Activated protein C protects from GvHD by inducing regulatory T-cell expansion and signaling *via* the PAR2/PAR3 heterodimer in T-cells

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Abstract

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative therapy for many malignant and nonmalignant hematologic diseases. The T-cells from the donor allograft are the critical component for the success of this therapy. However, the donor T-cells target not only the diseased cells, but also the recipient`s healthy cells, which the donor's T-cells recognize as "foreign" or "non-self". The latter can cause severe immune mediated toxicity called graft versus host disease (GvHD). GvHD is a major complication of and limitation to allogeneic hematopoietic stem-cell transplantation (HSCT) therapy. The pathophysiology of GvHD is a multistep process involving tissue damage and an inflammatory cytokine "storm" induced by the pre-transplantation conditioning therapy which involves full body irradiation and/or chemotherapy. This excessive inflammatory conditions result in activation of donor derived Th1 $CD4^+$, cytotoxic $CD8^+$ and Th17 cells, which play an important role in the effector phase of GvHD pathophysiology. While GvHD is primarily a T-cell driven disease recent insights emphasize a role of endothelial dysfunction. Here we show that the cytoprotective protease activated protein C (aPC), which is generated by a healthy endothelium (via its interaction with thrombomodulin), ameliorates GvHD. aPC directly targets adaptive immunity by restricting allogenic T-cell activation *via* a heterodimer of the protease activated receptors PAR2/PAR3 on T-cells. Concomitant or pretreatment of human or mouse pan T-cells with aPC reduces the proliferation of T-cells. *In vitro* inhibition of PAR3, but not of PAR1, PAR2, or PAR4 on human pan T-cells using antibodies targeting the N-terminal and protease sensitive end of the corresponding PAR abolished the inhibitory effect of aPC. Intriguingly, blocking of PAR2 signaling in human pan T-cells with a blocking peptide abolished the inhibitory effect of aPC. Collectively, these observations suggest that aPC cleaves the N-terminal region of PAR3 and that the neo-N-terminal PAR-3 derived end generated by aPC interacts with PAR2 to induce intracellular signaling. Accordingly, co-immunoprecipitation confirmed a direct interaction of PAR2/PAR3 in human pan T-cells. Similarly, the inhibitory effect of aPC is abolished upon using mouse PAR3 deficient T-cells *in vitro*. Notably, in an *in vivo* model of GvHD *ex vivo*

pretreatment of pan T-cells or Tregs with aPC prior to transplantation is sufficient to protect from GvHD in mice. Of note, *ex vivo* pretreatment of PAR3 deficient T-cells or blocking of PAR2 signaling abolished the protective effect of aPC against GvHD in mice. This corroborates a function of the PAR2/PAR3 heterodimer for the aPC mediated protective effect against GvHD *in vivo*. *Ex vivo* pretreatment of pan T-cells prior to transplantation significantly reduces the proportion of Th1 $CD4^+$ and Th17 T-cells and increases the frequency of activated Tregs (CD4+CD127+FOXP3+) in mice. Accordingly, the pro-inflammatory cytokines IFNγ, TNFα, IL17A were reduced while IL10 was induced in these mice. Pretreatment of human pan T-cells likewise reduces Th1 (T-bet+) and Th17 (ROR-γt+) T-cells while increasing activated Tregs, which is accompanied by a corresponding cytokine profile (reduced IFNγ, TNFα, IL17A and increased IL10 expression). Transplantation of human HLA-DR4$^-$CD4$^+$ T-cells into "humanized" NSG-AB°DR4 mice triggers a robust GvHD, which is efficiently prevented by aPC-pretreatment of HLA-DR4$^-$CD4$^+$ T-cells. The protective effect of aPC on GvHD does not compromise the GvL effect in C57BL/6 → BALB/c chimeric mice co-injected with MLL-AF9 leukemic cells. These results establish that aPC expands the Treg population and that aPC-PAR2/PAR3 signaling in T-cells ameliorates GvHD. *Ex vivo* pretreatment of T-cells with aPC or aPC-based therapies may foster tolerance, providing a safe and effective approach to mitigate GvHD.

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Activated protein C protects from GvHD by inducing regulatory T-cell expansion and signaling *via* the PAR2/PAR3 heterodimer in T-cells

Zusammenfassung

Die allogene hämatopoetische Stammzelltransplantation (HSCT) ist eine potentiell kurative Therapie vieler maligner und nicht-maligner hämatologischer Erkrankungen. Die T-Zellen des Spender-Allografts sind entscheidend für den Erfolg der Therapie. Die T-Zellen des Spenders richten sich jedoch nicht nur gegen die kranken Zellen, sondern auch gegen die gesunden Zellen des Empfängers, die als „fremd“ erkannt und angegriffen werden. Dies löst eine schwerwiegende immunvermittelte Toxizität aus, die Graft-Versus-Host Disease (GvHD) genannt wird. Die GvHD ist eine gravierende Komplikation und schränkt die Anwendung der allogenen Stammzelltransplantation bei malignen hämatologischen Erkrankungen wesentlich ein. Die Pathophysiologie der GvHD ist ein mehrstufiger Krankheitsprozess. Die Konditionierungstherapie mit Bestrahlung und/oder Chemotherapie, die vor der Transplantation durchgeführt wird, verursacht einen Gewebeschaden und einen inflammatorischen Zytokinsturm. Diese Entzündungsreaktion induziert eine Aktivierung der vom Spender stammenden Th1 $CD4^+$-, zytotoxischen $CD8^+$- und Th17-Zellen, welche eine wichtige Rolle während der Effektorphase der GvHD spielen. Obwohl GvHD zuvorderst eine T-Zell vermittelte Erkrankung ist, legen neuere Daten eine Rolle der endothelialen Dysfunktion nahe. Wir zeigen hier, dass die endothelzell-abhängige zytoprotektive Gerinnungsprotease aktiviertes Protein C (aPC) die GvHD eindämmt. aPC greift direkt die adaptive Immunität an, indem es die allogene T-Zellaktivierung über die Heterodimerisierung der Protease-aktivierbaren Rezeptoren (PAR) PAR2 und PAR3 reduziert. Sowohl die gleichzeitige als die vorhergehende Behandlung von humanen und murinen Pan-T-Zellen mit aPC reduziert die T-Zellproliferation. Eine *in vitro* Vorbehandlung von humanen Pan-T-Zellen mit einem Antikörpern, der die partielle Proteolyse (und damit Aktivierung) von PAR3 inhibiert, hebt den inhibitorischen Effekt von aPC auf, wohingegen korrespondierende Antikörper gegen PAR1, PAR2 oder PAR4 keinen Effekt zeigen. Darüber hinaus hebt die Blockierung des PAR2 vermittelten Signaltransduktion mit einem blockierenden Peptid die Effekte von aPC auf humanen Pan-T-Zellen auf. Zusammenfassend deuten diese

Befunde darauf hin, dass aPC das N-terminale Ende von PAR3 spaltet, welches dann zu einer Trans-aktivierung von PAR2 und somit zur intrazellulären Signalvermittlung führt. Eine Interaktion von PAR2 und PAR3 auf humanen T-Zellen konnte mittels Ko-Immunopräzipitations bestätigt werden. Darüberhinaus sind die inhibitorischen Effekte von aPC in PAR3 defizienten T-Zellen aufgehoben. Interessanterweise reicht eine Vorbehandlung mit aPC von Pan-T-Zellen oder von Tregs *ex vivo* vor der Transplantation aus, einen Schutz vor der GvHD zu vermitteln. Die protektiven Effekte von aPC bzgl. GvHD sind bei *ex vivo* mit aPC vorbehandelten PAR3-defizienten Maus-T-Zellen aufgehoben. Die Blockierung der PAR2 vermittelten Signaltransduktion in murinen T-Zellen hebt ebenso die protektiven Effekte von aPC auf die GvHD auf. Diese Befunde legen nahe, dass der PAR2/PAR3 Heterodimer für die protektiven Effekte von aPC bzlg. GvHD erforderlich ist. Die Vorbehandlung von Pan-T-Zellen mit aPC *ex vivo* reduziert in Mäusen signifikant den Anteil der Th1 $CD4^+$ und Th17 T-Zellen und erhöht den Anteil der aktivierten Tregs (CD4+CD127+FOXP3+). Gleichzeitig werden durch aPC die pro-inflammatorischen Zytokine IFNγ, TNFα und IL17A reduziert und IL10 erhöht. Ebenso reduziert die Vorbehandlung humaner Pan-T-Zellen mit aPC Th1 (T-bet+) und Th17 (ROR-γt+) T-Zellen, während aktivierte Tregs induziert werden. Dies geht mit einem entsprechenden Zytokinprofil einher (niedrige IFNγ, TNFα, IL17A und erhöhte IL10 Expression). Die Transplantation von humanen HLA-DR4-CD4+ T-Zellen in „humanisierte“ NSG-AB°DR4 Mäuse löst eine GvHD aus, welche durch die Vorbehandlung von HLA-DR4-$CD4^+$ T-Zellen mit aPC verhindert werden kann. Die protektiven Effekte von aPC auf die GvHD beeinträchtigen nicht den therapeutisch gewünschten Graft-versus-Leukemia (GvL) Effekt in chimären C57BL/6→BALB/c Mäusen, die mit MLL-AF9 Leukämiezellen ko-injiziert wurden. Die hier vorliegenden Ergebnisse belegen, dass aPC die Treg-Population expandiert und dass aPC via eine PAR2/PAR3 vermittelten Signaltransduktion in T-Zellen die GvHD abschwächt. Die *ex vivo* Vorbehandlung von T-Zellen mit aPC oder aPC-basierten Agenzien kann potentiell die körpereigene Toleranz gegenüber dem Transplantat erhöhen und somit das Auftreten einer GvHD vermindern.

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List of abbreviations:

AgPC	antigen presenting cells
aPC	activated protein C
APC^{high}	transgenic mice with constitutive elevated aPC plasma levels
ApoER2	apolipoprotein E receptor 2
α1-PI	α1-proteinase inhibitor
BM	bone marrow
BMT	Bone marrow transplantation
ECM	extracellular matrix
EPCR	endothelial protein C receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GP	glycoproteins
GPCRs	G protein-coupled receptors
GvHD	graft versus host disease
GvL	graft versus leukemia effect
HDAC	histone deacetylase
HLAs	human leukocyte antigens
HSCT	hematopoietic stem-cell transplantation
ICAM-1	Intercellular Adhesion Molecule-1
IFNγ	interferon γ
I/R	ischemic reperfusion
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
miH	minor histocompatibility
mRNA	messenger RNA
MLL-AF9	mixed lymphocyte lineage
PAI-1	plasminogen activator inhibitor 1
PAR	protease activated receptor
PC	protein C
PHA	Phytohaemagglutinin A

PMA	Phorbol myristate acetate
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
SEM	standard error mean
sTM	soluble thrombomodulin
SI	small intestine
S1P1	sphingosine-1-phosphate receptor 1
TBI	total body irradiation
TM	thrombomodulin
TNFα	tissue necrosis factor-α
TCR	T-cell receptor
Tregs	regulatory T-cells
VCAM-1	vascular cell adhesion molecule-1
vWF	von Willebrand factor
wt	wild type

1 Introduction

1.1 Graft versus Host Disease

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative therapy for many malignant and nonmalignant hematologic diseases. The T-cells derived from the donor allograft are the critical component for the success of this therapy. The donor T cells attack and eliminate the leukemic cells. This desired effect is called the "Graft versus Leukemia effect" (GvL). Unfortunately the recipient's healthy and normal tissues are likewise frequently recognized as "foreign" or "non-self" by donor T-cells and are hence being attacked by donor T-cells. This results in a severe immune mediated toxicity termed "Graft versus Host Disease" (GvHD). The success of HSCT is dependent upon the development of effective therapies, which mitigate GvHD without compromising the GvL effect. Unfortunately, the majority of efforts to reduce the incidence of GvHD also diminished the GvL response, increasing the tumor relapse rate. Therapies differentially targeting GvHD while sparing the GvL effect are missing [1, 2].

The graft versus host reaction was initially recognized after transplanting irradiated mice with allogeneic bone marrow and spleen cells [3]. Although the transplanted mice recovered from radiation injury and marrow aplasia, they subsequently died from a "secondary disease" [3], characterized by diarrhea, weight loss, skin injury, and liver abnormalities following the transplantation. This phenomenon was subsequently recognized as GvHD. For the development of GvHD three requirements were formulated by Billingham [4]. First, the graft must contain immunologically competent cells, which are now recognized as mature T-cells. It has been established that the severity of GvHD (but unfortunately also the efficacy of the GvL effect) in both experimental and clinical allogenic bone marrow transplant (BMT) correlates with the number of transplanted donor T-cells [5, 6]. Second, the recipients must be immunecompromised, as the allograft will be rejected by a patient with a normal immune system. Immunosuppression of the recipient is achieved with chemotherapy and/or radiation before stem cell infusion [7]. Third, the tissue antigens expressed by the recipient must be different from that of donor transplant.

The intense research in this area has led to the discovery of the major histocompatibility complex (MHC) [8]. Human leukocyte antigens (HLAs) are highly polymorphic proteins which are expressed on the cell surfaces of all nucleated cells in the human body and define the

immunological “self” of an individual. The HLAs mediate the allogeneic activation of donor T-cells [8, 9].

1.2 Pathophysiology of GvHD

Two important principles must be taken into consideration when looking into the pathophysiology of acute GvHD. First, acute GvHD represents an exaggerated but otherwise normal inflammatory responses by the donor’s lymphocytes that have been transplanted into a “foreign” (the host’s) environment. This inflammatory response is directed against antigens (alloantigens: MHC proteins) that are ubiquitously expressed by host tissues. Second, donor lymphocytes encounter tissues in the recipient that are typically profoundly damaged. Thus, the underlying disease, infections prior to the HSCT, and the intensity of the conditioning regimen all result in substantial changes not only of immune cells but also of endothelial and epithelial cells. Collectively, these pro-inflammatory changes in the host tissues prior to transplantation lead to the activation and proliferation of donor lymphocytes after they are infused into the “foreign” host body. Thus, the pathophysiology of acute GvHD can be considered as a distortion of normal inflammatory cellular responses that are mediated not only by donor T-cells, but also involve multiple other innate and adaptive cells and mediators [10].

The principal target organs of GvHD are skin, gut, and liver. These target organs are extensively exposed to endotoxins and other bacterial products that can trigger and amplify local inflammation. This exposure distinguishes them from organs like the heart and kidneys, which are not targeted in acute GvHD. Several studies suggest that the lung is an organ of controversy in this regard. While the lungs are not classic GvHD targets there is some accumulating evidence suggesting that the lung is susceptible to some extent along with the skin, gut, and liver in acute GvHD [7, 11]. Since skin and gut function as primary barriers to infection, these target organs have large populations of professional antigen-presenting cells (AgPCs), such as macrophages and dendritic cells, which enhance the graft-versus-host (GVH) reaction. Recent findings suggest that acute experimental and clinical GvHD is manifested and maintained by excessive production of cytokines, the central regulatory molecules of the immune system, as well as various other cellular effectors [12-14]. The above mentioned pathophysiologic factors in acute GvHD can be conceptually divided into three sequential phases (Figure 1, for details see the following sections) [12, 15].

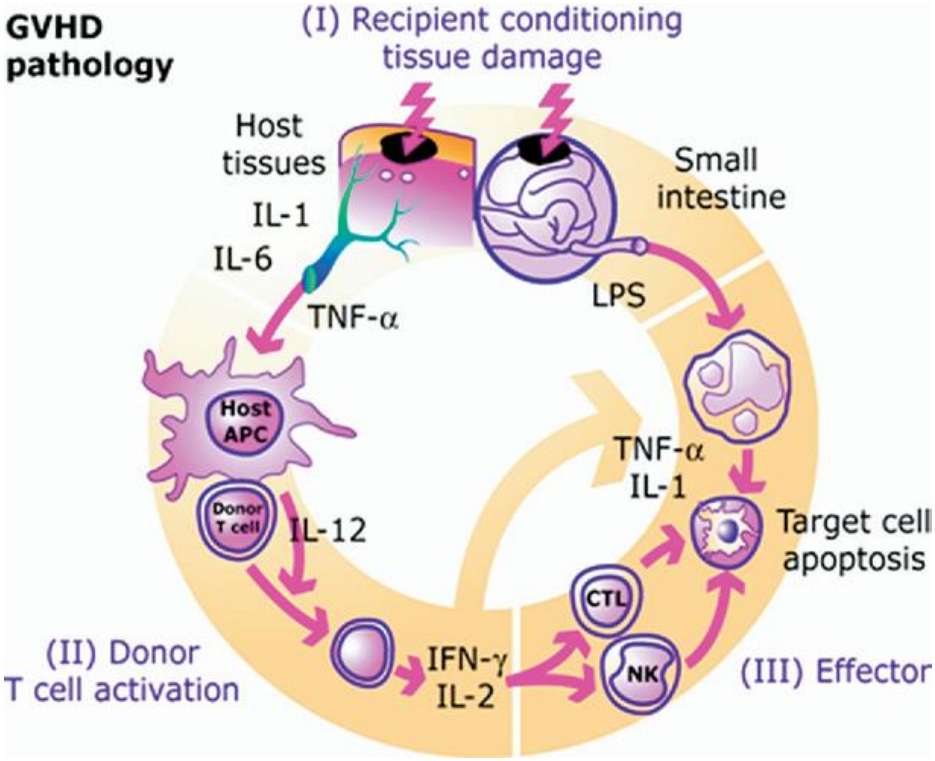

Figure 1: Illustration of the three phases in GvHD pathopysiology (Sun *et al*, 2007)

1.2.1 Phase 1: Conditioning regimen/activation of antigen presenting cells (AgPCs)

The earliest phase of acute GvHD commences before the transplantation of donor cells. The underlying disease, infections, and conditioning regimens prior to BMT, which include total body irradiation and/or chemotherapy, cause profound damage to the host tissues which in turn set into motion the initial phase of acute GvHD [16-20].

The BMT conditioning regimen is an important factor in initiating the pathogenesis of acute GvHD, as it can damage and activate host tissues, including the intestinal mucosa, liver, and other tissues. Upon activation these host cells secrete various inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin IL-1 [18], and growth factors such as granulocyte-macrophage colony stimulating factor (GM-CSF) [21-23]. Various inflammatory cytokines that are released during this phase activate AgPCs by upregulating adhesion molecules [10] and major histocompatibility complex (MHC) antigens [11–15]. This in turn enhances the recognition of host MHC or minor histocompatibility antigens by mature donor T-cells after they

are infused into the host. Various animal models and clinical studies support a relationship between conditioning intensity, inflammatory cytokines, and GvHD severity [24].

Dendritic cells (DC) are the most potent AgPC and play an important role in the induction of GvHD [25]. Experimental data also suggests that GvHD can be regulated both qualitatively and quantitatively by modulating distinct DC subsets [26]. Other professional AgPCs such as monocytes/macrophages or semiprofessional AgPCs might also play a role in this phase. Recent data suggest that host-type B cells might also play a regulatory role under certain conditions [27]. Several experimental evidence suggest that host APgCs are particularly important in GVH reactions, and eliminating host AgPCs of hematopoietic origin can promote tolerance and reduce GvHD [28, 29].

