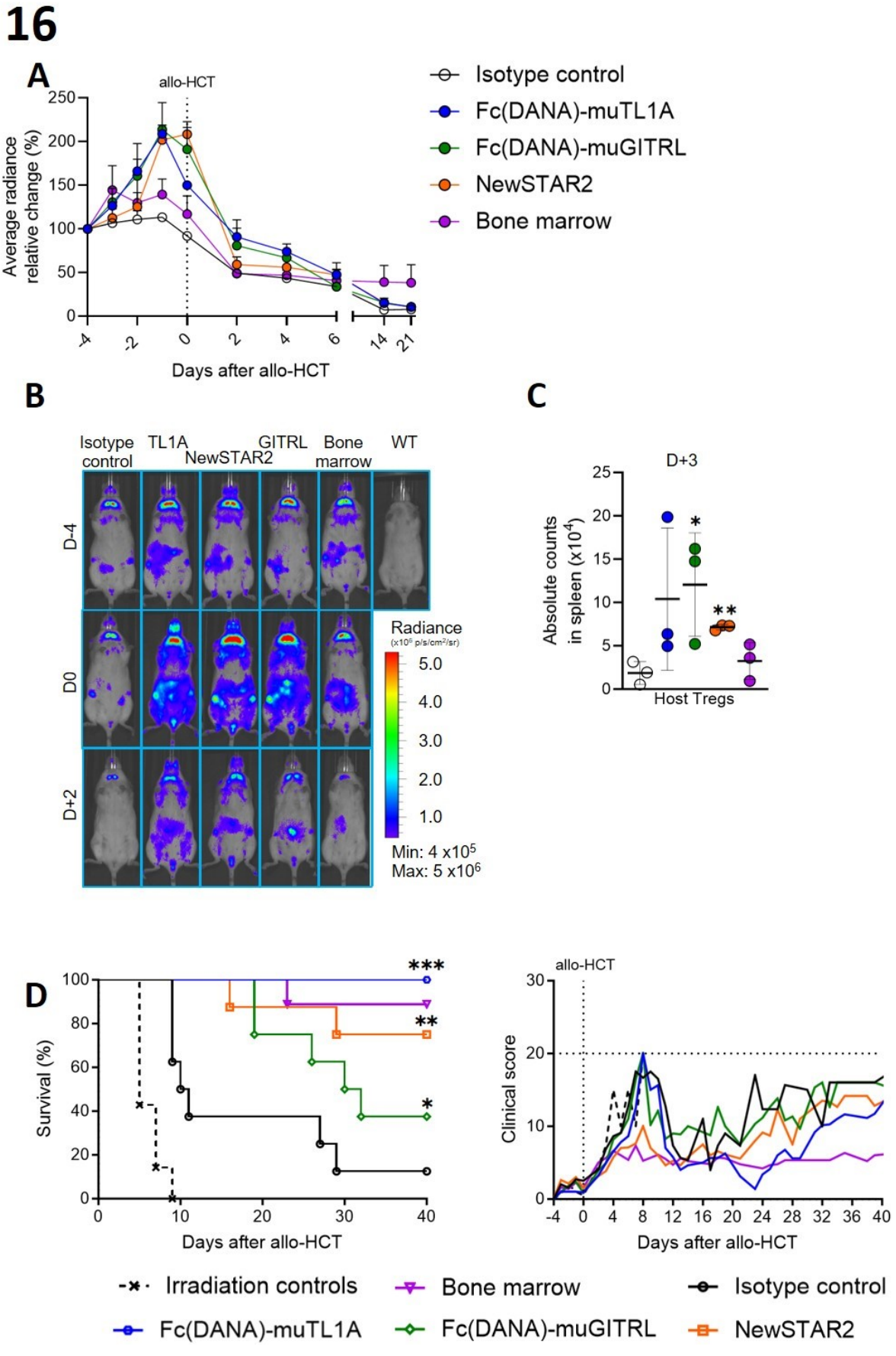
16
A
allo-HCT
Average radiance relative change (%)
Days after allo-HCT
Isotype control
Fc(DANA)-muTL1A
Fc(DANA)-muGITRL
NewSTAR2
Bone marrow
B
Isotype control
TL1A
NewSTAR2
GITRL
Bone marrow
WT
D-4
D0
D+2
Radiance
Min: 4 x10^5
Max: 5 x10^6
C
D+3
Absolute counts in spleen (x10^4)
Host Tregs
D
Survival (%)
Days after allo-HCT
Irradiation controls
Bone marrow
Isotype control
Fc(DANA)-muTL1A
Fc(DANA)-muGITRL
NewSTAR2
allo-HCT
Clinical score
Days after allo-HCT

Figure 16. Fc(DANA)-muTL1A induces an enhanced protection over Fc(DANA)-muGITRL or NewSTAR2 as prophylactic treatment. Other TNFRSF agonists given before transplantation induce a Treg expansion that persists during the first 2 days after transplantation and remains higher than untreated mice until around day +6. Despite higher DR3 expression on Tcons compared to Tregs, a DR3 agonist confers increased protection against GvHD over TNFR2 or GITR agonists. Experimental setup of allogenic hematopoietic cell transplantation done as described in Figure 12, but this time 140 µg of NewSTAR2, Fc(DANA)-muGITRL, Fc(DANA)-muTL1A or isotype control antibody were injected 4 days before transplantation. **A:** Relative average radiance change (day -4 baseline) from Tregs of recipient B6a.FoxP3.Luci.DTR injected i.p. on day 0 with 140 µg of the corresponding TNFRSF agonist or isotype control antibody. In vivo bioluminescence imaging from ventral view was taken on the shown time points. Dotted line denotes the day of allo-HCT. **B**: Representative bioluminescence images from the different treatment groups on day -4, 0 before allo-HCT and +2 after allo-HCT. **C**: Flow cytometry analysis of spleens on day +3 after allo-HCT showing absolute numbers of host Tregs. **D**: Kaplan-Meier survival graphs (left) and clinical score (right). Each datapoint represents one biological replicate. Data shown as mean +/- SD.Unpaired t-Test (**C**) or Kaplan-Meier estimator statistic (**D**), *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; not significant comparisons not shown.

