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Studies on immunosuppression using antigen-specific regulatory T cell in xenotransplantation

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博学之，审问之，慎思之，明辨之，笃行之.

-礼记·中庸

To this attainment there are requisite the extensive study of what is good, accurate inquiry about it, careful reflection on it, the clear discrimination of it, and the earnest practice of it.

- Book of Rites · The Doctrine of the Mean

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1 Summary

Generation of human porcine-specific regulatory T cells with high expression of IL-10, TGF-β1 and IL-35

Background: Organ transplantation remains the most effective treatment for patients with late stage organ failure. Transgenic pigs provide an alternative organ donor source to the limited availability of human organs. However, cellular rejection still remains to be the obstacle for xenotransplantation. Regulatory T cells (Treg) play an important role in maintenance of homeostasis in vivo. Natural Treg (nTreg) generation in vitro is laborious and expensive. Antigen-specific Treg are more effective and alleviate cellular rejection with fewer side effects. Here, we demonstrate the use of a fast method to provide tolerogenic dendritic cells (tolDC) that can be used to generate effective porcine-specific Treg cells (PSTreg).

Method: tolDC were produced within three days from human monocytes in medium supplemented with anti-inflammatory cytokines. Treg were generated from naïve $CD4^+$ T cells and induced to become PSTreg by cocultivation with porcine-antigen-loaded tolDC.

Results: PSTreg exhibited the expected phenotype, $CD4^+CD25^+CD127^{low/-}$ $Foxp3^+$, and exhibited a more activated phenotype. The specificity of PSTreg was demonstrated by suppression of the effector T cell (Teff) activation markers CD154 and CD25 at different stages and by inhibition of Teff cell proliferation. TolDC and PSTreg exhibited high expression of IL-10 and TGF-β1 at both protein and RNA levels, and PSTreg also highly expressed IL-35 at RNA levels. Upon restimulation, PSTreg retained the activated phenotype and specificity.

Conclusion: Taken together, the newly developed procedure allows efficient generation of highly suppressive PSTreg.

Generation of baboon PSTreg with high expression of IL-10 and TGF-β1

Background: The baboon, as a pre-clinical non-human primate experimental animal model, is widely used in xenotransplantation research. An effective method to generate baboon xeno-specific Treg would benefit research on immune tolerance in xenotransplantation using this model system.

Method: Baboon tolDC were generated in three days from monocytes isolated from baboon peripheral blood mononuclear cells (PBMC) in medium supplemented with anti-inflammatory cytokines as described for human tolDC. After loading with porcine-specific (PS) in vitro transcribed RNA (ivtRNA), tolDC were used to induce $CD4^+$ T cells to become baboon PSTreg in cocultures supplemented with IL-2 and rapamycin for 10 days. Anti-inflammatory and inflammatory cytokine expression was evaluated at the mRNA and protein levels in both baboon tolDC and PSTreg. PSTreg specificity was demonstrated by their capacity to suppress induction of activation markers on PSTeff at early (CD154) and intermediate (CD25) stages.

Results: Baboon tolDC generated with this method exhibited a tolerogenic phenotype, expressed CCR7, and produced high levels of IL-10, whereas IL-12p40 was not expressed. PSTreg were successfully generated in cocultures of $CD4^+$ T cells and PS ivtRNA-loaded tolDC. They exhibited the known $CD4^+CD25^+CD127^{low/-}CD45RA^{low}Foxp3^+$ phenotype, and were characterized by high expression of IL-10 mRNA and protein. They showed upregulated expression of *TGFB1*, *EBI3* and *GARP* mRNA. PSTreg exhibited highly suppressive effects towards PSTeff, secreting high amounts of IL-10 cytokine upon interaction with PSTeff.

Conclusion: Our fast 3-day method is also applicable to generate baboon-derived tolDC and allows subsequent induction of baboon PSTreg displaying high porcine-antigen specificity and expression of IL-10 and TGF-β1. Porcine-specific baboon Treg can be used in porcine solid organ or cell xenotransplantation studies through adoptive cell transfer into host baboons.

SUMMARY

Immune monitoring of baboons pre and post transplantation of multitransgenic pig hearts

Background: Immunosuppressive therapy was investigated to achieve clinical tolerance in transplantation. Immune monitoring of pig heart-transplanted baboons is an important component to provide a better understanding of the immune reactions during xenotransplantation.

Methods: Three groups of baboons receiving different immunosuppressive pre-treatments were set up in this project. In group 1, baboons received α-1,3-galactosyltransferase (*GGTA1*) homozygous knockout/heterozygous *hCD46* pig hearts, and baboon 49 obtained a *GGTA1* homozygous knockout/heterozygous *hCD46*/heterozygous *HLA-E* pig heart. In group 1, conventional immunosuppressive therapy with bortezomib and cyclophosphamide was used. In group 2, baboons received *GGTA1* homozygous knockout/heterozygous *hCD46* pig hearts, and anti-CD40 monoclonal antibody (mAb) was used to block antigen-presenting cell (APC)-T cell reaction instead of conventional immunosuppression. In group 3, baboons received *GGTA1* homozygous knockout/heterozygous *hCD46*/homozygous *hTM (thrombomodulin)* pig hearts, the baboons received anti-CD40 mAb or anti-CD40L mAb. In all groups, B cells were depleted by anti-CD20 mAb treatment. For immune monitoring during transplantation, different T cell populations, monocytes, B cells, natural killer (NK) and NKT cells were analyzed using flow cytometry.