1.2.2 Phase 2: Donor T cell activation and differentiation

The second phase of acute GvHD is marked by the activation of individual donor T-cells by antigen presenting cells and the subsequent proliferation and differentiation of these activated T-cells. After entering into the recipient blood stream the donor $CD4^+$ T-cells interact with MHC class II molecules (HLA-DR -DP, -DQ) of the host's AgPCs, whereas a $CD8^+$ T-cells interact with MHC class I antigens (HLA-A, -B, -C). In cases of MHC mismatch between donor and recipient, donor T-cells recognize host MHC molecules as foreign, and this can result in dramatic GVH reaction even against single mismatched MHC antigens. If the recipient's and donor's MHC are identical GvHD may still occur through recognition of different peptides, so-called minor histocompatibility (miH) antigens. Therefore, one potential area to interfere with signal recognition is at the level of MHC-peptide-TCR interaction [30, 31].

1.2.2.1 T cell adhesion and costimulation

After the transplantation of donor T-cells into the host these T-cells are transported within the host's vasculature. Here the T-cells potentially encounter the first alloantigen expressed within the capillary bed of blood vessels. The capillary bed provides an extensive contact area for donor-derived T-cells with alloantigen presenting cells. Consequently, vascular antigens have been studied as potential miH antigens, and various, albeit not all, studies suggest that they may be important in the pathogenesis of GvHD [32-34]. In order to get activated, the T-cells must recognize and adhere long enough to the antigen presenting surface. Various integrins and

selectins additionally play an important role in the adhesion of T-cells to the antigen presenting surface [30]. Furthermore, these molecular anchors provide the possibility for T-cells to egress from the circulation into lymph nodes, spleen, reticuloendothelial tissues, and other target organs. T-cell activation requires two signals. For donor T-cells, the first signal is provided by the interaction between TCR and the allo-peptide bound to the host or donor MHC. The second or costimulatory signal is provided by AgPCs [35, 36]. The latter (second) signal determines the outcome of the activation sequence, which may result in complete activation, partial activation, or a long-lasting state of antigen-specific unresponsiveness, termed "anergy". Costimulatory signals can be provided by various ligands for resting T-cells, antigen-primed T lymphocytes, and T-helper cell clones. B7 antigens are the best-characterized costimulatory molecules and they bind to two T-cell surface receptors, CD28 and CTLA-4. The combined signal from the TCR, a costimulatory signal from CD28, and an inhibitory signal from CTLA-4 determines the outcome of T-cell activation. Costimulatory requirements for T-cells depend on their state of activation-induced maturation. Vascular cell adhesion molecule 1 (VCAM-1) and intercellular activation molecule-1 (ICAM-1) (and possibly other molecules) have been demonstrated to provide a costimulatory signal or signals for resting (unprimed) CD4 T-cells. *In vivo* activation of T-cell is very complex and depends on the state of activation of the T-cell (resting vs. activated, naive vs. mature) as well as the nature of the AgPC (professional vs. nonprofessional, resting vs. activated). Interactions of CD40 and its ligand (CD40L) are also important costimulatory signals for T-cell activation. Human endothelial cells express CD40, and the interaction with CD40L on the T-cell can induce endothelial cell activation [35, 37, 38]. This activation can lead to increased expression of ICAM-1 (or CD54), E-selection (CD62E), and VCAM-1 on endothelial cells. These interactions suggest a mechanism whereby activated $CD4^+$ T-cells may increase their own response by causing increased expression of endothelial cell surface adhesion molecules.

1.2.2.2 Regulatory T cells

Tregs serve a crucial role in maintaining peripheral self-tolerance. Natural Tregs are characterized by intracellular expression of the transcription factor forkhead box P3 (FOXP3) [39]. Recent advances indicate that distinct subsets of regulatory $CD4^+CD25^+$, $CD4^+CD25^-$ $IL10^+$ Tr cells, γδT cells, double negative DN^- T-cells, natural killer NK T-cells, and regulatory DCs also control immune responses by either induction of anergy or by actively suppressing the

alloreactive T-cells [40, 41]. Importantly, donor $CD4^+CD25^+$ T-cells suppress the early expansion of alloreactive donor T-cells and mitigate acute GvHD without compromising the graft-versus-leukemia (GVL) effect [42, 43]. However, evaluating this in patients will require methods for generating high ratios of Tregs to T effectors that are sustainable in vivo. One approach is ex vivo expansion with adoptive transfer. In the setting of HLA-mismatched haploidentical HSCT, infusion of donor Tregs prior to stem cells resulted in very low rates of acute GvHD, without standard prophylactic immunosuppression [44]. Unfortunately the major limitation to a broader clinical application of Treg adoptive transfer is the failure to efficiently enrich Tregs for therapeutic purposes [45]. As further refinements occur, production of more stable populations of Tregs is anticipated, which will enable more definitive clinical studies of GvHD prevention.

Another approach to improve the availability of Tregs is the conversion of conventional T-cells into inducible Tregs (iTregs). The process of conferring antigen specificity to iTregs, as a means of improving the efficiency and selectively of their suppressive function is also being explored. For example, iTregs specific for the naturally occurring male Y chromosome minor histocompatibility antigen (miHAg), termed HY, can be generated to prevent experimental GvHD [46]. Whether iTregs and conventional T-cells require similar antigen specificity to optimize effectiveness and how Tregs discriminate between beneficial (i.e. leukemia specific) versus deleterious (i.e. GvHD-specific) mHAs is currently unknown.

1.2.2.3 T-cell cytokines in GvHD

Various experimental models have provided evidence that excessive or dysregulated cytokine production is associated with GvHD. Pro-inflammatory cytokines like interferon γ (IFNγ) and IL2 that T-cells secrete are critical mediators of acute GvHD. Lymphocytes from animals with GvHD secrete significantly greater amounts of IFNγ in comparison to controls without GvHD and the increased IFNγ serum levels are associated with acute GvHD [47-49]. Additionally the elevated levels of IFNγ prime the macrophages to secrete other pro-inflammatory cytokines [50] that induce injury to the skin and the gastrointestinal tract [51, 52].

Tissue necrosis factor α (TNFα) is another well-established pro-inflammatory cytokine that causes organ damage in experimental acute GvHD. Mice transplanted with allogeneic bone marrow and T-cells develop severe skin, and lung lesions that are associated with high levels of TNFα messenger RNA (mRNA) in these tissues [2]. Additionally, application of anti-TNFα antibodies ameliorate the injury of target organs in experimental mice with GvHD. Various

studies in humans have confirmed that elevated serum levels of TNFα in patients are associated with GvHD [53]. Various blood mononuclear cells also secrete TNFα that aggravates GvHD [30]. In humans anti-TNFα monoclonal antibodies temporarily mitigate steroid-resistant acute GvHD [54].

1.2.3 Phase 3: Effector phase

The effector phase is a very complex cascade of multiple cellular and inflammatory effectors that lead to the damage of GvHD target organs. Effector mechanisms of acute GvHD can be grouped into cellular effectors (e.g. $CD4^+$T-cells and cytotoxic $CD8^+$T-cells (CTLs)) and inflammatory effectors such as cytokines.

1.2.3.1 Cellular effectors

Upon activation donor $CD4^+$CTLs mediate damage in host tissue preferentially *via* the Fas-FasL pathway, whereas $CD8^+$CTLs primarily induce damage in the host tissues *via* the perforin–granzyme pathway [55].

1.2.3.2 Inflammatory effectors

Initially it was hypothesized that only the cytolytic function of cytotoxic T lymphocytes (CTLs) directly causes the majority of tissue damage in GvHD target organs [56]. But later in several animal models it was found that large granular lymphocytes (LGLs) or natural killer (NK) cells are also prominent components of the effector arm in GvHD, contributing to the pathologic injury in GvHD target organs [56, 57]. LGLs are recruited to the target organs by cytokines released by T-cells as LGLs do not recognize HLA proteins as targets. Mononuclear phagocytes are primed by Th1 cytokines like IFNγ during phase 2 and they also have an important role in this phase of acute GvHD. Monocytes also receive a second, triggering signal to secrete the inflammatory cytokines TNFα and IL-1. This stimulus is provided by lipopolysaccharide (LPS, endotoxin), which can leak through the intestinal mucosa damaged by the conditioning regimen and subsequently stimulate gut-associated lymphocytes and macrophages [50]. The LPS can also reach skin tissues and may further stimulate keratinocytes, dermal fibroblasts, and macrophages to produce similar cytokines in the dermis and epidermis [21-23]. Some experimental data suggest that donor cells can produce TNFα in response to LPS which is considered to be an important risk factor for GvHD severity independent of the T-cell response to host antigens [58]. TNFα can cause direct damage of GvHD target organs either by inducing necrosis of target cells,

or by inducing apoptosis (programmed cell death). Apoptosis is very critical in GvHD in the large intestine [76] and skin [59, 60]. In addition to these pro inflammatory cytokines, excess nitric oxide (NO) produced by activated macrophages may contribute to the deleterious effects on GvHD target tissues [53, 61, 62]. Thus, the induction of inflammatory cytokines may synergize with the cellular damage caused by CTLs and NK cells [56, 63], resulting in the amplification of local tissue injury and further promotion of an inflammatory response, which ultimately lead to the observed target tissue destruction in the BMT host.

Tissue injury is known to cause **coagulation activation**. The interaction of coagulation and innate immune response is well established. Importantly, coagulation proteases can directly modulate cellular function via receptor dependent mechanism. One coagulation protease known to regulate innate immune responses and being signaling competent is the coagulation protease **activated protein C (aPC)**. aPC is generated by activation of endothelial protein C receptor (EPCR) bound protein C zymogen which is accomplished by thrombomodulin-thrombin complex (details in section 1.5).

1.3 Thrombomodulin and its association in steroid-refractory GvHD

Thrombomodulin (TM), a type 1 transmembrane molecule expressed predominately on endothelial cells. The important function of TM in regulating the coagulation system, inflammation, fibrinolysis and cellular proliferation is well established. It has been reported that vascular endothelial cells can also be targeted and severely damaged during acute GvHD [64, 65]. Endothelial cell dysfunction can be detected by elevated serum thrombomodulin (sTM) and high sTM levels have been reported to be associated with complications after HSCT [64, 66]. Luft *et al.* has shown that in patients who do not clinically respond to standard steroid therapy to mitigate GvHD (refractory GvHD) have significantly high levels of sTM [64].

1.4 Blood Coagulation Sytem

The first known reference to blood coagulation was probably made by Hippocrates (ca. 460–377 B.C.), who observed that, when blood was collected from an animal, it congealed on cooling [67]. In humans and other vertebrates, the blood coagulation system is a first line of defense against trauma of the vascular system. The vertebrate blood coagulation system consists of cellular elements (blood platelets, white cells, to some extent red cells and microvascular

remnants or microparticles) and proteins (the coagulation enzymes and co-factors and a number of anticoagulant proteins). The blood coagulation system comprises three basic elements: 1. platelet adhesion, activation and aggregation, 2. fibrin formation, and 3. fibrinolysis. When blood coagulation is triggered, cells and membrane remnants interact with coagulation factors assembling effective macromolecular complexes that contribute to the formation of fibrin molecules [68, 69].

Injury to the blood vessel wall causes disruption of its endothelial layer, resulting in the exposure of the underlying extracellular matrix. Both von Willebrand factor (vWF) and collagen are present within the matrix, and following exposure they will bind to specific receptors, glycoproteins (GP), present on the surface of the platelets. Other glycoproteins are involved in the adhesion of platelets to the vessel wall depending on the flow within the vessel. Formation of the tissue factor-factor VII(a) complex drives the formation of thrombin and fibrin. Platelet adhesion, activation, and interactions with leukocytes accelerate the process of thrombin formation yielding activated coagulation proteases such as factor XIa and VIIIa that amplify thrombin generation [67].

1.5 The protein C pathway

The important components of the protein C system include protein C (PC), protein S, thrombomodulin, endothelial protein C receptor (EPCR), protease activated receptors (PARs), e.g. PAR1 and PAR3 [70, 71]. Protein C is a vitamin K-dependent zymogen and plays a key role in the regulation of blood coagulation. The molecule was discovered in 1976 by Johan Stenflo who purified a protein in “pool C” of a chromatography elute derived from bovine plasma. The function of this protein was revealed few years later when Charles Esmon and colleagues discovered that its activated form was a physiological anticoagulant. PC is derived from the human PROC gene on chromosome 2 (2q13-q14) which contains nine exons. Human PC is a 62 kDa protein and consists of 419 amino acids. There are four major moieties that make up the PC molecule; the Gla domain, two EGF-like regions, a small activation peptide, and the active serine protease domain [72]. Activation of the EPCR-bound protein C zymogen is accomplished by thrombomodulin-bound thrombin (Figure 2). After activation the active protease, referred to as activated Protein C (aPC), may directly engage into (EPCR-dependent) signaling or dissociate from EPCR and diffuse to other sites to interact with its substrates and other cellular receptors. It has been established that aPC induces 3 major types of activity (Figure 2).

- Antithrombotic,
- Cytoprotective, and
- Regenerative

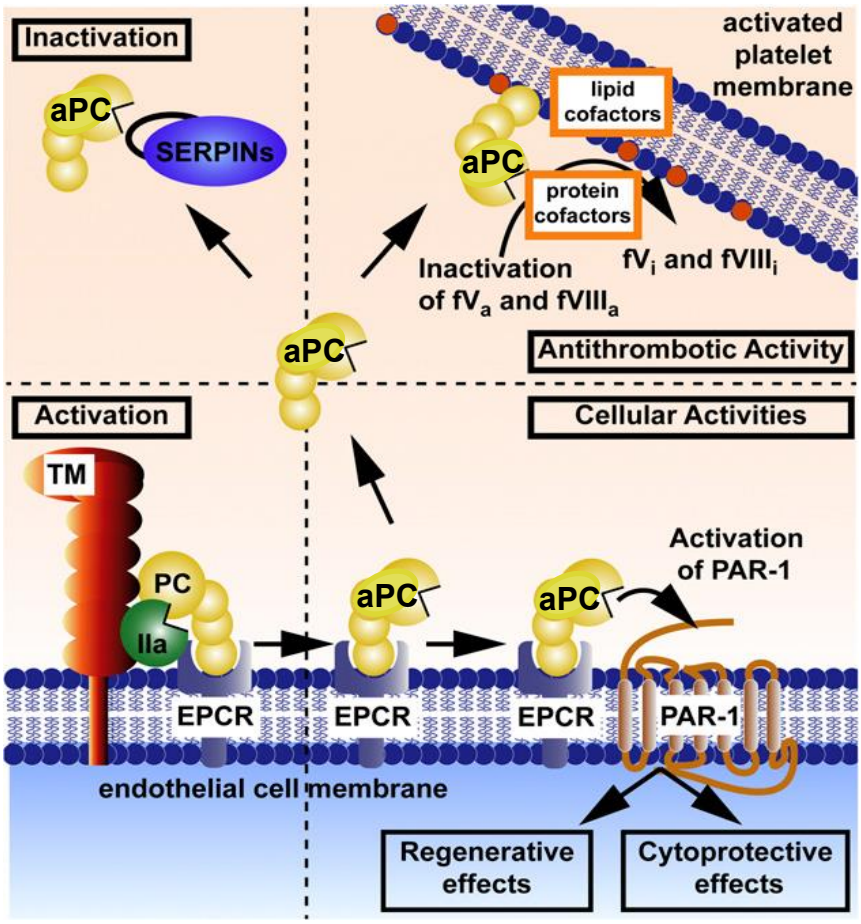

Figure 2: Protein C activation and aPC's multiple activities.

Activation of the EPCR-bound protein C (PC) zymogen (bottom left) is accomplished by thrombomodulin (TM)-bound thrombin (IIa). Anticoagulant activity (upper right) is based on limited proteolysis, causing irreversible inactivation of the activated clotting factors (f)Va and fVIIIa (resulting in fVi and fVIIIi). The anticoagulant function requires negatively charged lipid-surfaces (e.g. on activated platelets, as shown in the figure) and protein cofactors (e.g. protein S). Cytoprotective actions of aPC (bottom right) include its antiapoptotic and anti-inflammatory activities, its ability to stabilize endothelial barriers to prevent vascular leakage, and its ability to alter gene expression profiles. aPC's various cytoprotective activities and regenerative effects require frequently (e.g. on endothelial cells) EPCR and PAR1. Not depicted here is the fact that aPC's cytoprotective or regenerative actions may also require PAR3 and/or other receptors,

depending on the biological context, cell type, and organ. Inactivation of circulating aPC by plasma serine protease inhibitors (SERPINs; upper left) is a major clearance mechanism of aPC (Griffin *et al,* 2015).

The anticoagulant activity of aPC is dependent on limited proteolytic inactivation of the activated clotting factors Va and VIIIa generated during the coagulation process [73]. aPC can directly act on cells and mediate multiple cytoprotective effects that include (1) alteration of gene expression, (2) anti-inflammatory activities, (3) antiapoptotic activities, and (4) protection of endothelial barrier function that stabilizes the endothelial barrier and prevents vascular leakage. To carry out these cytoprotective activities aPC generally needs to be bound to EPCR in order to activate PAR1. In a cell, tissue and context specific fashion other receptors may also be required for aPC signaling. Such receptors include PAR3, sphingosine-1-phosphate (S1P) receptor 1 (S1P1), Mac-1, apolipoprotein E receptor 2 (ApoER2), epidermal growth factor receptor, Tie2, and other receptors. Mechanistically, the beneficial actions of aPC have been linked to altered gene and protein expression [74, 75]. In the context of aPC's regenerative properties it has been established that aPC requires additional receptors that include PAR3 and S1P1 for neurogenesis [76] and PAR2, epidermal growth factor receptor, and Tie2 for wound healing [77, 78]. Circulating aPC can be inactivated by several plasma serine protease inhibitors (serpins), in particular the protein C inhibitor (Figure 2, upper left), but also by α1-proteinase inhibitor (α1-PI), plasminogen activator inhibitor 1(PAI-1). These serpins result in a rapid inactivation of aPC.

In humans the normal level of plasma protein C is 70 nM, and human plasma contains approximately 40 pM of circulating aPC. The half-life of protein C in healthy subjects is 8 hours, whereas pharmacologic aPC has a half-life of 15 to 20 minutes and murine aPC has a half-life of 12 to 14 minutes [79].

1.6 Protease-activated receptors

Protease-activated receptors (PARs) belong to the family of G-protein coupled receptors (GPCRs) [80]. Four PAR-family members (PAR1–4) are known. As opposed to other GPCRs, which are typically activated by binding of a ligand, PARs are activated by proteolytic cleavage of their N-terminal extracellular end.

Approximately 1200 genes (4.5% of all human genes) encode proteases in the human genome, alluding to the importance of proteases in the human body under physiological and pathophysiological conditions. However, the role of protease signaling in innate and adaptive

immunity has not been investigated extensively so far. Proteases can initiate cell signaling by various ways, but PARs are believed to be crucial receptors through which proteases mediate the signaling events [81, 82]. Signaling via PARs involves a unique mechanism of receptor activation wherein the receptor`s N-terminal end is cleaved by the protease. This results in exposure of a new, previously cryptic sequence, which remains tethered to the receptor and acts as a receptor-activating ligand, named "tethered ligand" [81, 82]. The newly generated tethered ligand interacts with the receptor itself by binding to the extracellular loop 2 [83-86]. Importantly, the tethered ligand may also interact with the extracellular loop 2 of another PAR-receptor, resulting in cross-activation within a PAR-heterodimer. Furthermore, some proteases (e.g.) may cause receptor inactivation by cleaving the N-terminal end downstream of the tethered ligand sequence and thereby removing the tethered ligand sequence, making further proteolytic activation of PARs impossible. Thus proteases are involved in both activation and inactivation of PARs.