Agonist-treated mice navigated the first lethal inflammatory aGvHD wave, typically characterized by cytokine storms, increased intestinal permeability, and liver failure within the initial two weeks after allo-HCT. During this critical period, over half of the untreated mice succumbed to aGvHD (**Figure 16C**). After the second week, symptoms enhanced by host conditioning waned, and GvHD clinical manifestations took center stage. The Fc(DANA)-muGITRL group exhibited early-onset skin lesions, escalating weight loss, evident in the clinical score curves (**Figure 16C**). Conversely, the NewSTAR2 and Fc(DANA)-muTL1A groups experienced a delayed onset of symptoms, such as conjunctivitis and minimal skin damage. Median survival increased from 10 days in untreated mice to 30 and 40 days with Fc(DANA)-muGITR, and NewSTAR2 or Fc(DANA)-muTL1A, respectively. Between day 20 and 40 post allo-HCT, mice treated with Fc(DANA)-muTL1A or NewSTAR2 displayed worsening conditions, marked by diarrhea and weight loss, leading to the exclusion of some NewSTAR2-treated mice from the experiment upon reaching the humane endpoint. No significant difference was found between the survival curves of NewSTAR2 and Fc(DANA)-muTL1A or Fc(DANA)-muGITRL groups, although Fc(DANA)-muTL1A was significantly different from Fc(DANA)-muGITRL.

Day 3 analyses of the alloreactive T cell response in the spleen showed a marginal decrease in the average absolute numbers of donor CD4 and CD8 T cells in agonist treated mice, which did not reach statistical significance (**Figure 17A,B**). Donor T cells positive for the proliferation marker Ki67 were also slightly reduced on average in the Fc(DANA)-muTL1A and NewSTAR2 groups compared to untreated mice, while the average numbers of proliferating donor T cells in Fc(DANA)-muGITRL-treated mice increased to a small degree. Although CD44, a marker

upregulated on effector T cells after activation, relates to their ability to migrate, adhere, and release cytokines, neither the total number of $CD44^+$ donor CD4 nor donor CD8 T cells were lower in spleens of treated mice compared to untreated mice. The significant difference between biological replicates obscured any difference, if present, in the expansion of alloreactive T cells on day 3. Overall, this data suggests that the protective effect of prophylactic selected TNFSF agonists does not influence the alloreactive T cell priming phase of GvHD in secondary lymphoid organs.

Determination of acute-phase cytokine concentrations in the liver, intestine, and serum on day 6 after allo-HCT showed higher concentrations of IL-6 than IL-1β in serum and liver, but similar values between all groups except bone marrow controls (**Figure 17C**). The Th1 effector cytokine IFN-γ was found to be decreased in the serum of Fc(DANA)-muGITRL-treated mice and to a greater extent in NewSTAR2-treated mice. Similar trends were observed in serum and organs for each cytokine, with slightly lower average concentrations in treated than untreated mice, with almost always the lowest in the NewSTAR2 group. GM-CSF in the ileum of NewSTAR2-treated mice was significantly lower and even at similar levels to BM control mice. Cytokines known to be released after conditioning showed very similar concentrations to those in treated and untreated mice that received alloreactive T cells, suggesting that decreased tissue damage is not the reason why mice are protected after prophylactic endogenous Treg expansion. In contrast, cytokines known to be secreted after immune priming and sustain inflammation, and thus depend (directly or indirectly) on alloreactive T cells, showed a trend towards lower values in treated mice. Altogether, this supports that alloreactive T cell activation was unhampered while initial effector T cell functions appeared attenuated.

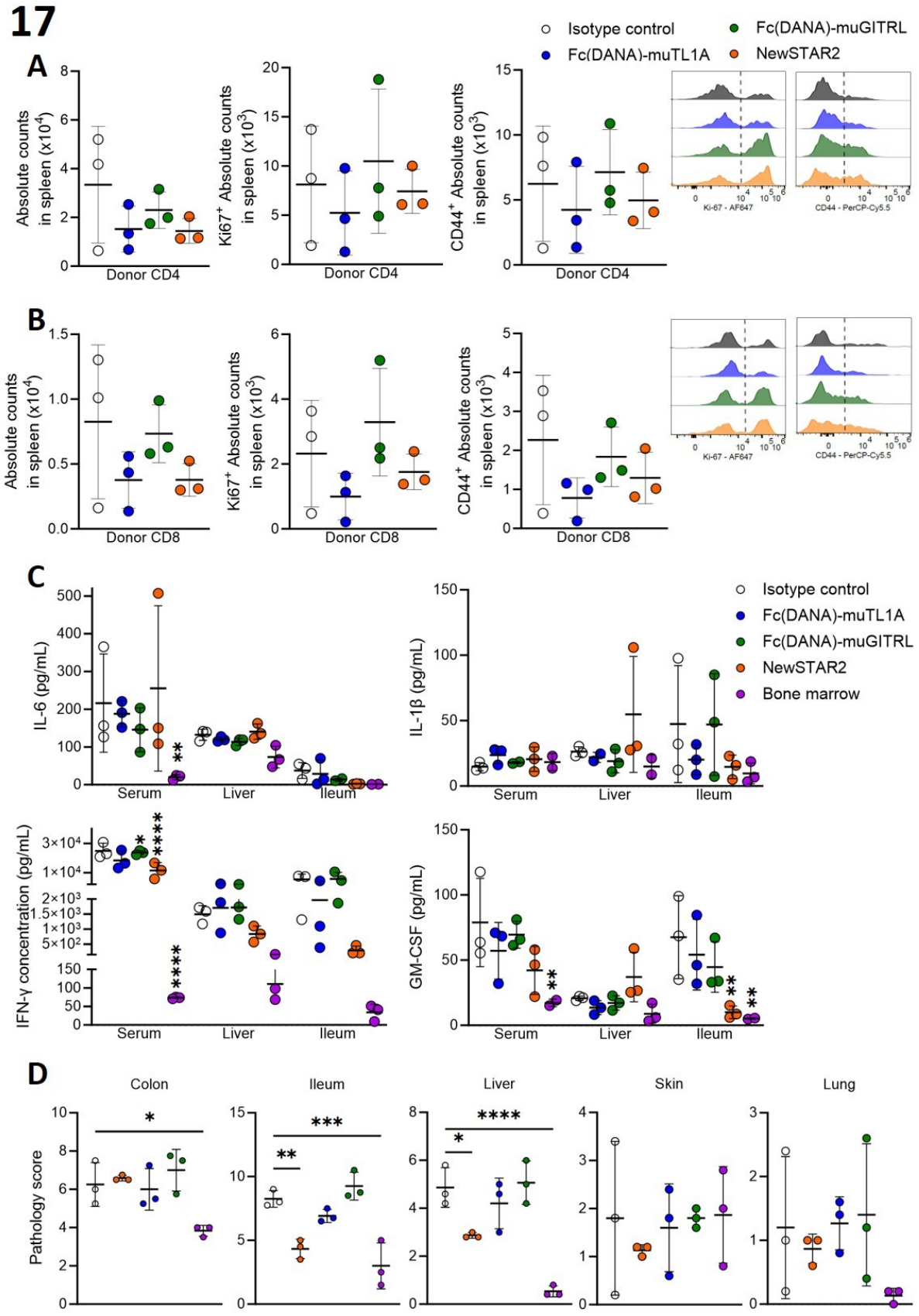
17
A
B
C
D
Isotype control
Fc(DANA)-muTL1A
Fc(DANA)-muGITRL
NewSTAR2
Bone marrow
Absolute counts in spleen (x10⁴)
Ki67⁺ Absolute counts in spleen (x10³)
CD44⁺ Absolute counts in spleen (x10³)
Donor CD4
Donor CD8
Ki-67 - AF647
CD44 - PerCP-Cy5.5
IL-6 (pg/mL)
IL-1β (pg/mL)
IFN-γ concentration (pg/mL)
GM-CSF (pg/mL)
Serum
Liver
Ileum
Pathology score
Colon
Ileum
Liver
Skin
Lung

Figure 17. TNFRSF agonists do not exacerbate activation and differentiation of alloreactive T cells. In the same experimental setup described in **Figure 16**, 3 additional mice per group were euthanized on day +3 to perform flow cytometry analysis of spleens and 3 more mice on day +6 to perform histopathological analysis of target tissues and cytokine bead array to study serum, liver and ileum concentrations of inflammatory cytokines. **A**: Flow cytometry analysis of spleens on day +3 after allo-HCT showing absolute numbers of donor CD4 T cell subsets, frequencies of $Ki67^+$ and $CD44^+$ donor CD4 T cell within the total donor CD4 population, and representative histograms of Ki67 and CD44 expression. **B**: Flow cytometry analysis of spleens on day +3 after allo-HCT showing absolute numbers of donor CD8 T cell subsets, frequencies of $Ki67^+$ and $CD44^+$ donor CD8 T cell within the total donor CD8 population, and representative histograms of Ki67 and CD44 expression. **C**: Blood from the heart was drawn to isolate serum and fragments of ileum and liver were dissected and homogenized (and diluted 1:2 for serum samples) on day +6 to perform cytokine bead array assay and evaluate concentrations of different inflammatory cytokines. IFN-γ, IL-1β, IL-6 and GM-CSF concentrations in the different organs from the different treatment groups are shown in the bar graph. Other cytokines were not found significantly altered. **D**: Histopathological score for the different isolated organs on day +6. Each datapoint represents one biological replicate. Data shown as mean +/- SD.Two-way ANOVA (**C**) or one-way ANOVA (**D**), *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$; not significant comparisons not shown.

5. Discussion

5.1. High TNFRSF expression does not necessarily translate into stronger outcome after engagement

Our phenotypic analyses show not only how the frequency of TNFR2, GITR or DR3-expressing cells varies between the main T cell subsets and how the intensity of their expression does not correlate with it, but also how the apparent less Treg exclusive marker, DR3, can have a potent effect particularly on Tregs and not in Tcons. In line with this, signal transduction after ligand binding has been reported to induce strong activation of JNK and IκBα making NF-κB activation by DR3 remarkable(Pobezinskaya et al., 2011). Studies from our group have described that the abundance of TNFR2 and GITR on the surface is also quite different, with approximately $5x10^4$ vs $3x10^4$ receptors on the surface, respectively. It is not known for DR3 how this abundance compares to these other two markers but from our indirect quantification (lower intensity in voltage as measured by the photomultiplier tubes) one can speculate that it is considerably lower. Even though GITR expression levels were the highest of the three markers on Tregs and also exhibited the biggest difference between its expression on Tregs and CD4 or CD8 T cells, the increase of Tregs four days after in vivo stimulation was actually the lowest. In contrast, the almost parallel expression levels of DR3 seemed to preferentially expand the Treg subset and inhibit CD8 T cells demonstrating counteracting effects in its agonism as also reported in literature (Bittner et al., 2017; Bittner & Ehrenschwender, 2017; Valatas et al., 2019). Overall, the strength of signal induction that each marker can develop is clearly an important factor that influences the outcome as much as the receptor abundance on the surface and the number of cells capable of responding to the ligand binding, which are certainly distinct between TNFRSF members.

In the more simplistic terms, we could evidence expansion of Tregs, increase in $ICAM1^+$ and $Helios^+$ with any of the three agonistic molecules making them appropriate potential therapeutics to modulate the immune system by means of higher and arguably more stable Treg numbers (driven by Helios (Lam et al., 2022; Thornton & Shevach, 2019)) and increased capability to extravasate to inflammation sites. At a closer glance though, the upregulation of other receptors hint to the aforementioned differential outcomes. The reduction of CD8 T cells with Fc(DANA)-muTL1A could be a response to the increased Treg numbers that can limit their expansion. Alternatively, engagement of DR3 on CD8 T cells can trigger apoptosis, after all this receptor shares the closest homology to TNFR1 and as its name implies, it was initially

described based on its ability to cause apoptosis (Valatas et al., 2019). As the analysis cannot distinguish between naïve or activate Tcons, it is not possible to confirm if the generation of new CD8 T cells was undermined or if the existing pool of CD8 T cells in spleen was reduced. An increase in sample size for the in vivo stimulation with these agonists can also elucidate if this same effect extends to the CD4 Tcon population, as the tendency is similar to that observed in CD8 T cells. The promising survival achieved with Fc(DANA)-muTL1A treatment is contended by the high costimulation that was observed in Tcons based on the upregulation of Helios and ICAM1. Even though the total number of cells in steady-state was in average reduced, the high expression of these markers would allow them to migrate, activate and differentiate stronger when challenged by an antigen (Akimova et al., 2011; Skadow et al., 2019).

Fc(DANA)-muGITRL may have a clear limitation in its application due to downregulation on Tregs after activation as proposed in our work, making them less responsive even when the frequencies of $GITR^+$ Tregs are unchanged. This can reduce the number of applications in which a positive effect on Tregs is observed, impeding any repetitive dosing. Hence, we propose that consecutive dosing but with agonists against different receptors can be an alternative to not saturate one pathway and keep the overall TNFRSF signaling ongoing before a negative feedback mechanism has started. Interestingly, a mild upregulation of GITR but at inconsistent degrees in the three T cell populations was observed when Fc(DANA)-muGITRL was given in combination with NewSTAR2 and Fc(DANA)-muTL1A, suggesting the disparate interplay that different receptors of the TNFRSF may have upon each other. Of note, our results indicate that the engineered GITR agonist can have a positive effect on Tregs without stimulating CD4 Tcons or CD8 T cells even though its role as costimulatory molecule on T cells has been reported (H. Li et al., 2021; Nocentini & Riccardi, 2009; Snell et al., 2011).