All four PARs have been cloned and characterized [81, 82]. PARs 1, 3, and 4 were initially identified as targets for thrombin but can also be activated by other proteases such as trypsin or cathepsin-G (CG). In contrast, PAR2 is not cleaved by thrombin but can be activated by trypsin, mast cell tryptase, leukocyte proteinase-3 (PR3), and bacteria-derived enzymes [81, 82, 87]. PARs can also be activated by synthetic agonist peptides (so-called PAR-APs) without involving their cleavage. PAR-APs have sequences homology to the tethered ligands, bind directly to the extracellular receptor loop 2 and thus cause activation of PAR-signaling [81, 82]. PAR-APs have been successfully employed to investigate the role of PAR signaling, as they allow specific PAR-activation without independent effects mediated by the corresponding protease. PAR1, -2, and -4 mediated signaling are now firmly established, but the role of PAR3 still remains to be investigated. Importantly, our group previously identified a novel signaling mechanism underlying the cytoprotective effect of aPC in podocytes. Here Thati *et al.* demonstrated that aPC binds to and cleaves PAR-3 in podocytes, which induces heterodimerization of PAR-3 with PAR-2 (human) or PAR-1 (mouse) in podocytes [88]. The tethered ligand of PAR-3 then cross-activates the other PAR within the receptor heterodimer. Others also demonstrated that PAR3 is signaling incompetent by itself and currently PAR3 is viewed as a co-receptor for PAR1, PAR2 or PAR4 [89-91].

1.7 aPC and protease-activated receptors

In the presence of EPCR, aPC can activate PAR1 on endothelial cells [92-95]. In endothelial cells and in the presence of EPCR aPC induces PAR1 dependent MAPK phosphorylation, increases intracellular calcium fluxes, and modulates PAR1 specific gene expression [74, 93, 95]. This establishes that aPC can induce biologically relevant intracellular signaling transduction through PAR1 in endothelial cells. These effects depend, however, on the presence of EPCR.

It has been demonstrated that PAR1 and EPCR are also required for *in vivo* anti-inflammatory and neuroprotective effects of aPC. Kerschen *et al*. demonstrated an essential role for EPCR and PAR1 on hematopoietic cells and identified EPCR-expressing dendritic immune cells as a critical target of aPC. aPC reduces the mortality of endotoxemia in mice, at least in part via EPCR and PAR1 on hematopoietic cells [75]. Furthermore, by using murine aPC and mice with targeted gene deletions of PARs or mice that were severely deficient in EPCR it has been established that PAR-1 and EPCR are required for pharmacological beneficial effects of aPC in *in vivo* mouse models for ischemic stroke [96-98].

aPC can also cleave PAR2 [95]. However, its physiological relevance function has yet to be fully determined. Kaneider *et a.l* [99] showed that the barrier-protective effects of aPC are abolished by silencing of PAR1 or PAR2 with small-interfering RNA (siRNA), suggesting that the barrier protective effects of aPC require both PAR1 and PAR2. While the majority of reports cast PAR2 as pro-inflammatory, others show that PAR2 agonists are beneficial in several mouse models that involve inflammation or ischemia [83].

1.8 Role of aPC in various diseases: preclinical studies

Severe sepsis is a fatal condition characterized physiologically by an aberrant systemic inflammatory response and microvascular dysfunction. In mice low levels of endogenous PC exacerbate endotoxic [100] and septic [101] responses. In septic human a reduction of circulating aPC is evident, which appears to be due to both decreased PC activation due to a loss of TM-function and lower PC levels. Accordingly, high plasma levels of soluble TM (reflecting loss of endothelial TM function), low PC blood levels, and decreased activation of PC to aPC are strongly correlating with sepsis prognosis [102, 103]. Evidence from the Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) and Evaluation of Recombinant Human Activated Protein C (ENHANCE) clinical trials suggests that administration of recombinant human aPC

may reduce mortality in a subset of patients with severe sepsis [104-106]. Based on these observation aPC was distributed as a drug for septic patients. However, due to an increased risk of hemorrhage aPC was later withdrawn from the market.

Numerous preclinical studies have been carried out using human or recombinant murine wt-aPC in various injury models like kidney ischemic reperfusion (I/R), cardiac I/R, nephropathy models, EAE etc. [107-113]. In these pre-clinical studies diverse and remarkable beneficial effects of aPC have been reported. It was found that pharmacologic aPC promotes *in vivo* tissue homeostasis and tissue repair in the brain, heart, lungs, kidney, gastrointestinal tract, spleen, eye, bone marrow, and skin [70, 96, 107, 113-116]. These preclinical research data further establish that healing and tissue homeostasis in almost every organ of the body is promoted by pharmacologic aPC. The broad beneficial effect of pharmacologic aPC in *in vivo* studies mirrors the *in vitro* beneficial effects of aPC on endothelial cells, epithelial cells, neurons, astrocytes, keratinocytes, podocytes, dendritic cells, osteoblasts, fibroblasts, and others. The ability of aPC to alter expression of genes in different cell types is also likely key to many of aPC's benefits [107]. Of note, the role of aPC in regulating the function of adaptive immune cells like T-cell and various T-cell mediated diseases has not been investigated in relevant disease models so far. Furthermore, studies on T-cells have been restricted to Jurkat cells, which are a leukemia derived immortalized T-cell line. Hence, the role of aPC for T-cell biology remains largely unknown.

1.9 The role of PAR activation in the regulation of functional responses of human and nonhuman immune cells

The schematic representation of effect of activation of PAR1 and PAR2 on the function of human immune cells is illustrated in figure 2 (PAR1) and figure 3 (PAR2).

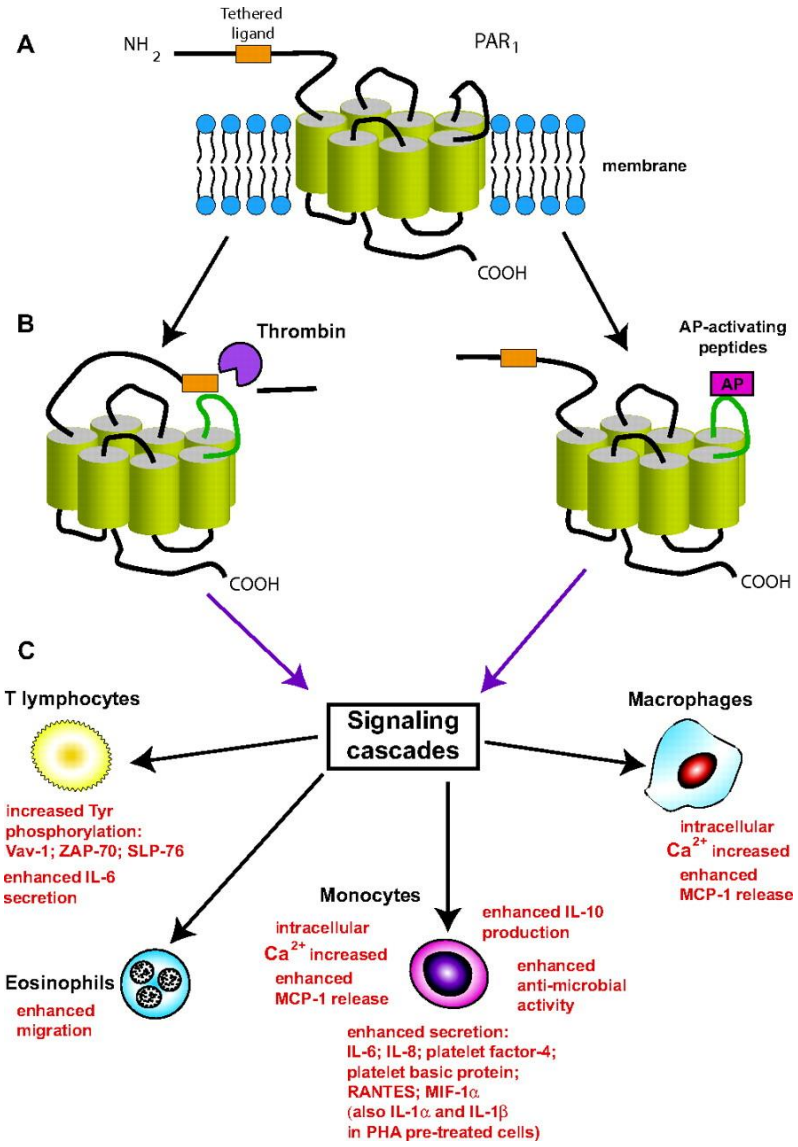

Figure 2: Scheme of PAR1 activation and PAR1-triggered responses in human immune cells

(A) In the inactive status the N-terminal-tethered ligand of PAR1 remains cryptic and cannot interact with the second extracellular loop of the receptor. (B) Suitable serine proteases (e.g. thrombin) cleave the N-terminal end of the receptor thus unmasking the tethered ligand. The unmasked tethered ligand interacts with the second extracellular loop of the same receptor, thus triggering signaling events (left). Synthetic activating peptides (PAR1-APs) activate PAR1 without proteolytic cleavage. PAR1-APs directly interact with the second extracellular receptor loop that results in activation of signaling pathways. (C) PAR1 activation on human immune cells leads to various responses as indicated. ZAP-70, ζ-associated protein 70; SLP-76, Src homology 2 domain-containing lymphocytic protein of 76 kDa; MIF-1α, macrophage migration inhibitory factor-1α (Shpacovitch *et al*, 2008)

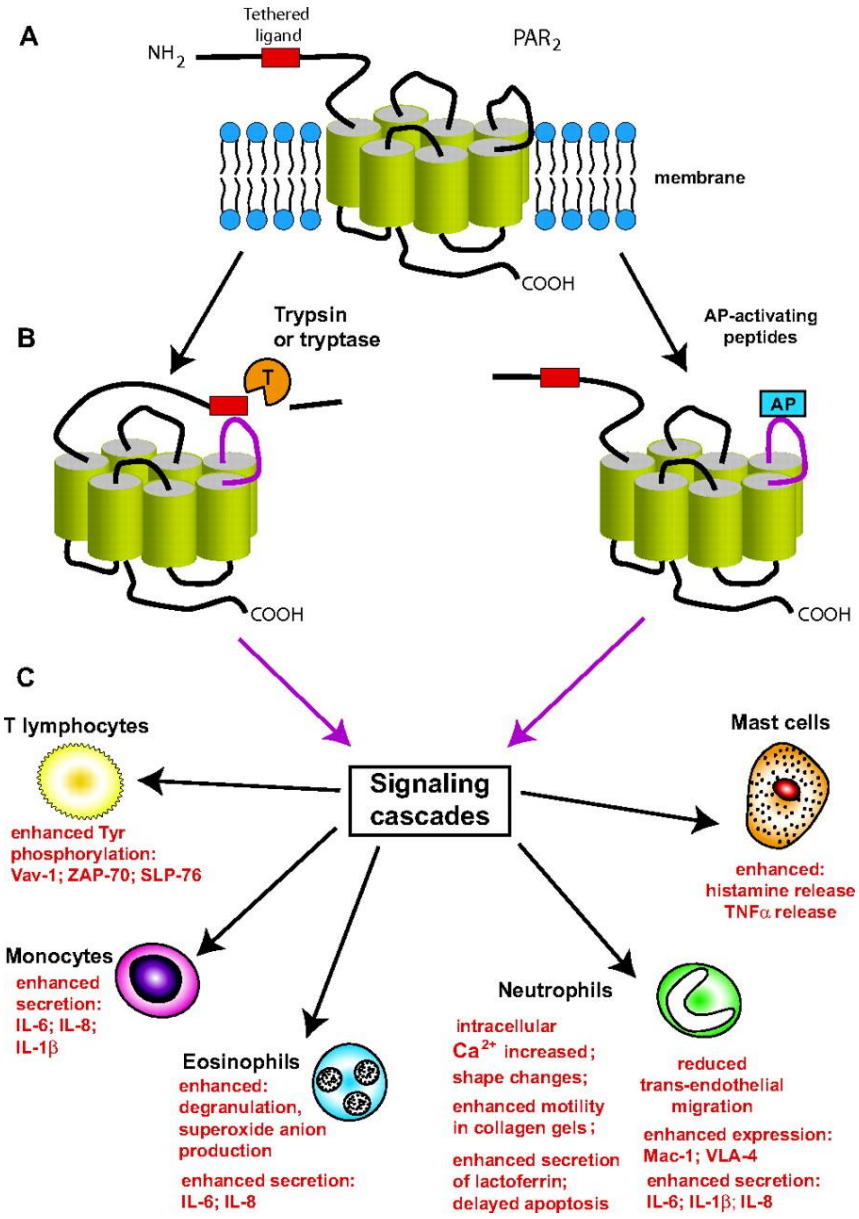

Figure 3: Scheme of PAR2 activation and PAR2-induced effects in human immune cells.

(A) Inactive PAR2 has an N-terminal-tethered ligand sequence, which remains cryptic and does not interact with the second extracellular loop of the receptor and does not trigger downstream signaling events. (B) Endogenous or exogenous serine proteases (e.g. trypsin, tryptase) cleave the N-terminal part of the receptor, unmasking the tethered ligand. The tethered ligand interacts with the second extracellular loop of the same receptor triggering signaling events. Synthetic activating peptides (PAR2-APs) activate PAR2 without proteolytic cleavage. PAR2-APs directly interact with the second extracellular loop of the receptor that results in activation of signaling pathways. (C) PAR2 activation on human immune cells results in various immune cell responses. Mac-1, Membrane-activated complex 1; VLA-4, very late activation Ag-4 (Shpacovitch *et al*, 2008)

1.9.1 Role of PARs activation in T lymphocytes

Human T-cell lines and human T-cells in tissues are known to express all four PARs [117-120]. Experimental evidence suggests that in peripheral blood lymphocytes the level of PAR2 mRNA expression is enhanced upon stimulation of these cells with phytohaemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA) [117]. Functional PAR2 is also expressed by mouse T lymphocytes, but its physiological or pathophysiological role still remains to be clarified [82]. Intracellular calcium (Ca^{2+}) levels are elevated in human Jurkat T-cells following stimulation with thrombin, trypsin, or PAR1- or PAR2-APs [117]. Bar-Shavit and colleagues [118] further demonstrated that the activation of PARs (PAR1, PAR2) in Jurkat T- cells induces tyrosine phosphorylation of Vav-1, ZAP-70, and SLP-76, suggesting an interaction with TCR signaling. Unfortunately a nonselective PAR1/PAR2 agonist (SFLLRNPNDK) was used in these studies and hence the observed effects might be due to simultaneous activation of PAR1 and PAR2 or activation of either receptor. Furthermore, in this study a functional role for PAR3 on T-cells was suggested but further work revealed that the PAR3-AP used (TFRGAPPNSF) does not activate PAR3 in Jurkat T-cells but rather stimulates PAR1 and PAR2 [119]. In human primary T-cells the level of IL-6 was found to be elevated upon treatment with thrombin, trypsin, tryptase, and PAR-APs [120]. However, the underlying signaling events and their role under physiological and pathophysiological conditions need to be further investigated. Similarly, in mouse $CD4^+$ T-cells an important role for PAR2 signaling has been found for cytokine production [121]. It was found that splenic $CD4^+$ T-cells secrete less IL4 during OVA-induced airway inflammation in PAR2-deficient mice, as compared with wild-type animals. IFNγ production was enhanced after antigen stimulation in PAR2 null mice as compared with wild-type mice. JNK1 phosphorylation appears to be associated with this PAR2-mediated regulation of T cell cytokine production [121]. The role of PAR2 in cytokine production and other responses of human and murine T-cells have been shown. However, the involvement of PAR1 in the regulation of T cell function is not investigated extensively and also and the role of PARs in T cell-mediated diseases need further investigation.

1.10 The PAR interactome: novel concepts for signaling selectivity and specificity

It has been argued that PAR3 is signaling incompetent receptor yet it shows remarkable signaling selectivity. This indicated that PAR3 requires other co-receptors for signal induction,

diversification, and regulation. It has been hypothesized that the formation of PAR-PAR heterodimers and homodimers results in the formation of PAR effector complexes [122], which may enable PAR induced transactivation of other PARs, integrate the transactivation of other GPCRs such as S1P1 [76, 123, 124], or incorporate cooperative cross talk with integrins such as Mac1 [125, 126] or other receptors such as ApoER2 [127, 128] or Tie2 [78, 129, 130].

1.11 Motivation/Objective of present study

It has been previously shown that human steroid refractory GvHD is associated with higher serum level of soluble thrombomodulin. Soluble thrombomodulin is a marker of endothelial dysfunction and associated with impaired protein C activation. Loss of thrombomodulin function and aPC generation is associated with vascular complications. Of note, T-cells express PARs, raising the question as to whether aPC may not only act on the vasculature, but directly modify T-cell activation and hence adaptive immunity in GvHD. Given that GvHD is associated with vascular dysfunction and elevated levels of soluble thrombomodulin, reflecting impaired PC-activation, we hypothesized that aPC regulates GvHD by modulating the allo-activation of T-cells which express protease activated receptors (PARs) that is required for aPC signaling.

2 Materials and Methods

2.1 Materials

FITC anti-mouse MHC class I (H-2Db)	eBioscience
FITC or APC-eFlour780 anti-mouse CD4	eBioscience
PE anti-mouse IFN-γ, IL17A, TNFα	Biolegend
PerCp-Cy5.5 anti-mouse IL-10	Biolegend
PerCp-Cy5.5 anti-human/mouse T-bet	Biolegend
Alexa Fluor 647 anti-human/mouse FOXP3	Biolegend
PE anti-human/mouse ROR-γt	eBioscience
FITC anti-human CD4	eBioscience
APC (Allophycocyanin) anti-human CD4	Biolegend
PE anti-human IFN-γ, IL17A, IL4, IL10, TNFα	Biolegend
Mouse anti- human PAR1 (ATAP2) and PAR2 (SAM11)	Santacruz, Heidelberg, Germany
rabbit polyclonal antibody to PAR3 (H103)	Santacruz, Heidelberg, Germany
goat polyclonal antibody to PAR4 (S-20)	Santacruz, Heidelberg, Germany
blocking peptides for PAR1 (FR 171113), PAR2 (FSLLRY-NH2) and PAR4 (ML 354)	Tocris Bioscience
HRP-conjugated secondary antibodies rabbit or mouse IgG	Cell Signalling Technology, Germany
RPMI 1640	Sigma-Aldrich, Germany
phorbol 12- myristate 13-acetate (PMA)	Sigma-Aldrich, Germany
Ionomycin	Sigma-Aldrich, Germany
Brefeldin A (BFA)	Sigma-Aldrich, Germany
protease inhibitor cocktail	Roche diagnostics GmbH, Mannheim, Germany
AIM V Medium	Thermoscientific, Germany
BCA reagent	Thermoscientific, Germany
BCA reagent	Millipore GmbH, Germany
immobilion enhanced chemiluminescence reagent	Millipore GmbH, Germany

FOXP3 / Transcription Factor Staining Buffer Set	eBioscience

2.2 Buffers

2.2.1General Buffers

10x PBS:

- 80 g NaCl
- 2 g KCl
- 14.4 g Na2HPO4 (2H2O)
- 2.4 g KH2PO4

Make up to 1 litre with dH_2O after adjust the pH to 7.4.

10x TBS:

- 24.2 g Tris base
- 80 g NaCl

Make up to 1 litre with dH_2O after adjust the pH to 7.6 with HCl.

0.5 M EDTA:

- 186.1 g EDTA
- 800 ml dH2O

Make up to 1 litre with dH_2O after adjusting pH to 8.0 with NaOH.