This study describes mostly effects seen on Tregs in steady-state and during an acute, one-shot, stimulation with our engineered TNFRSF agonists, thus one cannot dismiss the possibility that the enhanced Treg counts and upregulation of “Treg activation markers” is inferior or absent. TNFRSF members being described as modulators of the immune response are able to have opposing effects and this hinges on their context-dependent signaling and expression. The significance that our reagents may have during ongoing inflammation has not been explored and is a major point for future research. Although the constitutive expression of several receptors like TNFR2, GITR, OX40, 4-1BB (albeit at low levels in resting Tregs) and DR3 has been well described for Tregs, inducible expression in activate T cells is described extensively (Kumar et al., 2018; Lubrano di Ricco et al., 2020; Schreiber et al., 2010). It would

be interesting then to test how is the expression of TNFR2, GITR and DR3 within the different T cell subsets (as well as on myeloid, epithelial, and endothelial cells) in models of inflammatory disease or infectious disease. In a context where effector T cells are the predominant T cell population once again, we would need to determine if our reagents will act as costimulatory molecules increasing inflammation, or activate cell death pathways reducing the damage caused by the T cell response.

5.2. Complementary but not additive effects can succeed after concomitant TNFRSF agonism

Almost all members of the TNFRSF can activate NFκB but this is not because they are redundant, instead it is believed that this is the result of a conserved central defense mechanism regulated by high-order molecular complexes that sense a signal and given the cell's intrinsic threshold of activation and the strength of such signal, will then proceed with activation. Studies have described how the concentration of TNF-α is sensed to decide if a time delayed response should be mounted, or if the concentration is too high that immediate actions should ensue (Tay et al., 2010). In favor of this theory, other studies have shown how the TNF response threshold is indeed different between cells but at the end, regardless of high or low concentration, the end nuclear activity of NFκB is the same (Wu, 2013). Therefore, all the players involved in these molecular complexes can represent related highly-fitting blocks in different places that will intercommunicate to come together and decide if and how the immune response should be mounted and thus efficiently amplify and transmit this signal throughout different systems.

This explains the varying effects of NewSTAR2, Fc(DANA)-muTL1A and Fc(DANA)-muGITRL on Treg expansion observed in our in vivo data and also how engaging one receptor opened the possibility to sensitize the system to engage another one, as seen by the upregulation of GITR after NewSTAR2 (in vivo and in vitro) or Fc(DANA)-muTL1A treatment. Furthermore, the combination therapy with all the three agonists exemplifies how this effect is not simply added when compared to single treatments and how it can have a mild complementary effect on Treg frequencies but a higher-than-additive effect on Treg absolute numbers in spleen. Similarly, the expression of ICAM1 does not follow an additive pattern but rather an increased effect over the seemingly already potent Fc(DANA)-muTL1A or NewSTAR2 treatments. Mentioned before was also the expression of GITR which on Tregs was increased with Fc(DANA)-muTL1A and

NewSTAR2 but dramatically reduced with Fc(DANA)-muGITRL and partially restored with the combination therapy. The latter implies that for GITR, a lower threshold is set (perhaps dependent on its high expression in steady-state, allowing for broad sensing) that can be reestablished with additional concomitant signals from other TNFRSF probably in case a very strong stimulus is triggering an immune response that must be addressed also very strongly. In accordance, we also found that additional regulatory mechanisms can be induced only once a strong/broad stimulation of the TNFSF is given: $CD8^{+}FoxP3^{+}$ T cells were barely identified in untreated and single treatment groups but their frequencies within total CD8 T cells leaped to an almost 4-fold increase making this population stand out after combination therapy.

It is surprising that with the tested agonists we seem to observe mostly immunoregulatory mechanisms coming to play (increased Tregs and Treg stability, Treg activation and migration to inflamed tissues, increased CD8 Tregs, reduced CD8 Tcons). We hypothesize that this is due to the fact that no other immunogenic signals like TCR engagement or innate defense mechanisms are engaged. Most of the TNFRSF are described as costimulatory molecules that can boost the T cell response and have a net pro-inflammatory effect, however as we demonstrated, the different treatments seem to cause a mostly anti-inflammatory effect. Further evidence to this was the lack of any adverse effect seen on the mice during the 4 days after treatment but also until 3 weeks after treatment with NewSTAR2. Our work does not intend to challenge the general consortium that agrees that TNFRSF members are a potent inflammatory mediators, in lieu we show evidence, as others have showed already (Beldi, Bahiraii, et al., 2020; Chen et al., 2007; Leclerc et al., 2016; Mavers et al., 2019; Schreiber et al., 2010; Valatas et al., 2019), of how it is possible to engage the negative feedback on the immune response, already imprinted in the TNFRSF signaling pathways, without an initiating inflammatory signal.

5.3. Antibody backbone extends TNFR2 agonist half life boosting its single-dose effect, and domain architecture does not impact Treg stimulation

Serum retention analysis shows a clear improvement when fusing an irrelevant IgG domain to the TFN80 trimers, extending the detectable concentration of NewSTAR2 to almost double the time of STAR2 around 10^{-3} serum dilutions. Other than that, expansion of Tregs and upregulation of markers in vitro were comparable. For this work we wanted to test STAR2 and

NewSTAR2 on equal conditions and that is why we did not give repeated injections of STAR2, even though this has been the appropriate administration regime that induces Treg expansion and GvHD protection (Chopra et al., 2016). Anyway, previous work from our group determined that even when giving repeated injections of STAR2, the effect on Treg in vivo BLI signal is superior with NewSTAR2 (Steinfatt, 2019). We believe that this difference is again a consequence of the longer bioavailability of NewSTAR2 that extends its effect through time. Regarding the architecture of both reagents, it was observed that the effect on Treg expansion is observed when either a nonameric (STAR2) or a hexameric (NewSTAR2) TNFR2-binding structure is designed for the agonist, though it cannot be ruled out that partly the improved effect of NewSTAR2 is caused by more efficient clustering of TNFR2 that can be engaged further by circulating NewSTAR2 over several days, or by more flexible receptor-ligand binding complexes through the IgG backbone allowing pivoting of one trimer while the other has already been bound. Such a feature would explain why despite having less TNF trimers, NewSTAR2 had a higher TNFR2-binding affinity (Vargas et al., 2022).

The current accepted model for TNFSF ligand:receptor binding proposes that the THD of the different TNFSF ligands is responsible for the formation of homotrimeric molecules (except mouse GITRL which forms dimers) that then can cluster their corresponding TNFRSF and induce signaling. Additionally, their PLADs have different affinities allowing for homo- and oligomer assembly of the different TNFRSF allowing them to respond differently to the ligand trimeric clusters. (Prada et al., 2021; Wajant, 2015). Different receptors are properly activated when two or more of these trimeric receptor clusters come together, supporting the idea of a high-order molecular complexes and providing the fundament of developing reagents in oligomeric format that mimic the homotrimeric ligand clustering and engage one or more of these clusters to induce proper signaling. For TNFR2 it has been recently demonstrated that the domain architecture of these homotrimeric-containing molecules is more important than the targeted epitope or affinity of the trimers to TNFR2 (Anany et al., 2024). For GITR and DR3 exhaustive testing has not been performed yet, but these latest results are promising for the development of new reagents that can induce stronger signaling than the ones we tested in this study and for other TNFRSF members.

5.4. NewSTAR2 is not exclusively targeting Tregs

In vitro assays with different cell populations showed that Treg expansion was achieved in total T cell and CD4 T cell cultures. The secretion of IL-2 by Tcons is known to be required for IL-2-

induced Treg expansion, making other T cell populations possible enhancers of TNFR2-mediated Treg expansion. In addition, because TNFR2 engagement will lead to TNF-α secretion, it is proposed that autocrine TNF-α signaling from TNFR2-stimulated Tregs can be a main driver of the stimulatory effect observed. The fine balance of these highly sensitive signals (IL-2 with or without TNF-α) can be modified when cell population ratios differ from that in their natural niches, posing a possible reason why in splenocytes cell suspension the positive effect on Tregs is unseen. By design, NewSTAR2 is unable to bind FcγR or other antigen through its Fab domain in the biological system tested, this was further supported by our data, firstly by the detected concentration of NewSTAR2 in serum along several days, implying no clearance of the antibody, and secondly by the in vitro stimulations with TNFR2KO mice, in which Treg expansion was absent. Nonetheless, the engagement of TNFR2 on different cells that somehow can bind NewSTAR2 more efficiently than Tregs cannot be excluded.

In accordance, the flow cytometry analyses of myeloid cell populations show that at least neutrophils and monocytes in lymphoid organs can be some of the cells taking up NewSTAR2 and receiving stimulatory signals to increase their numbers within their niches. Compared to the published data on STAR2, our findings are agreeing with the neutrophil increase shown in spleens but a significant decrease in alveolar macrophages was not observed (Chopra et al., 2016). Functional assays to test phagocytosis, net formation or migration, and evaluation of increased differentiation and mobilization from bone marrow are needed to determine if neutrophils and monocytes are increasing their frequencies and their activities and thus represent an important population receptive to TNFR2 agonism. The slight increase in cDC2 can also have a big impact on the immune response as recent studies have shown that stimulated cDC2 can induce differentiation of T cells into Th1 and Th17 as well as enhance humoral responses by polarizing naïve T cells into Tfh subtype (Durand et al., 2019; Hatscher et al., 2021). In mucosal immunity cDC2 play a crucial role in commensal tolerance by initiating the development of Th17 cells (Akagbosu et al., 2022; Ngoi et al., 2022). These are arguments that grant additional examination of other cell populations after TNFRSF agonism in steady-state and inflammatory conditions.

One of our initial hypotheses based on the TNFR2 RNA expression levels on different cells led us to think that macrophages or monocytes would be much stronger responders than Tregs. And considering the robust response seen on Tregs (in this and previous works (Chopra et al., 2016; Vargas et al., 2022)) despite their relatively low TNFR2 RNA transcription, any changes in macrophages or monocytes, which express much higher levels of TNFR2 RNA would have

been evident in the different assays but this was not exactly the case. Notwithstanding the few organs analyzed and the general classification that we employed to identify these cell subsets, we failed to identify an effect on myeloid cells matching the reported RNA data. Several microRNAs (miRNA) have been found to interact with mRNA to block TNF signaling by targeting TNFA, TNFR1 and TNFR2 in gastric cancer and short hairpin RNAs (shRNA) can block TNFR2 in Lewis lung carcinoma cultures (Rossi et al., 2019; Sasi et al., 2012). Regulatory mechanisms at RNA level may account for these discrepancies and could position Tregs at the frontline of cells predominantly influenced by TNFRSF-based therapies.

5.5. TNFRSF agonists expand Tregs systemically but do not likely induce new Tregs from Tcons

The definition of induced, and natural Tregs is still contested and redefined constantly and is dependent on expression of surface markers, demethylation state and requirements for their differentiation. In the broader sense, a conventional CD4 T cell that expresses FoxP3 after stimulation by an APC and acquires suppressive capabilities will be considered for this work as an iTreg. Here a distinction between iTregs generated in vitro or in vivo won't be made. Our experimental setups did not include a way to stimulate TCRs and only a costimulatory signal was provided through the TNFRSF agonists, therefore the signal 1 is lacking and only signal 2 for T cell activation is present. On top of this, even though it was previously hinted that cDC2 could be increasing in numbers, a stimulation of APCs was not seen in our work or in previous work with STAR2 (Chopra et al., 2016). In agreement with this is also the fact that in vitro there is an increase of Tregs with enriched CD4 T cell cultures, neglecting the involvement of splenic B cells or DCs in this effect. Evaluation of this hypothesis in a pure Treg culture in presence of NewSTAR2 could fail to induce expansion for the reasons exposed in 5.4, making it unsuitable. However, we propose that in vitro mixed cultures of labeled or congenic-marker expressing Tregs and Tcons stimulated with NewSTAR2 or the other reagents could elucidate the conversion of Tcons into Tregs if expression of FoxP3 is detected in the labeled Tcons.

Ex vivo analyses of Tregs showed increase in various organs. Among them there are non-immune organs in which the predominant Treg population is believed to be pTregs (like colon or lung) but also secondary and primary lymphoid organs where nTregs are the most abundant subset. On that account, we could not establish a difference between organs that would suggest the expansion of only circulating Tregs or tissue resident Tregs. Contradicting this

however, were the absolute numbers of CD4 Tcons, that were slightly reduced after NewSTAR2, yet not significantly changed with either Fc(DANA)-muTL1A or Fc(DANA)-muGITRL compared to untreated mice but there was a trend towards reduced numbers. Together this evidence proves that the mechanism triggered by TNFRSF stimulation is a general expansion of FoxP3^{+} CD4 T cells. Nevertheless, evaluation of additional surface markers and TCR repertoire would prove if we triggered an in-situ expansion of only nTreg/pTregs or even if we are causing an increased mobilization of nTregs, supported by the upregulation of molecules like ICAM1. Demethylation status could help to understand if the changes induced are like those described in iTregs or if a more stable and Treg-intrinsic outcome is determined by the TNFRSF agonism (Gottschalk et al., 2012; Hill et al., 2021; Thornton et al., 2019).