5x TBE:

- 54 g Tris
- 27.5 g Boric acid
- 20 ml EDTA (0.5 M)

Make up to 1 litre with dH_2O. For TBE working solution (0.5 x) dilute stock 1:10 with dH_2O.

H&E Staining

Acid rinse solution:

- glacial acetic acid 2 ml
- dH2O 98 ml

Blueing solution:

- NH4OH 1.5 ml
- 70 % EtOH 98.5 ml

Cell lysate total protein

RIPA Buffer (final concentration) for whole cell and tissue lysates

- 50 mM Tris-HCl (pH 7.4)
- 1 % NP-40
- 0.25% Na-deoxycholate
- 150 mM NaCl
- 1 mM EDTA
- 1 mM PMSF
- 1 mM Na3VO4
- 1 mM NaF
- add 40 µl/ml protease inhibitor cocktail (Roche)

SDS-PAGE 2.5.6

Electrophoresis resolving-buffer:

- 90.825 g (1.5 M) Tris-HCL
- 20 ml (0.4 %) 10 % SDS
- Adjust pH – 8.8

Make up to **500 ml** with dH_2O

Stacking-buffer:

- 30.275 g (1 M) Tris-HCL
- 8 ml (0.4 %) SDS
- Adjust pH – 6.8

Make up to **200 ml** with dH_2O

1 x SDS Sample-buffer:

- 62.5 mM Tris-HCl (pH 6.8 at 25°C)
- 2 % w/v SDS
- 10 % Glycerol
- 0.5 M DTT
- 0.01 % bromophenol blue

5 x SDS Running-buffer:

- 15.1 g (0.125 M) Tris
- 94 g (1.250 M) Glycine
- 50 ml 10 % SDS

Make up to 1 litre with dH_2O

Blot-buffer:

- 2.42 g (0.02 M) Tris
- 11.25 g (0.15 M) Glycine
- 200 ml Methanol

Make up to 1 litre with dH_2O

Blocking buffer:

- 5 g Non-fat dried milk powder
- 0.05 % (v/v) Tween 20

Make up to 1 litre with 1xPBS or 1x TBS

2.3 Mice

C57BL/6 (B6;H-2b) and BALB/c (H-2d) were purchased from Janvier Labs. Humanized NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} H2-Ab1^{tm1Gru}Tg (HLA-DRB1)31Dmz/SzJ (NSG-Ab°DR4) mice [131] were purchased from Jackson Laboratory. These NSG-Ab^{o} DR4 mice lack expression of the murine *Prkdc* gene, the X-linked *Il2rg* gene, and MHC class II, but express the human leukocyte antigen DR4 gene. The expression of HLA-DR4 in these mice leads to the development of allo-graft-versus-host disease (GvHD) after engraftment of human DR4-negative $CD4^+$ T cells.

APC^{high} mice, which express a transgene resulting in expression of a human protein C variant (D167F/D172K) in the liver, which can be efficiently activated in the absence of thrombomodulin, resulting in high plasma concentrations of aPC, have been previously described and have been backcrossed onto the C57BL/6 (B6;H-2b) background for more than 10 generation [109]. PAR3-/-mice were obtained from Jackson Laboratory and have been backcrossed onto the C57BL/6 (B6;H-2b) background for at least 10 generation [88]. Mice were housed in pathogen-free conditions in individually ventilated cages in the central animal facility of the Medical Faculty of the Otto-von-Guericke University, Magdeburg. All animal experiments were conducted following standards and procedures approved by the local Animal Care and Use Committee (Landesverwaltungsamt Halle, Germany).

2.4 Preparation of activated protein C

Activated protein C (PC) was generated as previously described with slight modifications [132-134]. Briefly, prothrombin complex (Prothromplex NF600), containing all vitamin K dependent coagulation factors, was reconstituted with sterile water and supplemented with $CaCl_2$ at a final concentration of 10 mM. A column filled with Affigel-10 resin covalently linked to the calcium dependent monoclonal antibody to PC (HPC4) was used for PC purification. This column was equilibrated at 4°C with 1 column volume (CV) of washing buffer (0.1 M NaCl, 20 mM Tris, pH7.5, 5 mM benzamidine HCl, 2 mM Ca^{2+}, 0.02% sodium azide) at a flow rate of 0.5ml/min which was used during the whole purification step. After binding of the protein the column was washed first with 1 CV of washing buffer and then 1 CV with a buffer containing high salt concentration (0.5 M NaCl, 20 mM Tris, pH 7.5, 5 mM benzamidine HCl, 2 mM Ca^{2+}, 0.02% sodium azide). Then benzamidine was washed off the column with a buffer of 0.1 M NaCl, 20 mM Tris, pH 7.5, 2 mM Ca^{2+}, and 0.02% sodium azide using again 1 CV. To elute PC 1 CV of elution buffer (0.1M NaCl, 20 mM Tris, pH 7.5, 5 mM EDTA, 0.02% sodium azide, pH 7.5) was applied to the column. A subsequent fractionation of the eluate was done collecting the samples in 5 ml fractions. Peak fractions, identified by measuring absorbance at 280 nm, were pooled. The recovered PC was activated with human plasma thrombin (5% w/w, incubated for 3h at 37°C). To separate thrombin an ion exchange chromatography with FPLC (ÄKTAFPLC®, GE Healthcare Life Sciences) was used, yielding purified activated protein C (aPC). To this end a Mono Q anion exchange column (GE Healthcare Life Sciences) was equilibrated with 5 CV of 20 mM Tris pH 7.5, 100 nM NaCl. After applying the solution containing aPC and a washing step

with 5 CV of the solutions used for equilibration a 10-100% gradient using 5 CV of a 20 Mm Tris, pH 7.5, 1 M NaCl buffer was applied to the column to elute aPC. aPC eluted at 0.46 M NaCl (~36 mS/cm conductivity). Fractions of 0.5 ml were collected during the peak and pooled. Proteolytic activity, integrity, and purity of aPC was ascertained with the chromogenic substrate SPECTROZYME® PCa and by Coomasie-staining of the purified protein on 10% SDS-PAGE gel.

2.5 GvHD models

Pan T-cells were isolated from whole spleen by magnetic bead depletion of non-T-cells using mouse Pan T-cell isolation Kit II (Miltenyi Biotec) following the manufacturer's recommendations. Purity of T-cells was ascertained by FACS and ranged from 95% to 98%. Bone marrow (BM) was prepared by isolating tibia and femur bones and flushing out BM from them using RPMI complete medium. RBCs in BM were lysed using RBC lysis buffer (Buffer EL; Qiagen).

To induce MHC-mismatched GvHD we transplanted either C57BL/6 or BALB/c BM along with C57BL/6 or BALB/c splenic T-cells into BALB/c or C57BL/6, respectively, recipient mice. Recipient mice, 8 to 10 weeks of age, were conditioned with total body irradiation (TBI) of 11Gy (single dose) for BALB/c and 13Gy (single dose) for C57BL/6 mice on Day 0. For TBI the BioBeam 8000 (Gamma Service Medical GmbH, Germany) providing gamma irradiation (137Cs) was used. Mice were immobilized by anaesthesia with intra-peritoneal injection of 100µl Ketavet and Rompun solution (Ketavet 20mg/ml and Rompun 1mg/ml) and kept in a radiation chamber. Four hours after irradiation recipient mice received intravenously 5×10^6 mismatched (C57BL/6→BALB/c or BALB/c→C57BL/6) BM cells with purified 0.5×10^6 mismatched pan T-cells or 0.5×10^6 PAR3-/- pan T-cells. In a subset of experiments T-cells were pretreated with aPC (20nM, 1h, 37°C in AIM V serum free medium). Following 1h incubation with aPC cells were washed with PBS. Control T-cells were exposed to the same medium without aPC. In some experiments T-cells were incubated with the selective PAR2 antagonist FSLLRY-NH2 (30 µM, 30 min, 37°C) prior to incubation with aPC [135, 136]. In further experiments regulatory T-cells were isolated using the CD4+CD25+ Regulatory T cell Isolation Kit (Miltenyi Biotec) and purity was ascertained by FACS analysis and ranged from 95% to 98%. The pooled T-cells obtained after isolation of regulatory T-cells in the previous step were used as T-cells excluding Tregs. Tregs or T-cells excluding Tregs were separately pretreated with aPC (20nM, 1h, 37°C) prior to

BM transplantation. Following 1h incubation with aPC cells were washed with PBS. To induce allogenic GvHD with human T-cells in mice we used the "humanised" NSG-Ab° DR4 mice (see above). For preconditioning these mice received TBI of 2 Gy. After 4h these mice were intravenously injected with 4×10^6 of human CD4+ T-cells (HLA-DR4$^-$) without or with aPC-pretreatment (20nM, 1h, 37°C).

2.6 HLA-DRB1-04 genotyping

To isolate HLA-DRB1-04 (HLA-DR4)-negative T-cells blood samples were obtained from volunteers after getting their written informed consent. Expression of DR4 was assessed by real time polymerase chain reaction (PCR) using a Light Cycler (CFX Connect, Real Time System, BioRad). Briefly, genomic DNA was extracted from donor`s PBMC (1×10^6) using the phenol chloroform extraction. The HLA-DRB1-04 gene was amplified using the following forward primer: 5′ GTTTCTTGGAGCAGGTTAAACA-3′ and two reverse primers in the same reaction: 5′-CTGCACTGTGAAGCTCTCAC-3′, 5′-CTGCACTGTGAAGCTCTCCA-3′ [137]. The following cycling parameters were used: initially 2 min at 95°C, followed by 40 cycles of 95°C, 10s; 68°C, 10s; 72°C, 23s with a single acquisition per cycle at 72°C. All temperature transitions were 20°C/s. Samples were then subjected to a melting curve analysis with the following conditions: 95°C, 0 s (slope 20°/s); 65°C, 10 s (slope 20°/s) and then heated to 95°C with a slope of 0,3°/s using step acquisition. Positive and negative samples were distinguished by the presence or absence of fluorescence signal during the PCR reaction and the presence of a melting peak (~91°C).

2.7 Assessment of GvHD

Individual weights of transplanted mice were obtained and recorded on day +l and weekly thereafter until the time of analysis. Survival was checked once daily. The clinical score of GvHD was assessed by a scoring system described in Table 1 that incorporates five physical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity. Every week mice were evaluated and graded from 0 to 2 for each criterion. A clinical index was subsequently generated by summation of the five criteria scores (maximum index = 10) [138].

Table 1: Assessment of clinical GvHD in transplanted animals

Criteria	Grade 0	Grade 1	Grade 2
Weight loss	<10 %	>l0% to <25%	>25%
Posture	Normal	Hunching noted only at rest	Severe hunching impairs movement
Activity	Normal	Mild to moderately decreased	Stationary unless stimulated
Fur texture	Normal	Mild to moderate ruffling	Severe ruffling/poor grooming
Skin integrity	Normal	Scaling of paws/tail	Obvious areas of denuded skin

Representative samples of GvHD target organs (gut, liver, and skin) were excised from recipients 14 days post-BM transplantation. Formalin-fixed tissues were paraffin embedded and sectioned (5-μm-thick sections). Sections were stained with hematoxylin and eosin for histologic examinations. Pathological scoring was conducted by an experienced pathologist (TK) blinded to the groups. Intestinal GvHD was scored based on the frequency of crypt apoptosis (0, rare to none; 1, occasional apoptotic bodies per 10 crypts; 2, few apoptotic bodies per 10 crypts; 3, the majority of crypts contain an apoptotic body; 4, the majority of crypts contain >1 apoptotic body) [139]. The severity of skin GvHD was assessed by a scoring system that incorporates following parameters: epidermic atrophy, hair follicle loss, increased collagen density in dermis, and inflammation. The slides were graded from 0 to 2 for each parameter [139]. The severity of liver GvHD was assessed by a scoring system that incorporates following parameters: Bile ducts infiltrated by lymphocytes (0: not present, 1: one or more lymphocytes in one bile duct, 2: lymphocytes in more than 1 bile duct, 3: lymphocytes in all bile ducts) and portal inflammation (0: not present, 1: inflammatory cells in some portal tracts, 2: inflammatory cells in most portal

tracts, 3: packing of inflammatory cells with or without spill-over into adjacent parenchyma in some or most portal tracts) [140].

2.8 Cell isolation and mixed lymphocyte culture

For *ex vivo* assessment of allogenic T-cell activation mixed lymphocyte reaction (MLR) were conducted by incubating pan T-cells with non-T-cells, containing antigen-presenting cells. For isolation of human T-cells fist PBMCs were obtained from peripheral blood using Ficoll-Paque (GE Healthcare) gradient and then pan T-cells were isolated by magnetic bead depletion of non–T-cells using the human pan T-cell Isolation Kit (Miltenyi Biotec) following the manufacturer`s recommendations. In a subset of experiments regulatory T-cells were isolated using the human CD4+CD25+CD127dim/- regulatory T cell Isolation Kit II (Miltenyi Biotec) and untouched CD4+CD25-CD127hi cells obtained during regulatory T-cells isolation were used as effector Tcells. Purity of cells were ascertained by FACS and ranged from 95% to 98%. Non-T-cells were irradiated (30Gy) and used as antigen presenting cells (AgPC). Pan T-cells and non-T-cells from two genetically distinct (non-related) individuals were combined to trigger allogenic T-cell activation. For *ex vivo* assessment of murine allogenic responses BALB/c and C57BL/6 splenocytes were isolated by disrupting the spleen with a 100µm cell strainer. Pan T-cells were separated from splenocytes by magnetic bead depletion of non-T-cells using mouse Pan T-cell Isolation Kit II (Miltenyi Biotec) following the manufacturer`s recommendations. Mismatched non-T-cells, used as antigen presenting cells, were irradiated with 30 Gy and combined with pan T-cells to trigger the MLR response.

Both human and mice T-cells were cultured in AIM V serum free medium (Life Technologies) for 2h (37°C, 5% CO2) before performing MLR. aPC pretreatment of pan T-cells (both human and mice) were done in AIM V medium for 1h at 37°C and following 1h incubation cells were washed with PBS. For both human and mice MLR 1×10^5 pan T-cells without (PBS control) or with aPC-pretreatment (20nM, 1h, 37°C) were incubated with 3×10^5 irradiated allogenic non-T-cells for 96h. Proliferation of the human and mice T-cells were assessed by measuring [^{3}H] thymidine incorporation during the last 16h of the incubation time and [^{3}H] thymidine was added at 0.2 Ci/well. At the end of the incubation period cells were harvested and radioisotope incorporation was measured as an index of lymphocyte proliferation by betaplate liquid scintillation counter (MicroBeta, Wallac, Turku, Finland) [141].

2.9 Immunoblotting

To determine PAR-expression on T-cells cell lysates of purified human T-cells were prepared using RIPA buffer containing 50 mM Tris (pH7.4), 1% NP-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF supplemented with protease inhibitor cocktail. Lysates were centrifuged (13,000 g for 10 min at 4°C) and insoluble debris was discarded. Protein concentration in supernatants was quantified using BCA assay. Equal amounts of protein were electrophoretically separated on 10% or SDS polyacrylamide gel, transferred to PVDF membranes and probed with primary antibodies at a desired concentration of PAR1 (1:200), PAR2 (1:200), PAR3 (1:200), PAR4 (1:200), GAPDH (1:20000). After overnight incubation with respective primary antibodies at 4°C, membranes were washed with TBST and incubated with anti-mouse IgG (1:2000) or anti-rabbit IgG (1:2000) horseradish peroxidaseconjugated antibodies for 1h at room temperature. Blots were developed with the enhanced chemiluminescence system. To compare and quantify levels of proteins the density of each band was measured using Image J software. Equal loading for total cell lysates was determined by GAPDH western blot.

2.10 Immunoprecipitation

For immunoprecipitation total cellular proteins were isolated from human primary T-cells with RIPA lysis buffer containing Protease/Phosphatase Inhibitor Cocktail 1X (Cell Signalling Technology, Germany). Lysates (200 µg) were combined with 2µg of PAR2 antibody (SAM11) and incubated overnight at 4°C. Immunoprecipitates were purified with protein A/G agarose beads and washed with PBS. Immunoprecipitates were fractionated by SDS-PAGE (10%), transferred to membranes, and subjected to immunoblotting with PAR3 antibody (H103) and secondary antibodies as described above.

2.11 Functional PAR-signaling *in vitro* assays

To evaluate the functional relevance of PARs on human pan T-cells complimentary approaches were used. Human pan T-cells were either (1) incubated with N-terminal blocking anti-PAR1 (ATAP-2, 10µg/mL), anti-PAR2 (SAM-11, 10 µg/mL), anti-PAR3 (H-103, 20µg/mL) or anti-PAR4 (S-20, 20µg/ml) antibodies for 1h, or (2) with inhibitors blocking receptor activation by the tethered ligand (FR1113 for PAR1, FSLLRY-NH2 for PAR2, ML354 for PAR4; all from

Tocris) [88, 97, 135, 136, 142-144]. Following incubation of pan T-cells with N-terminal blocking antibodies or signaling inhibiting compounds pan T-cells with aPC-pretreatment (20nM, 1h, 37°C) or without aPC-pretreatment were combined with allogenic antigen-presenting cells and co-cultured for 96h for MLR.

2.12 Flow cytometry and intracellular cytokine staining

Murine splenocytes were isolated and stimulated *ex vivo* with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 100 ng/mL ionomycin (Sigma-Aldrich) at 37°C for 6h. Brefeldin A (BFA; Sigma-Aldrich) was added at a final concentration of 10 mg/ml 4h before intracellular cytokine staining. Cells were stained with FITC conjugated H-2b (eBioscience), FITC or APC-eFlour780 conjugated CD4, (eBioscience), PE conjugated IFN-γ, IL17A, TNFα (Biolegend), PerCp-Cy5.5 conjugated IL-10 (Biolegend), PE conjugated IL4 (eBioscience), PerCp-Cy5.5 conjugated T-bet, PE conjugated GATA3 (eBioscience), or Alexa Fluor 647 conjugated FOXP3 (Biolegend, using the FOXP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Intracellular staining for cytokines in human MLR was done 48h after MLR. Cells were stained with FITC conjugated CD4 (eBioscience) or APC (Allophycocyanin) conjugated CD4 (Biolegend), PE conjugated IFN-γ, IL17A, IL4, IL-10, TNFα (Biolegend), PerCp-Cy5.5 conjugated T-bet, PE conjugated GATA3 (eBioscience), Alexa Fluor 647 conjugated FOXP3 (Biolegend), using the FOXP3 / Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Cells were analyzed using FACS Canto II (BD Biosciences) and FlowJo software (TreeStar).

2.13 Leukemia/lymphoma models

Retroviral infection of hematopoietic progenitor cells was performed as previously described [145] with minor modifications. In brief, lineage-negative, Esam-1-positive, Kit-positive (L-E+K+) cells were sorted from Balb/C bone marrow and infected with an MSCV-MLL-AF9 construct. Primary, sublethally irradiated (7 Gy) 6-8 week old female Balb/C recipients were injected with up to $5x10^4$ pre-leukemic progenitors. Secondary, lethally irradiated BALB/c recipient mice received $5x10^3$ GFP/Kit-co-expressing (GFP^+Kit^+) MLL-AF9 leukemic cells (BALB/c background) along with 5×10^6 C57Bl/6 BM cells and purified 0.5×10^6 C57Bl/6 pan T-cells without or with aPC-pretreatment (20nM, 1h, 37°C, in AIM V serum free medium). Tumor

load was determined in peripheral blood samples obtained 4 weeks following BM-transplantation and injection with leukemic cells. Tumor load was determined as the integrated Mean Fluorescence Intensity (iMFI) of GFP+ leukemic cells using FACS Conto II (BD Biosciences) and FlowJo software (TreeStar).