5.6. NewSTAR2 boosts the general suppressive activity of Tregs

As described before, a deeper analysis with NewSTAR2 was carried out because of the availability of this reagent for testing and the general direction of the project that was paved as it was being developed and new findings from this and other projects were discovered. In vivo NewSTAR2-stimulated Tregs showed a marked increased suppression on Tcons extending the findings of higher numbers of Tregs to also functionally superior Tregs. However, our miniature suppression assay is not able to evaluate the full Treg suppression repertoire since the indirect mechanisms relying, for example, on Treg-APC interaction are not replicated in our model. One can conclude that the increased suppressive activity seen by NewSTAR2 therapy in our data is just a fraction of the total suppressive potential. Among the mechanisms that we speculate are responsible for the increased suppressive activity and that we may not observed directly in our results are: 1) reduced activation of Th1 through competition of TIGIT with CD226 for CD155 expressed on DCs, and potential secretion of fibrinogen like 2 protein that also engages inhibitory FcγRIIB on DCs and polarizes macrophages towards M2 phenotyping, all this depending on TIGIT upregulation on Tregs (Hou et al., 2021; Joller et al., 2014). 2) bind MHCII on APCs through Lag3 to prevent activation and maturation of T cells (Do et al., 2016; Liang et al., 2008). 3) prime Tregs to additional costimulation through GITR to increase proliferation and IL-10 secretion (detected in our in vitro culture supernatants (Kanamaru et al., 2004)). 4) increased migration to inflamed sites and more stable formation of immune synapse with Tcons through ICAM1 (Haydinger et al., 2023).

As it has been previously described in the literature, TNFR2 serves as a costimulatory molecule for Tregs but is a receptor expressed on other cells and part of the multidimensional TNF axis, central defense mechanism and regulator of the immune response. As such, its effects span over multiple cell types to induce activation/inhibition or pro-/anti-inflammatory signals, therefore assuming that it can act at a level of specific cellular functions among, for example, favoring only certain suppressive mechanisms is impractical. This same broad response of the TNFRSF to either induce or extinguish inflammation in a seemingly flexible yet polarized manner is what propels these family of molecules to the spotlight of development of novel immunomodulatory therapeutics.

5.7. One single intervention with prophylactic TNFRSF agonists proved the efficacy of TNFRSF-based in reducing transplant related mortality

As advantage over other Treg-based therapies is of importance the simplicity and applicability of this approach. When endogenous Tregs are expanded, there is no need to find and genotype haplotypes until finding an appropriate donor for cell transfusions, nor complicated in vitro modifications that must meet GMP standards and require costly machinery or resources. The lack of any genetic modification dismisses concerns about long-term effects amplified by single mutations over time or unpredictable genetic interactions or de novo oncogene development. Generation and purification of ligand-based or antibody-based constructs that can be offered to patients as simple i.v. infusions exemplify best the “from bench to bedside” process that describes translational immunology and can derive in reduced required testing of therapeutics until approval making them accessible sooner.

Specifically, against aGvHD we demonstrated that NewSTAR2 increases the surviving Tregs after conditioning, though at a much lower grade than what it initially expands them before transplantation. This single-dose effect can still be observed within the host Treg subset 7 days after injection and is not overshadowed by myeloablative TBI or allo-HCT. Assessment of the alloreactive T cell response showed no impact on T cell activation and differentiation into effector T cells with NewSTAR2. Notwithstanding this, a reduced damage in ileal intestinal crypts accompanied by reduced infiltration of inflammatory cells were the main difference that was found between treated and untreated mice. This indicates that the pathophysiology of

aGvHD cannot be subverted by TNFR2, GITR or DR3 agonism, however it can be lessened enough to considerably improve survival and development of clinical symptoms.

Given the complexity of GvHD is highly doubtful that a single approach will completely cure this disease. And this is what is currently seen in the clinics where first line and other consequent treatments rely on concomitant medications. Complementary therapies should be then considered to treat GvHD when TNFRSF agonists are implemented. Alternatively, the optimization of the therapeutic regime can reduce the dependency of concomitant medications if repetitive dosing, higher doses or pre-/post-transplant infusions are shown to improve the therapeutic efficacy and interfere on additional pathological mechanisms like PAMPs/DAMPs release, APC priming, T cell activation, Th1/17 differentiation or effector functions, and tissue repair. This study should open the door for the diverse treatment possibilities with TNFRSF agonists that can be explored to extend the effect seen with only one administration or maximize its efficacy by adjusting the dosages.

5.8. Is the effect of TNFRSF agonists in steady-state a reflection of the observed protection against GvHD?

Fc(DANA)-muTL1A and Fc(DANA)-muGITRL pretransplant treatment were comparable to NewSTAR2 on the basis of an imperceptible reduction of the T cell activation and a persistence of host Tregs after transplantation. Concentrations of acute phase cytokines in liver and ileum were similar between untreated and treated mice, while effector cytokine IFN-γ was in average reduced in serum (significantly with NewSTAR2 or Fc(DANA)-muGITRL) and not target organs (except in NewSTAR2-treated mice). Reduced inflammation in liver and ileum observed only after NewSTAR2 treatment, raises questions of which other mechanisms can be playing a role in protection by Fc(DANA)-muTL1A, although the differences when compared to the pathology score in NewSTAR2 samples are not that big as with Fc(DANA)-muGITRL hinting to a shared but weaker effect. Survival and clinical score parameters show the greatest differences between treatments with Fc(DANA)-muTL1A protecting 100% of the mice and exhibiting the stronger delay and attenuation of symptoms. Fc(DANA)-muGITRL treatment improved initial survival with mice overcoming the acute mortality shortly after transplantation but then failed to prevent clinical symptoms as seen by the strong skin manifestations and reduced survival after 3 weeks of transplantation. Taken together, our data reveal how these different TNFRSF

are not created equal and their differential expression levels and frequencies within T cell subsets have direct consequences in disease that are not easy to predict.

As described before the dramatic difference in expression of GITR suggests that Fc(DANA)-muGITRL would mostly target Tregs and have lesser costimulatory effects on Tcons in comparison, but in our aGvHD model the reduced expansion of Tregs in steady-state with Fc(DANA)-muGITRL is consistent with worse survival after transplantation. The narrower window of effectiveness of Fc(DANA)-muGITRL therapy can relate to the downregulation of the receptor, implying that a threshold had been reached faster and the system is accordingly reestablishing itself also sooner than with the other agonists, concluding the beneficial effect. In contrast, Fc(DANA)-muTL1A had outstanding survival rates despite the similar expression levels in T cell subsets discussed throughout the last sections. In hindsight, the higher ICAM1 upregulation on Tregs after Fc(DANA)-muTL1A treatment in steady-state mice could have foretold that the survival and clinical score outcome would have been better with this agonist. However, Tcons also showed increased ICAM1 upregulation after treating the mice in steady-state, suggesting possible Tcon costimulation and an expected stronger GvHD.It is striking how DR3, which is described as sharing close homology to TNFR1, had very similar effects after agonistic Fc(DANA)-muTL1A treatment as NewSTAR2 on TNFR2 given the differences in signaling and expression of TNFR1 vs. TNFR2. Treg expansion in steady-state was very similar between both treatments and the average values of absolute donor T cells, $Ki67^+$-, $CD44^+$ CD4 and CD8 T cells on day 3 after transplantation were also similar. In conclusion, neither RNA expression data nor the effect of TNFRSF agonists in steady-state could fully predict the outcome in the transplantation setting, which calls for a deeper characterization of the agonism effects to be able to predict the effects while inflammation is ongoing. Additionally, finer characterization of the time window in which each TNFRSF is exerting Treg stimulation exclusively or at its fullest, will establish the settings where any of the tested therapies becomes the preferred one. Since these agonists are not mutually exclusive, this characterization through time can also reveal the optimal therapeutic regime when combining several TNFRSF agonists at different time points can maintain a beneficial Treg expansion by engaging more than one pathway extending the net effect before one pathway is shut down.

To further explore the effectiveness of TNFRSF-based therapeutics, the recent developments in TNFRSF antibody-based agonists can be exploited to combine different TNFSF ligands to provide dual costimulatory signals or provide anchoring domains to the TNFRSF agonists and deliver this molecule to specific organs like those affected in GvHD. Anchoring domains would guarantee that the effect from stimulated Tregs is delivered to a target cell like alloreactive T

cells or CD103^{+} DCs, and thus avoiding dilution of its function by a systemic spread and engagement, reducing also off-target stimulation of other cell populations. Restricting the area of effect of such therapy can interfere with the signals in intestine that fuel GvHD pathophysiology while sparing other organs that can be susceptible to opportunistic infections and where a strong immunosuppression can not be afforded. This applies as well to the bone marrow niche or others where malignant cells would benefit from immunosuppression. Accordingly the impact of the proposed therapeutics in GvL or tumor models should also be carefully analyzed to understand how the regulation by TNFRSF would call for agonists or antagonists depening on the conditions.

To conclude, TNFRSF are specific regulators of the immune system that possess different sensitivities and exert different effects on immune cells that can be harnessed to treat inflammatory diseases and cancer. What can be considered a simple change, namely Treg expansion through engagement of these receptors, has profound implications at a higher scale as seen by the ability to protect the mice from a complex and multifactorial disease like GvHD. Ligand-based with antibody domain constructs that target TNFRSF can effectively exploit their immunomodulatory properties and represent simple and effective therapies with high translational potential.

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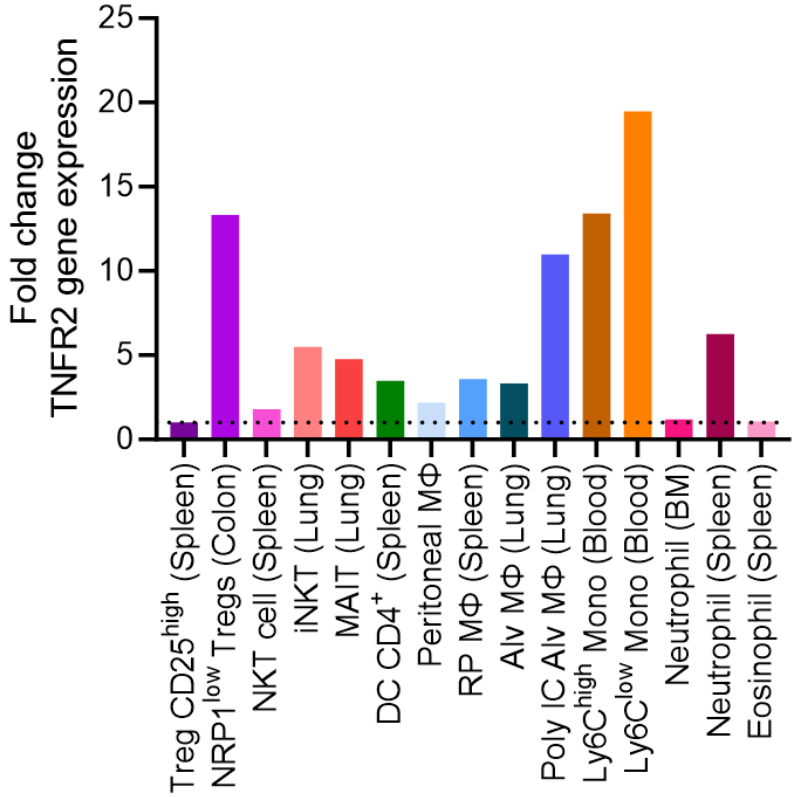
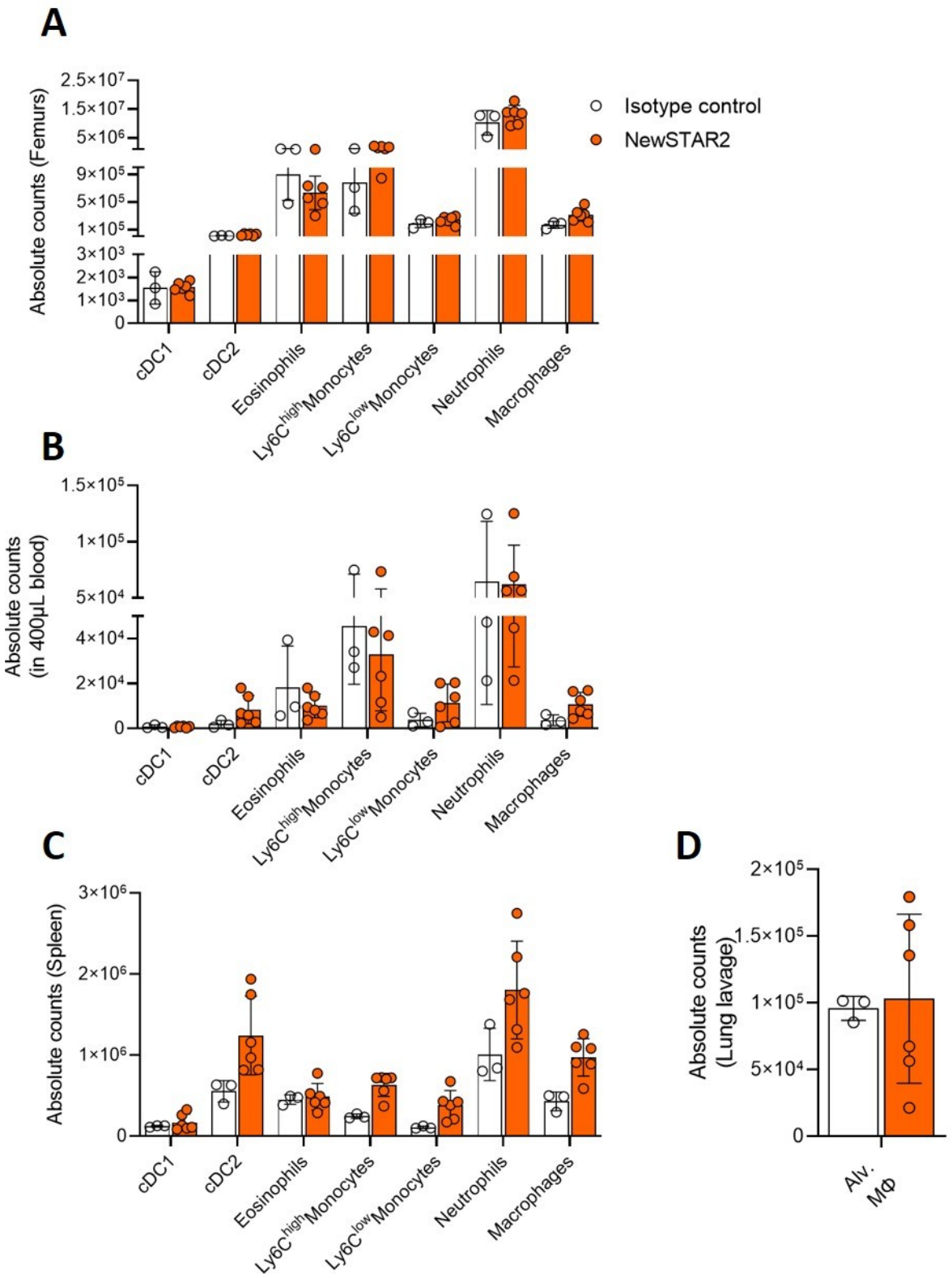
7. Annexes