2.14 Statistics

Survival was ascertained by Kaplan Meyer log-rank analyses. The Kolmogorov–Smirnov test was used to determine whether the data are consistent with a Gaussian distribution. Statistical analyses were performed with the Student t test, ANOVA or Mann–Whitney-U test, as appropriate. Prism 5 software (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Values of P <0·05 were considered statistically significant.

3 Results

3.1 aPC ameliorates murine GvHD in mice with higher levels of endogenous aPC

To investigate whether endogenous aPC could reduce acute GvHD we transplanted lethally irradiated (13Gy) APC^{high} mice (transgenic mice expressing a human protein C variant in the liver, resulting in high plasma concentrations of aPC) and C57BL/6 mice with 5×10^6 bone marrow cells along with 2×10^6 splenic T-cells from BALB/c mice. The survival of hPC mice (**Figure 3.1.1a**) was significantly improved in comparison to C57BL/6 mice. Likewise, the overall clinical scores (including weight loss, mobility, hunched posture, ruffled fur and skin integrity) of hPC mice were significantly reduced in comparison to C57BL/6 mice (**Figure 3.1.1b**). These data suggest that endogenous high levels of aPC ameliorate acute GvHD.

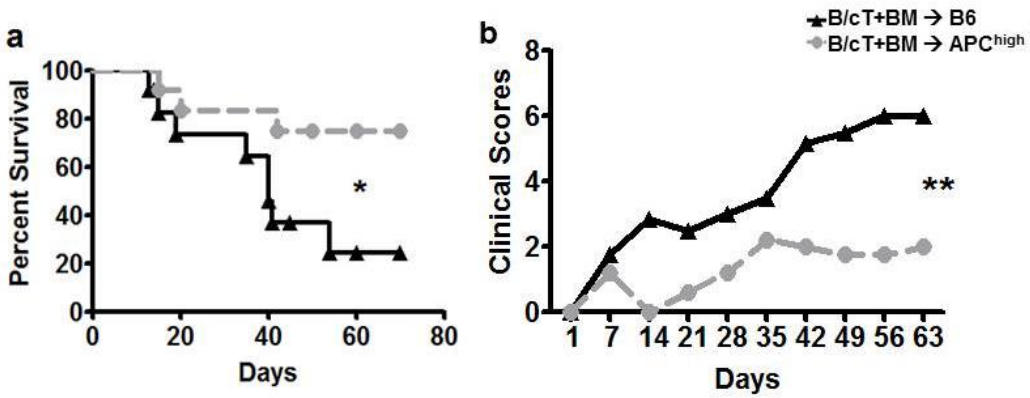

Figure 3.1.1: Endogenous high levels of aPC ameliroatge acute GvHD in mice

(a,b) Recipient C57BL/6 wild-type (B6) mice or C57BL/6 mice with endogenous high levels of aPC (APC^{high}) were lethally irradiated (13Gy) and transplanted with 5×10^6 whole bone marrow (BM) and 2×10^6 T-cells from donor BALB/c mice (B/cT+BM). Recipient mice were monitored for survival (**a**, Kaplan-Meyer curve) and physical parameters (including weight loss, mobility, hunched posture, ruffled fur and skin integrity), yielding a composite clinical score (**b**); pooled data from 3 independent experiments each with 4 recipient mice per genotype. (a: log-rank test; b: ANOVA)

Compatible with improved survival, histopathological analysis of the bowel (small and large bowel) demonstrated a significantly decreased disease score and a lower number of apoptotic cells in the crypts in APC^{high} mice in comparison with C57BL/6 mice. Similarly the histopathological damage in other target organs, i.e liver and skin, was significantly less in

APC^{high} mice in comparison to C57BL/6 mice as demonstrated by the histology (**Figure 3.1.2a**) and the histological scores (**Figure 3.1.2b**).

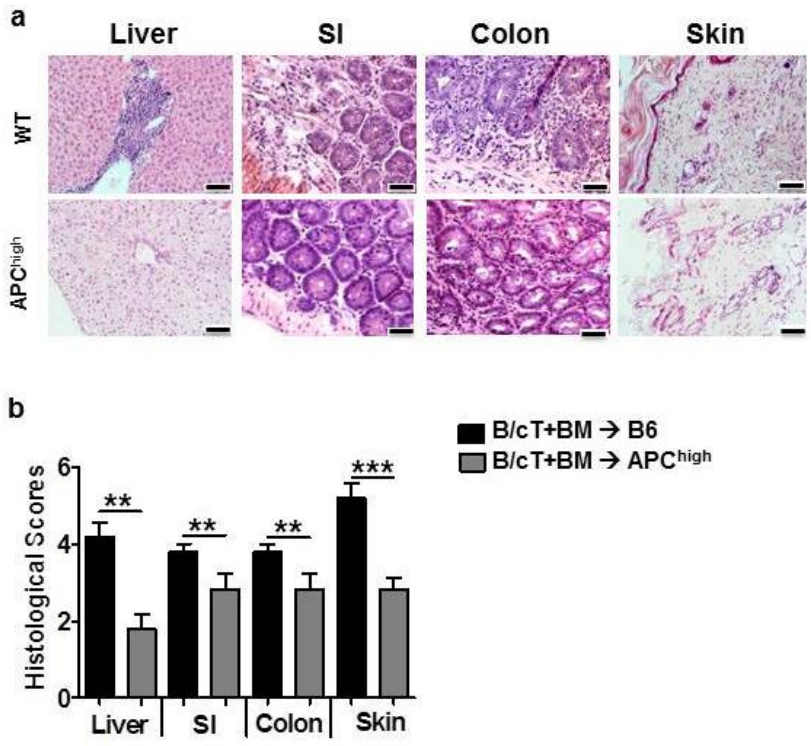

Figure 3.1.2 Endogenous high levels of aPC improves the survival of mice with GvHD
(a,b) Photomicrographs depicting typical morphology in liver, small intestine (SI), colon, and skin (**a**) and bar graph summarizing histological disease scores (**b**) Mean value ± SEM (b) haematoxylin and eosin stained sections (**a**, size bar: 50 μm); *P<0.05, **P<0.01, ***P<0.001 (b: t-test).

3.2 Pretreatment of mice pan T-cell with aPC reduces allogenic T-cell activation

As GvHD is a primarily T-cell driven disease we next explored aPC's effect on T-cell activation using *in vitro* mixed lymphocyte reactions (MLRs) [42]. Co-culture of C57BL/6 (wt) pan T-cells with irradiated allogenic antigen presenting cells (AgPC,BALB/c) for 96h induced T-cell proliferation, which was markedly blunted in the presence of aPC (20nM, every 12h, **Fig. 3.2a**), establishing that aPC directly inhibits MLR-driven allogenic T-cell activation. Next we applied the same assay system to examine whether the anti-proliferative effect of aPC was mediated directly via T-cells. To this end, wt T-cells were only pretreated once with aPC (20nM, 1h, 37^{o}C) prior to the MLRs. T-cells (with and without aPC-pretreatment) were then incubated with irradiated (30Gy) antigen presenting cells for 96h and proliferation was measured by thymidine

incorporation during the final 16h of co-culture. Intriguingly, pretreatment of T-cells with aPC (20nM, once, 1h, 37°C) was sufficient to inhibit allogenic T-cell proliferation (**Fig. 3.2b**).

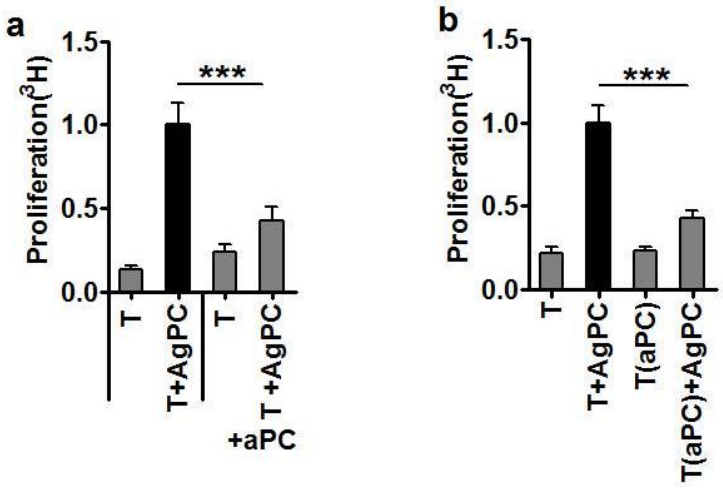

Figure 3.2 T-cell pretreatment with aPC reduces allogenic T-cell activation

(a,b) Splenic T-cells isolated from C57BL/6 wild-type (wt) mice (T) were co-cultured with BALB/c allogenic antigen presenting cells (AgPC) for 96h and proliferation was measured by thymidine incorporation during the final 16h. Concomitant treatment with aPC (**a,** +aPC, 20 nM, every 12h) or T-cell pretreatment with aPC (**b,** T(aPC), pretreatment once, 20nM, 1h, 37°C) reduces T-cell proliferation as compared to stimulated but non-treated cells (T+AgPC); results of 3 independent experiments, each in triplicates.

3.3 T-cell pretreatment with aPC ameliorates GvHD

To assess the *in vivo* effect of aPC-pretreatment on allogenic T-cells driven GvHD, donor T-cells were exposed to aPC (20nM, 1h, 37°C) prior to transplantation. We transplanted 5×10^6 BM cells and 0.5×10^6 splenic T-cells (both C57BL/6 derived) without (B6T+BM) or with (B6T(aPC)+BM) aPC-pretreatment into lethally irradiated (11Gy) BALB/c recipients. Mice transplanted with allogenic bone marrow cells along with allogenic T-cells pre-treated with aPC appeared healthy and survived significantly better than mice transplanted with allogenic bone marrow cells and allogenic T-cells without aPC-pretreatment (Fig. 3.3a). Mice that received allogenic bone marrow cells along with allogenic T-cells pre-treated with aPC developed significantly lesser signs of GvHD. Thus, mice receiving aPC-pretreated T-cells showed reduced weight loss, better mobility, reduced hunched posture, and less ruffled fur than mice transplanted with allogenic bone marrow cells and allogenic T-cells without aPC-pretreatment (Fig. 3.3b). Furthermore, a detailed histopathologic analyses of GvHD target organs (liver, skin, and

gastrointestinal tract) confirmed that recipient mice receiving aPC pre-treated T-cells demonstrated significantly reduced histopathologic damage in all target organs (Fig. 3.3c,d). Collectively, these data demonstrate that pretreatment of allogenic T-cells with aPC markedly improved survival, physical appearance, and histopathology in mice with GvHD.

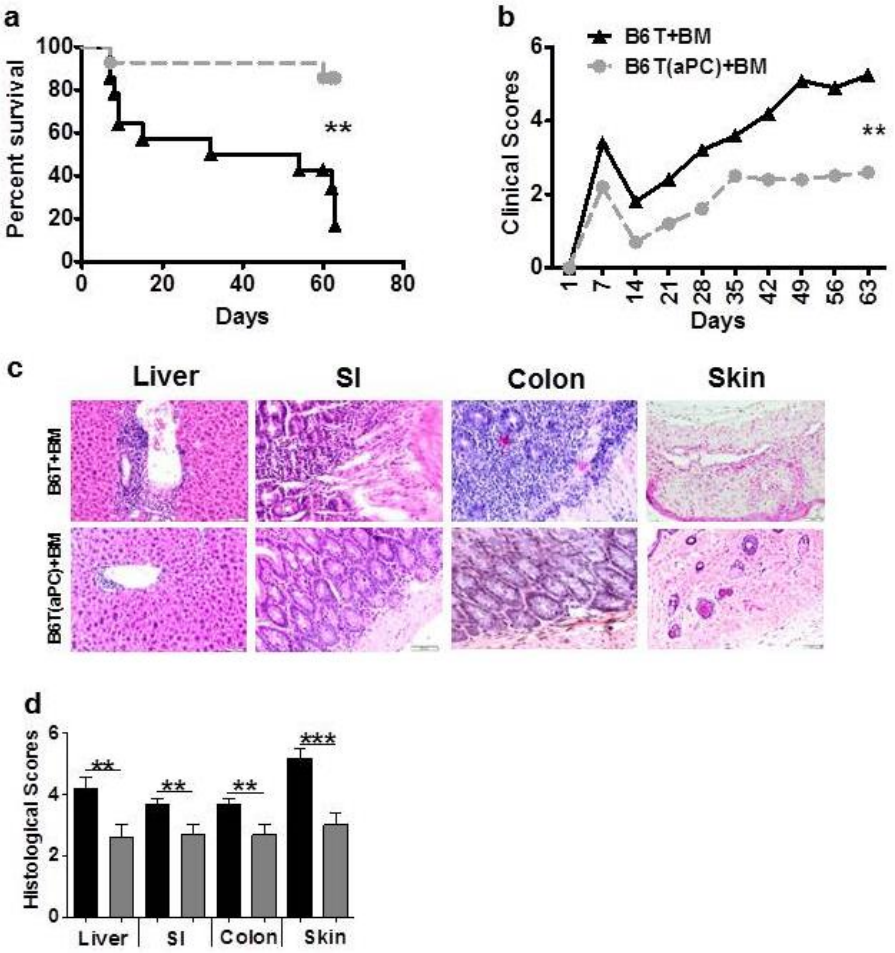

Figure 3.3 T-cell pretreatment with aPC ameliorates GvHD

(a,b) Recipient BALB/c mice were lethally irradiated (11Gy) and transplanted with 5×10^6 BM and 0.5×10^6 T-cells without (B6T+BM) or with (B6T(aPC)+BM) aPC-pretreatment (20 nM, 1h, 37°C) from donor C57BL/6 wt mice. Recipient mice were monitored for survival (**a**, Kaplan- Meyer curve) and physical parameters (**b,** clinical score); pooled data from 3 independent experiments each with 4 recipients per group. **(c,d)** Photomicrographs depicting typical morphology in liver, small intestine (SI), colon, and skin (**c**) and bar graph summarizing histological disease score (**d**)

3.4 T-cell pretreatment with aPC ameliorates GvHD by inducing Tregs and reducing Th1 and Th17 cells

To evaluate the mechanism underlying the highly protective effect of T-cell pretreatment with aPC we characterized splenocytes from GvHD mice on day 14 post transplantation *ex vivo*. aPC-pretreatment of T-cells prior to transplantation increased the frequency of donor-derived activated regulatory T-cells (B6-H-2^b+$CD4^+CD127^+FOXP3^+$, Tregs) about two-fold, while that of donor derived Th1 (B6-H-2^b+$CD4^+$T-bet^+) and Th17 (B6-H-2^b+$CD4^+$ROR-γt^+) T-cells were markedly reduced (**Fig. 3.4a,b**).

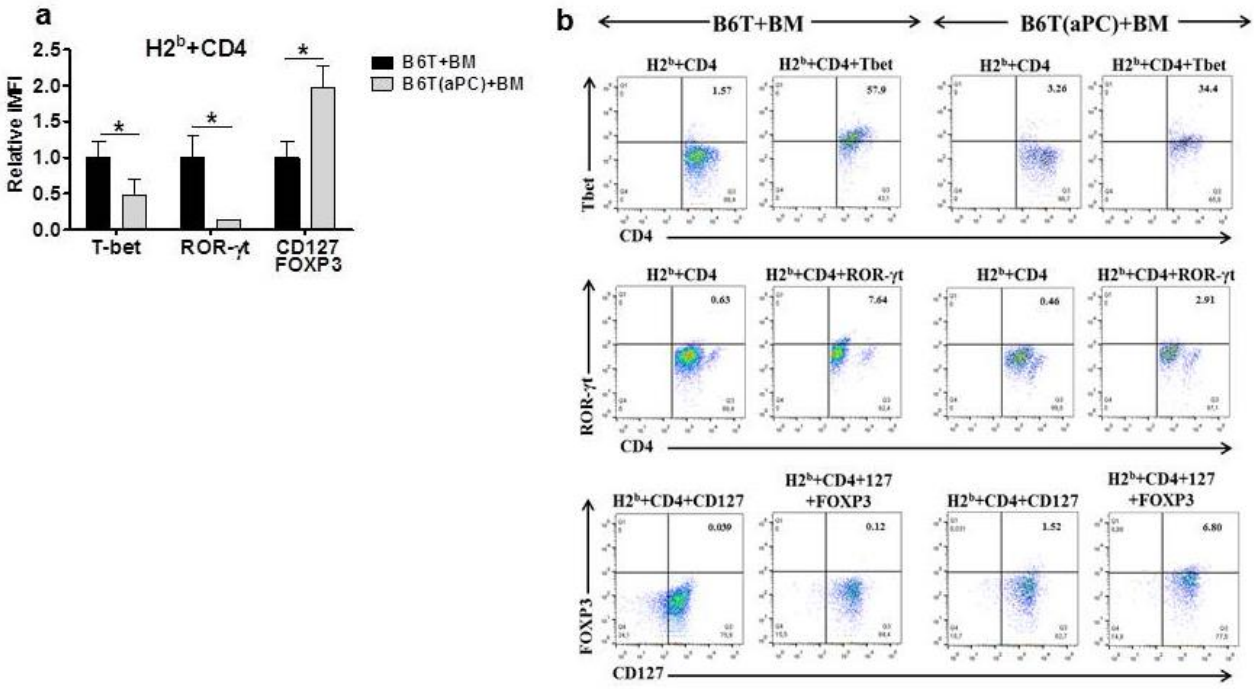

Figure 3.4 T-cell pretreatment with aPC ameliorates GvHD by inducing Tregs and IL10 while reducing Th1 and Th17 cells and pro-inflammatory cytokines

(a,b) Recipient BALB/c mice were lethally irradiated (11Gy) and transplanted with 5×10^6 bone marrow and 0.5×10^6 T-cells without (B6T+BM) or with (B6T(aPC)+BM) aPC-pretreatment (20nM, 1h, 37°C) from donor C57BL/6 wt mice. Recipients were sacrificed 2 weeks post transplantation, splenic T-cells were harvested and stained for H-2^b, CD4, T-bet, ROR-γt, CD127, FOXP3and analysed by flow cytometry. For T-bet and ROR-γt cells were gated on H-2^b+ CD4 cells, while for FOXP3 cells were gated on H-2^b+ CD4+CD127 cells.

3.4.1 T-cell pretreatment with aPC ameliorates GvHD by inducing IL10 while reducing pro-inflammatory cytokines

Concomitant with the change in Th1 (T-bet), Th17 (ROR-γt) and Treg (FOXP3) population, $CD4^+$ T-cells expressed less IFNγ, TNFα, and IL17A, but more IL10 in mice that received aPC-pretreated T-cells (**Fig. 3.4.1a,b**). These results establish that protective effects of aPC-pretreatment of donor T-cells were sustainable throughout the effector phase of acute GvHD and this protective effect could be mediated by activation and expansion of Treg cells, reduction of Th1 effector cells, and a protective cytokine profile [146].

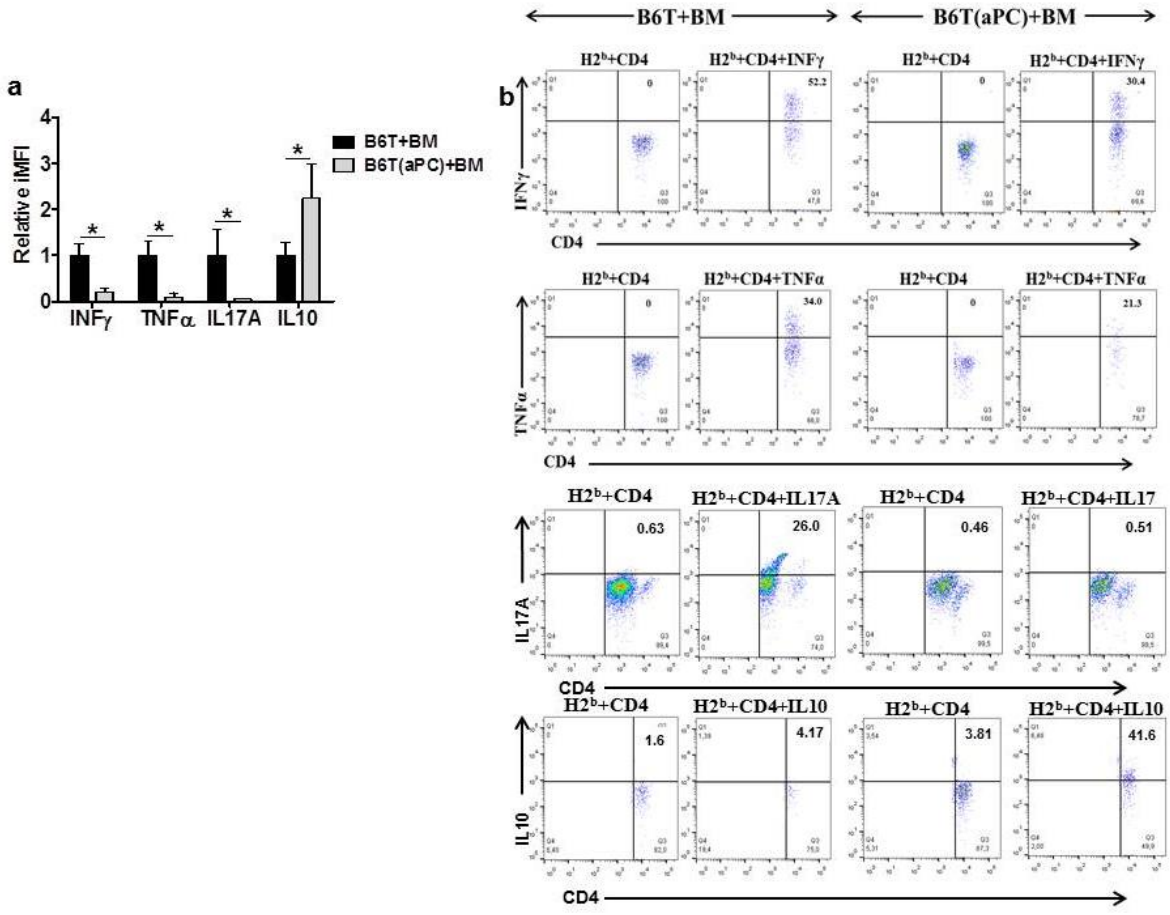

Figure 3.4.1 T-cell pretreatment with aPC ameliorates GvHD by inducing IL10 while reducing Th1 pro-inflammatory cytokines

(a,b) Recipient BALB/c mice were lethally irradiated (11Gy) and transplanted with 5×10^6 bone marrow and 0.5×10^6 T-cells without (B6T+BM) or with (B6T(aPC)+BM) aPC-pretreatment (20nM, 1h, 37°C) from donor C57BL/6 wt mice. Recipients were sacrificed 2 weeks post transplantation, splenic T-cells were harvested and stained for $H\text{-}2^b$, CD4, INFγ, TNFα, IL17A and IL10 and analysed by flow cytometry. For INFγ, TNFα, IL17A and IL10 cells were gated on $H\text{-}2^b$+CD4 cells.

3.5 Concomitant and pretreatment of human pan T-cell with aPC reduces allogenic T-cell activation

The effect of aPC on allogenic activation of human T-cells was next assessed using the MLR [147]. Co-incubation and pre-incubation of human pan T-cells, but not of human AgPC, with aPC significantly reduced T-cell proliferation in the MLR (**Fig. 3.5a,b**).

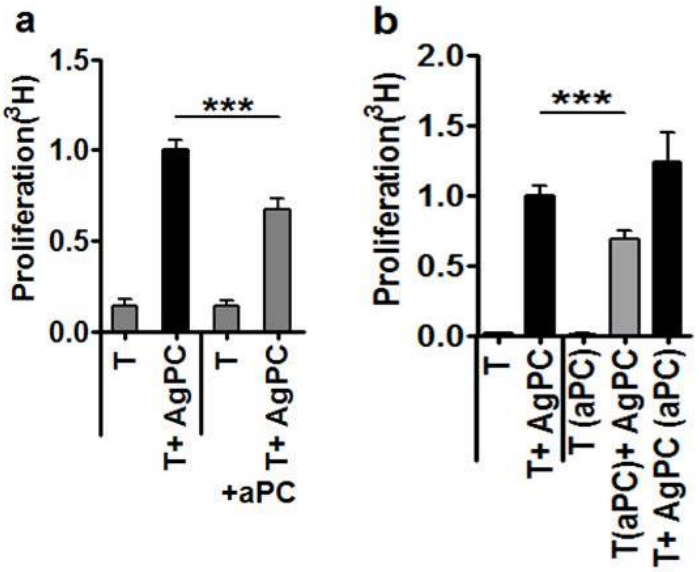

Figure 3.5 Concomitant and pretreatment of human pan T-cell with aPC reduces allogenic T-cell activation

(a) Mixed lymphocyte reaction (MLR) of human peripheral blood T-cells (T) with allogenic antigen presenting cells (AgPC) for 96h. Proliferation was measured by thymidine incorporation during the final 16h. Concomitant treatment with aPC (+aPC; 20nM, every 12h) reduces T-cell proliferation; results of 3 independent experiments, each in triplicates.

(b) aPC-pretreatment of human pan T-cells (T(aPC)+AgPC), but not of human antigen-presenting cells (T+AgPC(aPC)) reduces T-cell proliferation in the MLR (pretreatment once, 20nM, 1h, 37°C); results of 3 independent experiments, each in triplicates.

3.6 Pretreatment of human pan T-cell with aPC reduces Th1, Th17 cells and induces activated Tregs

Pre-incubation of human pan T-cells with aPC reduced the frequency of Th1 ($CD4^+T\text{-}bet^+$) and Th17 ($CD4^+ROR\text{-}\gamma t^+$) T-cells, while increasing the abundance of Tregs ($CD4^+CD127^+FOXP3^+$) following allogenic stimulation (**Fig. 3.6a,b**).

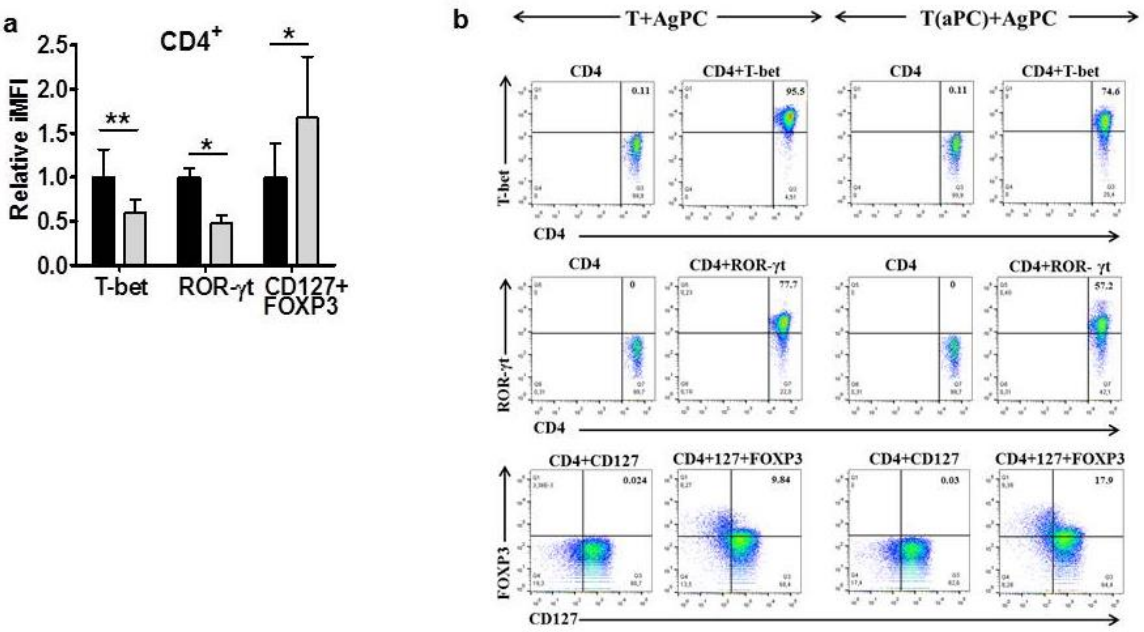

Figure 3.6 Pretreatment of human pan T-cell with aPC reduces Th1, Th17 cells and upregulates activated Tregs

(a,b) aPC-pretreatment of human pan T-cells reduces the frequency of T-bet and ROR-γt positive $CD4^+$ T-cells, while increasing $CD4^+$+$CD127^+$+$FOXP3^+$ regulatory T-cells, as measured by flow cytometry after 48h of MLR. For T-bet and ROR-γt cells were gated on CD4 cells, for FOXP3 cells were gated on CD4+CD127 cells.

3.6.1 Pretreatment of human pan T-cell with aPC reduces pro-inflammatory cytokines (IFNγ, TNFα, and IL17A) while inducing IL10

Concomitantly, pre-incubation of human pan T-cells with aPC reduces the expression of pro-inflammatory cytokines IFNγ, TNFα, and IL17A in T-cells, while expression of IL10 was increased (**Fig. 3.6.1a,b**).

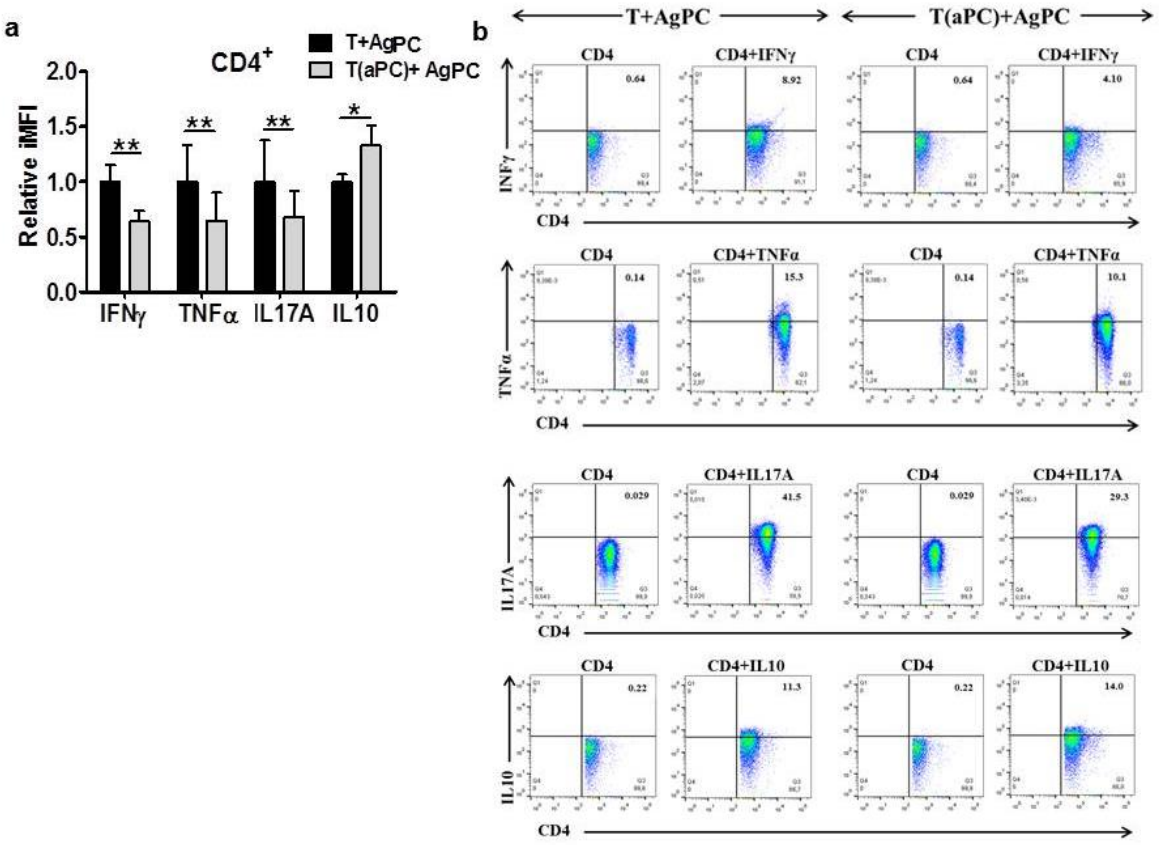

Figure 3.6.1 Pretreatment of human pan T-cell with aPC reduces pro-inflammatory cytokines (IFNγ, TNFα, and IL17A) and upregulates IL10

(a,b) aPC-pretreatment of human pan T-cells reduced expression of the pro-inflammatory cytokines interferon γ (INFγ), TNFα, and IL17A, while that of IL-10 is increased as measured by flow cytometry after 48h of MLR. For INFγ, TNFα, IL17A and IL10, cells were gated on CD4 cells.

3.7 aPC expands pre-existing regulatory T-cells and pretreatment of Tregs with aPC is sufficient to suppress T-cell proliferation

To determine whether aPC induces Treg differentiation from non-Treg cells or expands pre-existing Tregs we separated Tregs by MACS and conducted MLR without aPC-pretreatment (T+Tr+AgPC), MLR using Tregs without, but remaining T-cells with aPC-pretreatment (T(aPC)+Tr+AgPC), or MLR in which only Tregs were preincubated with aPC (T+Tr(aPC)+AgPC). Pre-incubation of Tregs with aPC, but not of the non-Treg T-cell population, increased the frequency of $CD4^{+}FOXP3^{+}$Tregs (**Fig. 3.7a**). To assess whether aPC induced Treg expansion is sufficient to reduce T-effector cell activation we separately isolated Teffector cells (Teff; $CD4^{+}CD25^{-}CD127^{hi}$) and Tregs ($CD4^{+}CD25^{+}$) by MACS. MLR-

experiments were then conducted in the absence (Teff+Treg+AgPC) or with aPC-pretreatment specifically of Tregs (Teff+Treg(aPC)+AgPC). Pre-incubation of Tregs with aPC was sufficient to suppress T-cell activation (**Fig. 3.7b**).

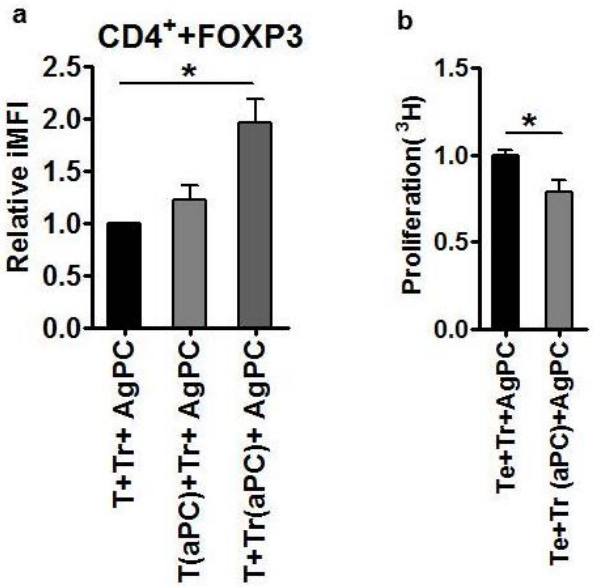

Figure 3.7 aPC expands pre-existing regulatory T-cells and pretreatment of Tregs with aPC is sufficient to suppress T-cell proliferation

(a) Pretreatment of pre-existing Tregs (Tr) with aPC (T+Tr(aPC)+AgPC), but not of non-Treg T-cells (T(aPC)+Tr+AgPC), increases Tregs as measured by flow cytometry after 48h of MLR.

(b) aPC-pretreatment of Tregs (Tr(aPC)) is sufficient to reduce the proliferation of T-effector cells (Te) in the MLR.

3.8 aPC signals via the PAR2/PAR3 heterodimer on T-cells

Jurkat T-cells express PARs, which are the pivotal receptors for aPC-dependent signaling [107, 119]. Similarly, primary human and mouse pan T-cells express all four PARs (**Fig 3.8a**) [118, 121]. The inhibitory effect of aPC on allogenic human T-cell activation was lost following incubation with an N-terminal blocking antibody to PAR3, while N-terminal blocking antibodies to PAR1, PAR2, or PAR4 had no effect **(Fig. 3.8b).** The requirement of PAR3 for aPC's inhibitory effect on T-cell activation was confirmed using T-cells isolated from PAR3-deficient mice (**Fig. 3.8c**). Intriguingly, blocking extracellular loop of PAR2 on human T-cells by blocking peptide abolished aPC's effect, while PAR1 or PAR4 inhibition had no effect (**Fig. 3.8d**), indicating the requirement of a PAR2/PAR3 heterodimer for aPC's inhibitory effect. Furthermore, co-expression and potential interaction of PAR2 and PAR3 in human pan T-cells

was confirmed by co-immunoprecipitation (**Fig. 3.8e).** In addition, PAR3 was readily detectable on human Tregs (CD4+CD25+) (**Fig. 3.8f**). These observations suggest that aPC cleaves PAR3 but signals via PAR2 in T-cells.

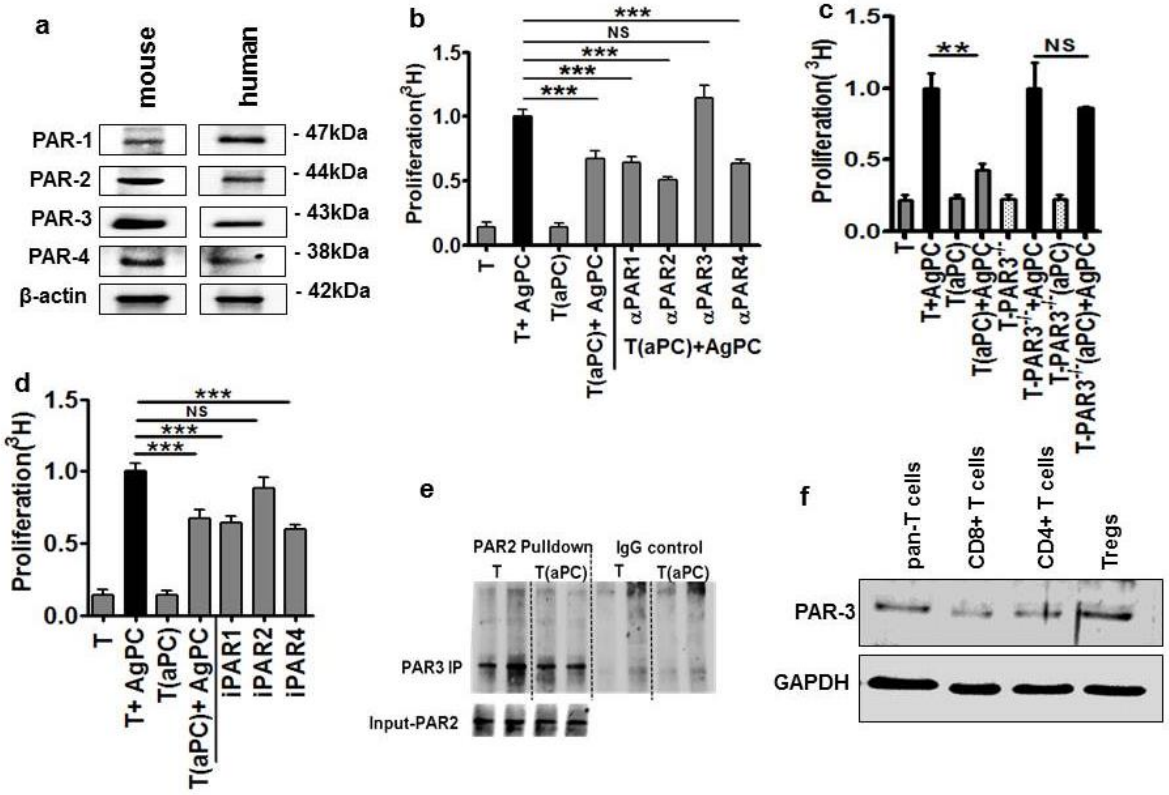

Figure 3.8 aPC signals via the PAR2/PAR3 heterodimer on T-cells

(a) Exemplary immunoblot showing expression of PARs on mouse and human primary T-cells. β actin was used as loading control.