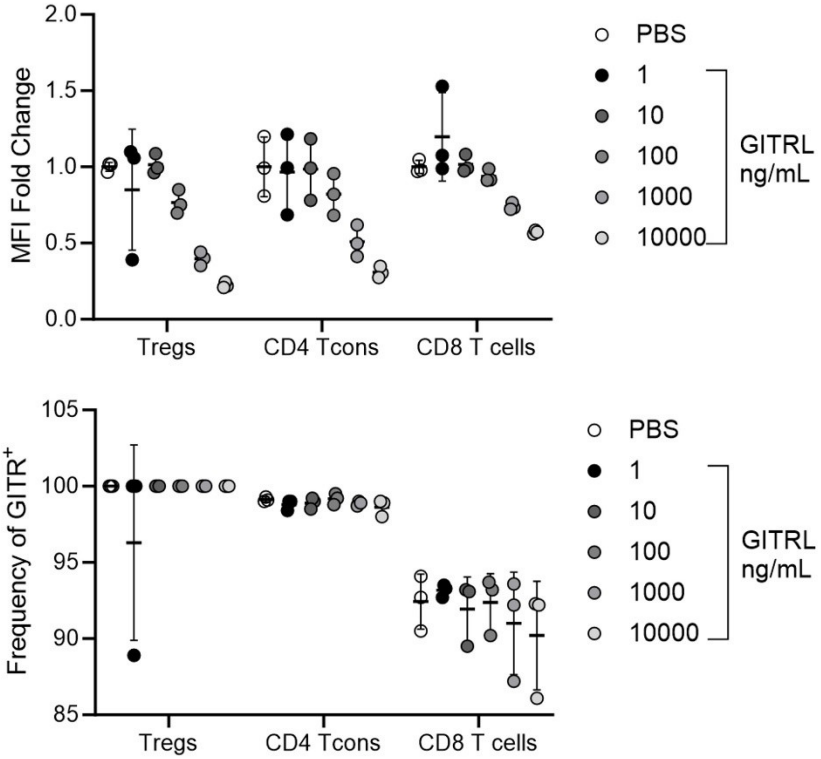
Supplementary data



Supplementary figure S1. RNA expression of TNFRSF1b in different cell types. mRNA expression data after RNA sequencing of TNFR2 obtained from the ImmGen Gene Skyline database. Expression levels normalized to CD25^{high} splenic Tregs.



Supplementary figure S2. Absolute number of myeloid cells in different organs 4 days after in vivo NewSTAR2 stimulation. Absolute numbers of the cell populations defined in Figure 10.



Supplementary figure S3. Fc(DANA)-muGITRL decreases the intensity of GITR expression detected by flow cytometry in a dose dependent manner without impact in cell frequencies positive for GITR. Splenocytes of B6a mice were isolated and $2x10^5$ cells were plated in 96-well plates. Fc-blocking with 1:20 NRS and different concentrations of Fc(DANA)-muGITRL was done for 15 minutes before staining for flow cytometry

Curriculum Vitae (next page)

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8. Publication list

Muhammad Shaikh, MSc, Maria Ulbrich, MSc, **Juan Gamboa Vargas, BSc**, Katja J Ottmüller, PhD, Duc Dung Le, PhD, Zeinab Mokthari, PhD, Joern Pezoldt, PhD, Hermann Einsele, MD, Burkhard Ludewig, PhD, Jochen Huehn, PhD, Andreas Beilhack, MD; Non-Hematopoietic Lymphoid Stromal Cells Prime Alloreactive CD4+ T Cells in Acute Graft-Versus-Host Disease, Blood, 2019, 134: 4421

Muhammad Haroon Shaikh, MSc, **Juan Gamboa Vargas, MSc**, Josefina Peña Mosca, MSc, Duc Dung Le, PhD, Hermann Einsele, MD, Burkhard Ludewig, PhD, Andreas Beilhack, MD; MHC Class II on Ccl19+ Reticular Cells Mitigates Graft-Versus-Host Disease Via Maintenance of Regulatory T Cells, Blood, 2020, 136: 23

Haroon Shaikh, **Juan Gamboa Vargas**, Zeinab Mokhtari , Katja J Jarick, Maria Ulbrich, Josefina Peña Mosca, Estibaliz Arellano Viera, Caroline Graf, Duc-Dung Le, Katrin G Heinze, Maike Büttner-Herold, Andreas Rosenwald, Joern Pezoldt, Jochen Huehn, Andreas Beilhack; Mesenteric Lymph Node Transplantation in Mice to Study Immune Responses of the Gastrointestinal Tract, Frontiers in Immunology, 2021, 12

Juan Gamboa Vargas, Jennifer Wagner, Haroon Shaikh, Isabell Lang, Juliane Medler, Mohamed Anany, Tim Steinfatt, Josefina Peña Mosca, Stephanie Haack, Julia Dahlhoff, Maike Büttner-Herold, Carolin Graf, Estibaliz Arellano Viera, Hermann Einsele, Harald Wajant Andreas Beilhack; A TNFR2-Specific TNF Fusion Protein With Improved In Vivo Activity, Frontiers in Immunology, 2022, 13

Mohamed A. Anany, Stefanie Haack, Isabell Lang, Julia Dahlhoff, **Juan Gamboa Vargas**, Tim Steinfatt, Lea Päckert, Daniela Weisenberger, Olena Zaitseva, Juliane Medler, Kirstin Kucka, Tengyu Zhang, Tom Van Belle, Luc van Rompaey, Andreas Beilhack and Harald Wajant; Generic design principles for antibody based tumour necrosis factor (TNF) receptor 2 (TNFR2) agonists with FcγR-independent agonism, Theranostics, 2024, 14(2), 496-509.