(b) Pre-incubation of human peripheral blood T-cells (T) with N-terminal blocking antibodies against PAR3 (αPAR3), but not against PAR1 (αPAR1), PAR2 (αPAR2), or PAR4 (αPAR4), prior to aPC-pretreatment and MLR, abrogates aPC's inhibitory effect in regard to T-cell proliferation.

(c) Pretreatment of T-cells from C57BL/6 PAR3-deficient (PAR3$^{-/-}$) mice with aPC and subsequent allogenic stimulation (T-PAR3$^{-/-}$(aPC)+AgPC) abrogates aPC's inhibitory effect in regard to T-cell activation (T-cell proliferation).

(d) Blocking PAR1 (iPAR1, FR 171113) or PAR4 (iPAR4, ML 354) on human peripheral blood T-cells prior to aPC-pretreatment and MLR does not abrogate aPC's inhibitory effect, while blocking PAR2 (iPAR2, FSLLRY-NH2) abolishes aPC's inhibitory effect.

(e) Exemplary immunoblot showing interaction of PAR2 and PAR3 in human primary T-cells analysed using immunoprecipitation for PAR2 followed by immunoblotting of PAR3. Immunoprecipitation using IgG was used as negative control. Immunoblot of PAR2 was done as a loading control.

(f) Exemplary immunoblot showing PAR3 expression on human T cell subsets; Tregs: $CD4^+CD25^+$ Regulatory T-cells.

3.9 PAR2 and PAR3 on T-cells convey aPC's protective effect in GvHD

To assess whether PAR2 and PAR3 are required for aPC's ameliorating effect on GvHD we transplanted lethally irradiated BALB/c mice with allogenic (C57BL/6) 5×10^6 BM and 0.5×10^6 T-cells. First, a subset of T-cells was treated with a PAR2 blocking peptide (iPAR2) followed by incubation with aPC (iPAR2(aPC)+BM) prior to transplantation. The GvHD protective effect of aPC was lost if PAR2 was blocked on T-cells (**Fig. 3.9a,b**). This demonstrates that PAR2 is required for the aPC's inhibitory effect on T-cells in GvHD *in vivo*.

Next, we transplanted lethally irradiated BALB/c mice with 5×10^6 BM cells and 0.5×10^6 T-cells derived from allogenic C57BL/6-wt or C57BL/6-$PAR3^{-/-}$ donor mice without (B6T+BM) or with (wtT-cells:B6T(aPC)+BM or $PAR3^{-/-}$T-cells: $PAR3^{-/-}$T(aPC)+BM) aPC-pretreatment. The GvHD protective effect of aPC was lost in mice transplanted with $PAR3^{-/-}$ T-cells (**Fig. 3.9a,b**). Of note, expression of PAR3 specifically on Tregs is sufficient for the GvHD protective function of aPC (**Fig. 3.9c)**. Taken together, these data establish that PAR2 and PAR3 on T-cells convey aPC's protective effect in GvHD *in vivo*.

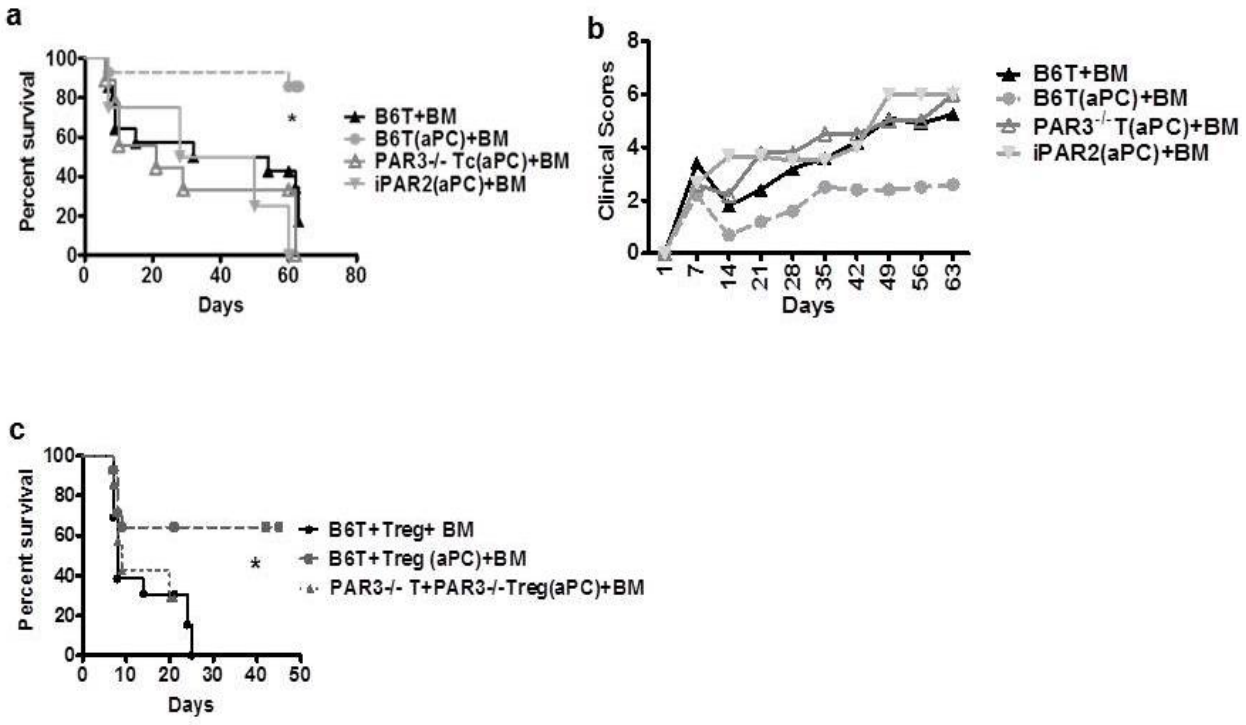

Figure 3.9 PAR2 and PAR3 on T-cells convey aPC's protective effect in GvHD

(a,b) Lethally irradiated (11Gy) recipient BALB/c mice were transplanted with C57BL/6 wt derived 5×10^6 BM cells and 0.5×10^6 T-cells without (B6T+BM) or with (B6T(aPC)+BM) aPC-pretreatment (20nM, 1h, 37°C). Alternatively, lethally irradiated BALB/c mice received 5×10^6 C57BL/6 wt derived BM cells and 0.5×10^6 C57BL/6 PAR3$^{-/-}$ T-cells (PAR3$^{-/-}$T(aPC)+BM) or 0.5×10^6 C57BL/6 T-cells treated with a PAR2 blocking peptide (FSLLRY-NH2) prior to aPC treatment (iPAR2(aPC)+BM; aPC treatment in both cases: 20nM, 1h, 37°C). aPC's protective effect is lost in mice transplanted with PAR3 deficient or PAR2 blocked T-cells; Survival (**a,** Kaplan-Meyer analyses) from two (iPAR2(aPC)+BM) or three (all other groups) independent experiments each with 4 mice per group are shown.

(c) Lethally irradiated (11Gy) recipient BALB/c mice were transplanted with C57BL/6 derived 5×10^6 BM cells and the following combination of C57BL/6 derived T-cells: (i) 0.4×10^6 T-cells and 0.1×10^6 Treg-cells (B6T+Treg+BM), (ii) 0.4×10^6 T-cells and 0.1×10^6 Treg-cells with aPC-pretreatment (B6T+Treg(aPC)+BM), or (iii) with 0.4×10^6 PAR3$^{-/-}$T-cells and 0.1×10^6 PAR3$^{-/-}$ Treg-cells with aPC-pretreatment (PAR3$^{-/-}$T+PAR3$^{-/-}$Treg(aPC)+BM). aPC-pretreatment was conducted as before (20nM, 1h, 37°C). Loss of PAR3 on Treg-cells abrogates the GvHD protective effect of aPC. Survival (Kaplan-Meyer analyses) from three independent experiments, each with 4 mice per group, is shown.

3.10 aPC mitigates human CD4$^+$ T-cell mediated GvHD

The above data suggest that *ex vivo* pre-incubation of pan T-cells or Tregs prior to transplantation may be an easy, efficient, and safe new therapeutic strategy to mitigate GvHD. To corroborate the translational relevance we transplanted NSG-Ab°DR4 mice, which lack expression of the murine *Prkdc* gene, the X-linked *Il2rg* gene, and MHC class II while expressing the human leukocyte antigen DR4 gene[64], with 4×10^6 human CD4$^+$ T-cells (HLA-DR4$^-$) without (hCD4$^+$) or with (hCD4$^+$(aPC)) aPC-pretreatment. Survival, physical appearance, histological damage of NSG-Ab° DR4 mice transplanted with aPC-pretreated human CD4$^+$ T-cells (hCD4$^+$ (aPC)) was markedly improved compared to control (hCD4$^+$) mice (**Fig. 3.10a-e**).

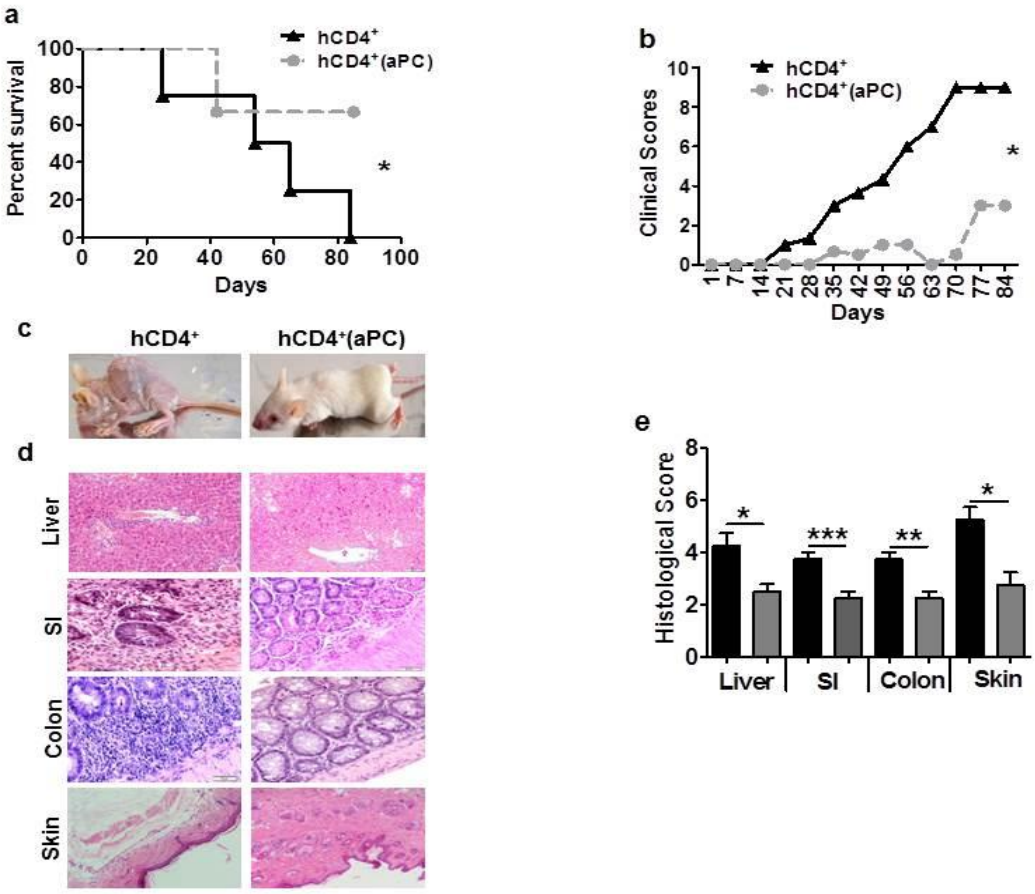

Figure 3.10 aPC mitigates human CD4$^+$ T-cell mediated GvHD

(a-e) Recipient NSG-Ab° DR4 mice were irradiated with 2 Gy and transplanted 4h later with 4×10^6 of human CD4$^+$ T-cells (HLA-DR4$^-$) without (hCD4$^+$) or with (hCD4$^+$(aPC)) aPC-pretreatment. Survival (**a**, Kaplan Meyer analyses), physical parameters (**b,** clinical score), and gross-appearance (**c**) were markedly improved in mice transplanted with aPC-pretreated human CD4$^+$T-cells; pooled data from 3 independent experiments each with 2 recipients. **(d,e)** Photomicrographs depicting typical morphology in liver, small intestine (SI), colon, and skin (**d,** haematoxylin and eosin stained section, size bar 50 μm and **e**, bar graph summarizing histological disease scores).

3.11 aPC mitigates GvHD without impairing the GvL effect

To evaluate aPC's effect on the graft-versus-leukemia (GvL) we transplanted lethally irradiated BALB/c mice with syngenic 5×10^3 GFP positive mixed lineage leukemic cells (MLL-AF9-BALB/c background) [148], 5×10^6 allogenic BM cells, and 0.5×10^6 allogenic T-cells without (B6T+BM+MLL-AF9) or with (B6T(aPC)+BM+MLL-AF9) aPC-pretreatment. The survival of mice transplanted with aPC-pretreated T-cells (B6T(aPC)+BM+MLL-AF9) was markedly

improved compared to B6T+BM+MLL-AF9 mice and similar (long-term survival ~40%) to that observed in mice without concomitant syngenic 5×10^3 GFP positive MLL-AF9 leukemic cells (**Fig. 3.11a**). Importantly, the peripheral leukemic load determined 4 weeks post transplantation was markedly reduced in B6T(aPC)+BM+MLL-AF9, but not in B6T+BM+MLL-AF9 mice (**Fig. 3.11b,c**). Hence, *ex-vivo* pretreatment of T-cells with aPC ameliorates GvHD without compromising the GvL effect.

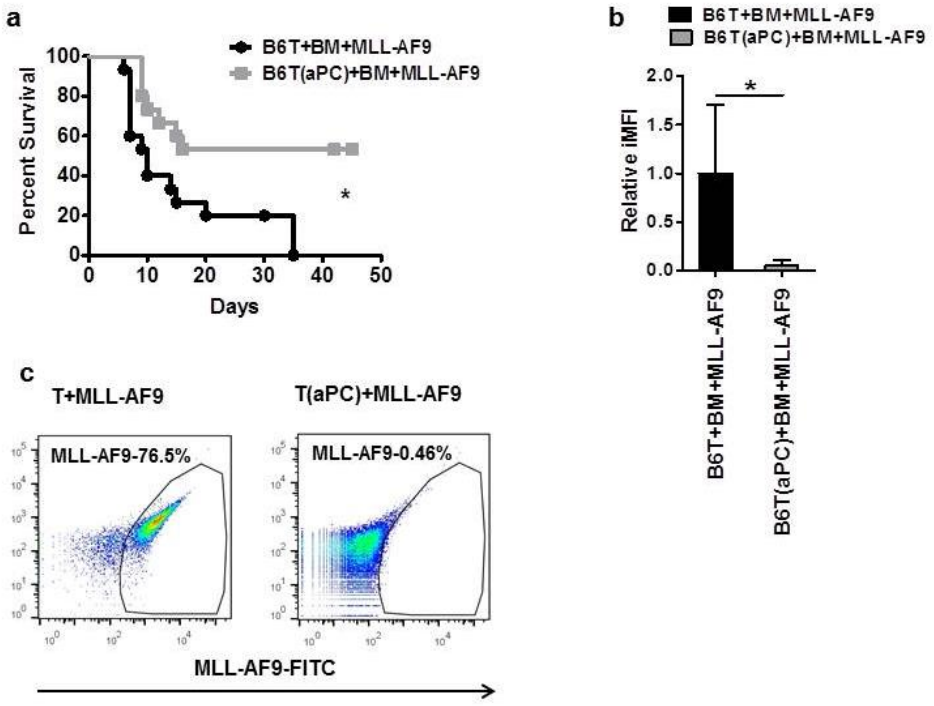

Figure 3.11 aPC mitigates GvHD without impairing the GvL effect

(a) Lethally irradiated (11Gy) recipient BALB/c mice were transplanted with 5×10^6 C57BL/6 derived BM cells, 5×10^3 GFP+MLL-AF9 leukemic cells, and 0.5×10^6 C57BL/6 T-cells without (B6T+BM+MLL) or with (B6T(aPC)+BM+MLL) aPC-pretreatment. In mice transplanted with aPC-pretreated T-cells survival is improved (**a**, Kaplan Meyer analyses) and leukemic load (determined by flow cytometry 4 weeks after transplantation in peripheral blood samples) is markedly decreased (**b,c**); pooled data from 3 independent experiments each with 4 recipients. Mean value ± SEM; iMFI: integrated mean fluorescence intensity, calculated as percentage of positive cells times mean fluorescence intensity of positive cells.

4 Discussion

Endothelial cell dysfunction characterized by loss of cell surface bound thrombomodulin (resulting in less aPC generation) has been reported to be associated with complications after HSCT [64, 66]. Luft *et al.* has demonstrated in human patients that high levels of sTM are associated with refractory GvHD [64]. Ikezoe *et al*. previously demonstrated that the use of recombinant thrombomodulin (rTM) alleviated acute GvHD in association with a decrease in the plasma levels of inflammatory cytokines and high-mobility group B1 DNA-binding protein (HMGB1) in a murine acute GvHD model [149]. Direct evidence that the coagulation protease aPC ameliorates acute GvHD has been lacking so far. The protective effect of endogenous high levels of aPC in acute GvHD in APC^{high} mice provides the first time evidence that aPC can alleviate acute GvHD in mice. The present study does not imply an association of endothelial dysfunction with acute GvHD in mice, but the current data strongly suggests that loss of thrombomodulin, which subsequently results in lesser aPC, can be compensated for by exogenous aPC in patients with acute GvHD. Further studies are required to establish the correlation of endothelial dysfunction with GvHD in mice and to study the role of exogenous aPC in regulating GvHD in these cases.

GvHD is primarily a T-cell driven disease and most of the current therapies aiming to mitigate GvHD employ suppression of allo-reactive donor T-cells. The role of coagulation protease in general and in particular of aPC in regulating T-cell reactivity has not been studied in detail so far. The first direct evidence that aPC conveys an anti-proliferative effect in both human and mouse allogenically stimulated pan T-cells is provided within the current study. Furthermore, our data demonstrates that aPC directly acts on T-cells and reduces their proliferation in MLRs. This data identify a novel immune-regulatory function of the coagulation protease aPC and further suggests that the anti-proliferative activity of aPC in T-cells may represent one of the mechanisms to regulate immune cells in GvHD.

Expression of PARs on T-cells has been reported before, but their physiological relevance in adaptive immunity remained unknown hitherto [107]. The current study demonstrates that the inhibitory effect of aPC is abolished by blocking proteolytic cleavage of the N-terminal end of PAR3 using an inhibitory antibody or by blocking the extracellular loop of PAR2 using blocking

peptide on human pan T-cells. Accordingly we observed co-localization of PAR2/PAR3 on T-cells by co-immunoprecipitation assay. The differential effect of N-terminal binding antibodies and signaling inhibitors (peptides) together with the co-localization of PAR2/PAR3 on T-cells strongly imply that aPC conveys signals in T-cells through cleavage of PAR3 and cross-activation of PAR2 via the tethered ligand of PAR3. Further studies are required to identify the molecular structures required for this receptor heterodimer on T-cells, the dynamics of heterodimer formation, and the intracellular signaling intermediates.

The acute phase of GvHD is primarily driven by T-helper (Th)1 inflammatory response marked by the increase in Th1-$CD4^+T\text{-}bet^+$ T-cell frequency and increased production of IFNγ [150]**.** Yu Y *et al.* have established that combined blockade of Th1 and Th17 differentiation is a promising strategy to improve outcome of allogeneic HSCT by inhibiting GvHD, while preserving GVL activity of donor T-cells [151]. The role of aPC in regulating T-cell differentiation following allogenic stimulation has not been studied so far. Our data demonstrates that pre-incubation of human pan T-cells with aPC not only reduced their proliferation but also diminished the frequency of Th1 ($CD4^+T\text{-}bet^+$) and Th17 ($CD4^+ROR\text{-}\gamma t^+$) T-cells in the MLRs. Concomitantly, the expression of pro-inflammatory cytokines IFNγ, TNFα, and IL17A in T-cells was reduced, while expression of anti-inflammatory cytokine IL10 was significantly increased. The increased IL10 levels were associated with an increased frequency of Tregs. The latter finding is in agreement with the previous observations showing that Treg exert their immunoregulatory functions through various mechanisms requiring production of immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF)-β [152]. Concomitant with the *in vitro* findings, the frequency of Th1 ($CD4^+T\text{-}bet^+$) and Th17 ($CD4^+ROR\text{-}\gamma t^+$) T-cells in mice transplanted with pre-treated aPC T-cells was reduced, which is in agreement with the findings of Yu Y *et al*.

Xue *et al*. demonstrated that intraperitoneal injection of aPC in non-obese diabetic (NOD) mice reduces the incidence of type 1 diabetes in these mice. This effect was associated with an increase of Tregs in the spleen, pancreatic islets, and pancreatic lymph nodes of NOD mice following aPC treatment [153]. However, the direct effect of aPC in Treg expansion was not addressed within this study. The present study demonstrates that the frequency of activated human Tregs ($CD4^+CD127^+FOXP3^+$) increases following aPC-pretreatment and allogenic stimulation

suggesting that aPC expands the pre-existing Treg cells. Furthermore, the current data demonstrates that pretreatment of Tregs with aPC only once is sufficient to suppress the proliferation of effector T-cells ($CD4^{+}CD25^{-}CD127^{hi}$). Expansion of Treg is a major limitation in Treg based therapy of various diseases and here we uncovered a novel method of Treg expansion. It has been known that adoptive transfer of freshly isolated natural occurring regulatory T-cells prevents GvHD in several animal models and following hematopoietic cell transplantation in clinical trials [154]. Unfortunately the major limitation to a broader clinical application of Treg adoptive transfer is the failure to efficiently enrich Tregs for therapeutic purposes [45]. Here we have demonstrated a novel mechanism to address this challenge. Future studies are required to evaluate the translational relevance of these finding in the clinical setting. Furthermore, it will be interesting to determine whether the mechanisms identified here provide protection not only in the setting of acute GvHD, but potentially also in other T-cell dependent disease processes.

Tregs have been shown to convey protection from GvHD [154] but the direct effect of aPC in regulating Treg expansion and its protective role in GvHD has not been studied so far. Our finding demonstrates that pretreatment of mouse T-cells with aPC ameliorate GvHD. This demonstrates that aPC has a direct effect on Tregs. Importantly, the increased frequency of Tregs and the associated cytokine IL-10 indicates that aPC's protective effect in murine acute GvHD is mediated *via* Tregs. The protective effect of aPC in T-cells requires PAR2/PAR3 complex. These findings uncover a new pathway targeting Tregs and ameliorating GvHD. The expansion of Tregs by aPC-PAR2/PAR3 signaling and the efficacy of aPC to improve GvHD following *ex vivo* pretreatment of T-cells provide an effective approach to expand Tregs and to ameliorate GvHD in mice. Whole body irradiation and immunosuppression, which is used prior to HSCT, is known to reduce endothelial TM-mediated PC activation [155]. We propose that aPC-mediated Tregs expansion may be severely impaired in this setting thus promoting GvHD. Endothelial protective therapies have been proposed to convey beneficial effects in GvHD, but their translation was only partially successful [156, 157]. In these approaches the effect of the endothelial protective therapies on aPC-generation has never been considered. By reconstituting aPC we restore a very specific, but well established endothelial dependent cyto-protective mechanism. Importantly, reconstitution of aPC's effect *ex vivo* may be a safe yet efficient approach to compensate for the inevitable impairment of endothelial- and TM function during preconditioning of patients, allowing amelioration of GvHD.

In the current study aPC pretreatment of PAR3 deficient T-cells abolished the protective effect of aPC in mice. Furthermore, inhibiting PAR2 signaling on mouse T-cells using a blocking peptide abolished the protective effect of aPC. These findings suggest that aPC cleaves PAR3 but requires PAR2 for intracellular signaling to mediate its protective effect in T-cells and in GvHD. These *in vivo* observations are entirely congruent with the observations made *in vitro* using the MLR and the interaction of PAR2 and PAR3 on T-cells. Previously Thati *et al*. demonstrated that aPC binds to and cleaves PAR-3 in podocytes, which induces heterodimerization of PAR-3 with PAR-2 (human) or PAR-1 (mouse) in podocytes [88]. But signaling via PARs and involvement of PAR2/PAR3 in T-cells was unknown hitherto. The current data provides for the first time evidence that aPC signals *via* PARs in T-cells and that this effect depends on PAR2/PAR3 heterodimers.

Another important finding in the current study is that pretreatment of T-cell only once with aPC is sufficient to translate its protective effect in mice. The high efficacy despite pretreatment of T-cells only once suggests that aPC may induce epigenetic modification in T-cells. Various lysine residues at the amino-terminus of histone H3 and H4 tails are acetylated by histone acetyltransferase (HAT) enzymes or deacetylated by histone deacetylase (HDAC) which leads to modifications in the accessibility of DNA. Previously, in both an experimental model of diabetic nephropathy and glucose-stressed podocytes we demonstrated that aPC epigenetically controls expression of the redox-regulating protein $p66^{Shc}$. aPC epigenetically suppresses glucose-induced $p66^{Shc}$ expression by enhancing methylation while diminishing acetylation of the $p66^{Shc}$ promoter. Glucose-induced H3 hyperacetylation is mediated—at least in part—by the H3 acetyltransferase GCN5 [158]. Accordingly, we observed in these studies evaluating aPC in diabetic nephropathy that glucose-dependent induction of GCN5 in podocytes was prevented by aPC [159]. Since GCN5 in particular, but epigenetic mechanisms in general, likewise modulate T-cell differentiation [160] the role of aPC in modulating GCN5 and other HATs or HDAC in T-cells needs to be investigated in detail in future studies.

One of the key questions that arise from our data is whether the protective effect of aPC pretreatment on T-cells seen in mouse GvHD models can be translated into the human situation and whether this may constitute a safe and effective therapy to ameliorate GvHD in humans. In

order to gain some insights into the translational relevance of the current findings we induced GvHD in a "humanized" mouse model (NSG-Ab°DR4 mice) by injecting human $CD4^+$ T-cells. Pretreatment of human $CD4^+$ T-cells with aPC prior to $CD4^+$ T-cell transplantation resulted in a marked improvement of the GvHD. This establishes that aPC does not only act on mouse, but also human T-cells in an *in vivo* model of GvHD. These results imply a translational relevance of the current results and suggest that pretreatment of T-cells prior to transplantation may be a safe and effective therapeutic approach to GvHD-prevention. Clinical studies are now required to determine the efficacy of aPC in humans receiving an allogeneic transplant. One potential limitation of any aPC-based therapy is the inherent risk of hemorrhage. Various clinical studies showed that recombinant wt-aPC therapy increases the risk of serious bleeding [104, 161, 162]. Importantly, in our study T-cells were washed prior to transplantation to remove the aPC. Hence, only traces of aPC, which can be expected to have no impact on the bleeding risk, will be transferred. This approach provides a safe and effective therapy to mitigate GvHD without an increased risk of hemorrhage and can be extended to clinical trials.

An alternative strategy is the generation and employment of aPC mutants which are signaling competent (and hence cytoprotective) but lack anticoagulant function. Extensive work by several groups demonstrated that the anticoagulant and cytoprotective functions of aPC are molecularly disjunct. Accordingly, aPC mutants with reduced anticoagulant activity but retaining cell-signaling function have been developed. Thus, various signaling-selective aPC mutants (5A-aPC, 3K3A-aPC, aPC-2Cys, and K193E-aPC) and an anticoagulant-selective aPC mutant (E149A-aPC) have been developed and their preclinical studies have proved invaluable to clarify which of aPC's 2 broad types of actions, namely anticoagulant or cytoprotective, is more important for reducing injury in preclinical injury models. Currently, studies in humans are being conducted testing aPC mutants lacking anticoagulant effects in patients with stroke.

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative therapy for many malignant and nonmalignant hematologic diseases. The T-cells from the donor allograft are the critical component for the success of this therapy by targeting the leukemic cells and mediating GvL effect. Unfortunately these cells also mediate GvHD by targeting the healthy tissues in the host. Maintaining balance between GvHD and the GvL effect is a major challenge for the success of HSCT. In our current study we demonstrate that aPC not only mitigate GvHD

in mice but also maintains the GvL effect, providing a safe and effective method of ameliorating GvHD without hampering the efficiency to eradicate the residual malignant cells. The exact mechanism of this effect is currently not fully understood but similar protective effects against GvHD and enhanced GvL effect have been observed in some human studies and murine models [42, 44, 163, 164]. Importantly, it has been demonstrated that Tregs mitigate GvHD without compromising the GvL effect [164]. This finding is entirely congruent with our observation that aPC increases the frequency of Tregs. Further studies are required to understand the mechanism through which aPC enriches Tregs, and how Tregs can mitigate GvHD and at the same time maintains GvL effect.

5 Conclusion

In the present study we establish a novel function of aPC on adaptive immune cells. Pretreatment of T-cells with aPC is sufficient to inhibit allogenic T-cell proliferation in both human and mouse T-cells. *In vivo* studies further showed that pretreatment of allogenic T-cells with aPC markedly improved survival, physical appearance, and histopathology in mice with GvHD. Furthermore, the population of Th1 and Th17 cells, which are known to mediate the GvHD pathophysiology in mice, were significantly reduced in mice transplanted with aPC pretreated T-cells. The inflammatory cytokines like TNFα and IFNγ, which are also known to mediate the inflammatory phase of acute GvHD, were also significantly reduced in mice transplanted with aPC pretreated T-cells. It has been demonstrated that regulatory T-cells mediate a protective effect in GvHD and correspondingly we also observed that the Treg population was increased in mice transplanted with aPC pretreated T-cells. This protective effect of aPC was mediated by the PAR2/PAR3 complex on T-cells. In the present study we also observed that endogenous high level of aPC prevents against acute GvHD in APC^{high} mice. Taken together these data provide evidence that aPC inhibits *in vitro* T-cell proliferation and prolonged animal survival despite GvHD *in vivo*. This newly characterized immunomodulatory activity of aPC is associated with an expansion of Tregs in GvHD mice. Further aPC ameliorates GvHD in NSG-Ab°DR4 mice when transplanted with aPC pre-treated human $CD4^+$ T-cells suggesting a translational relevance of this approach. These findings and the sustained GvL-effect identify aPC-PAR2/PAR3 signaling in T-cells as a novel approach to effectively ameliorate GvHD.

6 Future Outlook

I: To determine the role of aPC in protecting the ISCs and Paneth cells in GvHD

Intestinal GvHD is characterized by severe villous atrophy and crypt degeneration, but it has been shown that the crypt cell degeneration represents only the initial lesions of intestinal GvHD [8-10, 16, 165-167]. Various experimental data suggests that intestinal stem cells (ISCs) and their niche Paneth cells reside within the crypts and these cells have been known to play a pivotal role in both physiologic tissue renewal and regeneration of the injured epithelium. Teshima and others have tested the hypothesis that ISCs and their niche could be primary targets in GvHD in mice [11-13, 168, 169]. It has been observed that apoptosis is induced in the ISCs within 24 hours following irradiation while sparing the Paneth cells, but in the absence of GvHD during this phase, the ISCs rapidly recover and restore the normal architecture of the small intestine [12, 14, 170, 171]. However, after the onset of GvHD both ISCs and Paneth cells fail to recover, indicating that the ISC-niche system is the target in intestinal GvHD (Figure 1) [11-13, 168-170]. Secretory cells, such as Paneth cells and goblet cells, play a critical role in maintaining intestinal microbial ecology and protecting hosts from pathogens. Paneth cells secrete a range of antimicrobial peptides, such as α-defensins [15], which help in shaping the microbial composition in the host and in GvHD the loss of Paneth results in a reduced secretion of α-defensins, leading to intestinal dysbiosis [13, 16-19, 169, 172-175]. Goblet cells also secrete mucin that helps in shielding the epithelium of GvHD mice [20, 176]. In our study we observed significantly reduced apoptosis in the intestine of APC^{high} mice and furthermore the crypt loss was significantly reduced supporting the hypothesis that the ISCs and Paneth cells residing in the crypts of these mice were less damaged in comparison to wt mice. However, the role of aPC in protecting the ISCs and Paneth cells need to be investigated further and will be part of future studies.

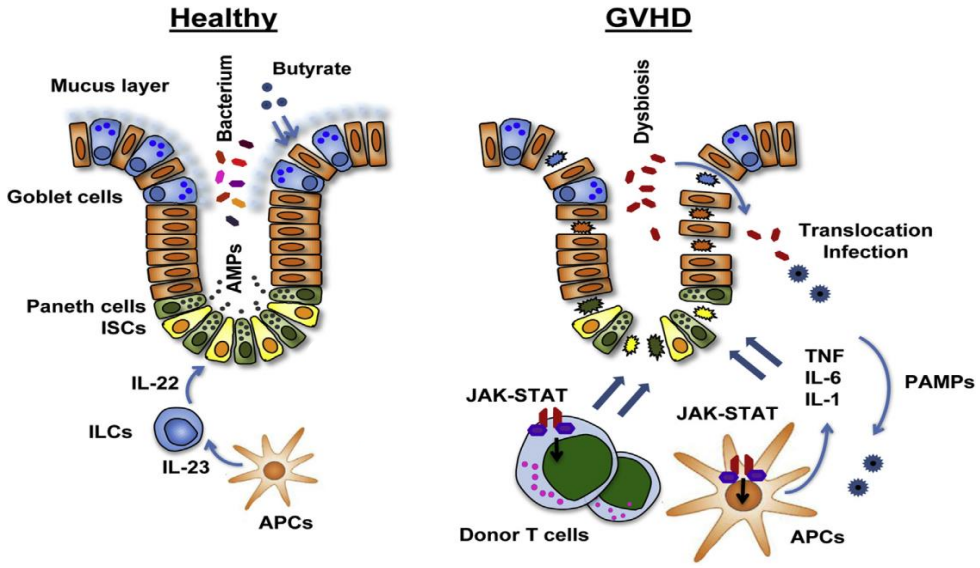

Figure 1: Homeostasis of self-renewing small intestinal epithelium results from ISCs, which locate at the base of the crypts and are interspersed between their niche Paneth cells. IL-22 produced from innate lymphoid cells (ILCs) upon stimulation of IL-23 from antigen-presenting cells (AgPCs) and R-spondins play a role in self-renewal and differentiation of ISCs. Paneth cells maintain intestinal microbial ecology by secreting antimicrobial peptides (AMPs) into the lumen. Goblet cells shield the epithelium from luminal bacteria by secreting mucin. Intestinal homeostasis depends on proper interaction between the mucosal immune system and intestinal microbiota and its metabolites, such as butyrate. After allo-SCT, donor T-cells and inflammatory cytokines, such as TNF, IL-6, and IL-1, damage the ISC-niche system. Paneth cell loss results in a reduced secretion of AMPs, leading to intestinal dysbiosis. Tissue injury permits translocation and dissemination of dominant luminal pathogens and pathogen-associated molecular patterns, which further accelerates GvHD by propagating a pro-inflammatory cytokine milieu. JAK-STAT pathways are required for responses to multiple cytokines and, thus, represent potential new targets in GvHD (Teshima *et al.* 2016).

II: To determine the role of aPC on the induction of "anergy" in T-cells

It has been known that the activation of T-cells is tightly controlled by many positive and negative regulatory processes. One of the mechanisms to negatively regulate T-cell activation is clonal anergy. Anergy is a hyporesponsive state that occurs when T-cells are activated through the T-cell antigen receptor in the absence of appropriate co-stimulatory signals. It has been observed that anergy can be induced both *in vitro* and *in vivo*. Schwartz laboratory in the 1980s for the first time characterized *in vitro* anergy using $CD4^+$ T helper 1 (Th1) cell clones [177]. They found that T-cells stimulated with chemically fixed AgPCs—which presumably failed to

upregulate co-stimulatory ligands—showed significantly less proliferation and secretion of interleukin-2 (IL-2) in comparison to T-cells that were stimulated with non-fixed AgPCs. The proliferation of T-cells is nearly completely blocked in anergic T-cells and at the same time secretion of IL2 is also significantly reduced. Also the anergic T-cells secrete very less pro-inflammatory cytokines like interferon-γ (IFN-γ) and IL-3 [178]. In the current study we observed that both concomitant and pretreatment of T-cells by aPC significantly reduces the proliferation of T-cells when stimulated with allogenic antigen presenting cells. Further, we observed that the secretion of the pro-inflammatory cytokine IFNγ was significantly reduced in allogenic stimulated aPC pretreated T-cells after 48h of MLC. Taken together, these findings suggest that aPC may induce anergy in T-cells. The induction of T-cell anergy would provide a mechanistic framework for the observed effects of aPC on T-cells. The role of aPC in the induction of anergy in T-cells needs to be further investigated and validated.

6 References

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8 Curriculum Vitae

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9 Declaration

Hiermit erkläre ich, dass ich die von mir zur Promotion eingereichte Dissertation mit dem Titel

Activated protein C protects from GvHD by inducing regulatory T-cell expansion and signaling *via* the PAR2/PAR3 heterodimer in T-cells

im Institut für Klinische Chemie und Pathobiochemie

mit Unterstützung durch Prof. Dr. med. Berend Isermann

selbständig verfasst und ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, den

Satish Ranjan

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