Results: In group 1, under conventional immunosuppressive therapy, T cells were suppressed successfully before transplantation. However, activated T cells were increased after transplantation. In Baboon 49 who received a HLA-E-expressing pig heart, the NK cells remained at low levels. Baboon 49 showed a prolonged survival in comparison to the other baboons. In group 2, blockade of CD40 signaling resulted in downregulation of T cells, and monocytes, especially in baboon 54 who also showed a longer survival. In group 3, no significant difference between blocking CD40 and blocking CD40L was observed. However, rather than depletion of T cell populations, blocking APC-T cell reaction inhibited T cell activation compared to group 1. In group 2 and group 3, the NK cell and NKT cell numbers decreased after transplantation in all baboons. Baboon 64 in group 3 showed the longest survival, the major difference in

immune monitoring was observed in the first week. Therefore based on the results seen in baboon 64, the decrease in activated T cells together with the increase in Treg in the first week after transplantation could be an indicative of longer transplant survival.

Conclusion: Immunosuppression by blocking CD40:CD40L signaling pathway prolonged the survival compared to conventional immunosuppression. Suppression of NK/NKT cells, B cells and an increased level of Treg cells help to prolong the survival of xenotransplants.

Zusammenfassung

Generierung humaner Schwein-spezifischer regulatorischer T-Zellen, die eine hohe Expression von IL-10, TGF-β und IL-35 aufweisen

Hintergrund: Organtransplantation ist die effektivste Behandlungsmethode für Patienten, bei denen das Organ so schwer geschädigt ist, dass es keine andere Möglichkeit der Behandlung mehr gibt. Organe transgener Schweine bieten eine Alternative zu humanen Organen, die nur begrenzt verfügbar sind. Zelluläre Abstoßung bleibt jedoch auch bei der Xenotransplantation ein Hindernis. Regulatorische T-Zellen (Treg) spielen eine bedeutende Rolle in der Erhaltung der Homöostase in vivo. Generierung ausreichender nTreg ist in vitro aufwendig und teuer. Abhilfe könnten Antigen-spezifische Treg schaffen, die effizienter sind und deren Einsatz zudem weniger Nebenwirkungen aufweisen würden. In dieser Arbeit zeigen wir eine schnelle Methode der Generierung tolerogener dendritischer Zellen (tolDC), mit deren Hilfe man Schwein-spezifische Treg (PSTreg) herstellen kann.

Methode: TolDC konnten innerhalb von drei Tagen aus humanen Monozyten generiert werden unter Verwendung eines Mediums mit anti-inflammatorischen Zytokinen. Anschließend wurden diese tolDC mit Schweineantigen beladen und mit naiven $CD4^+$ T-Zellen cokultiviert, um PSTreg zu erzeugen.

Ergebnisse: PSTreg zeigten den erwarteten Phänotyp ($CD4^+CD25^+CD127^{low/-}$ $FoxP3^+$) und wiesen einen aktivierten Zustand auf. Die Spezifität der PSTreg konnte anhand der Suppression der Aktivierungsmarker CD154 (*early marker*) und CD25 (*intermediate marker*) auf Effektor-T-Zellen (Teff) und der Suppression der Proliferation demonstriert werden. TolDC and PSTreg zeigten sowohl auf Protein- als auch auf RNA-Ebene eine hohe Expression von IL-10 und TGF-β. Auf RNA-Ebene konnte bei PSTreg auch eine Expression von IL-35

nachgewiesen werden. Auch nach Restimulation behielten die PSTreg ihre Aktivität und Spezifität.

Schlussfolgerung: Die hier neu entwickelte Methode erlaubt eine effiziente Generierung hoch suppressiver PSTreg.

Generierung PSTreg aus Pavianen, die eine hohe Expression von IL-10 und TGF-β zeigen

Hintergrund: Der Pavian ist ein weithin in der Xenotransplantation gebräuchliches präklinisches nicht-humanes Primaten-Tiermodell. Eine effiziente Methode der Herstellung xeno-spezifischer Pavian Treg würde vorteilhaft sein, um Immuntoleranz auch in diesem Xenotransplantationsmodell zu erzeugen.

Methode: Wie im humanen System sollten tolDC generiert werden, um Schwein-spezifische Pavian Treg zu induzieren. Auch Pavian tolDC konnten innerhalb von drei Tagen aus Monozyten durch Kultivierung in einem Medium mit anti-inflammatorischen Zytokinen erzeugt werden. Nach Beladung mit Schwein-spezifischer *in vitro* transkribierter RNA (ivtRNA) wurden auch diese tolDC dazu verwendet, um in Gegenwart von IL-2 und Rapamycin aus $CD4^+$ T-Zellen PSTreg zu induzieren. Anti-inflammatorische und inflammatorische Zytokine wurden sowohl in tolDC als auch in PSTreg auf mRNA- und Protein-Ebene analysiert. PSTreg Spezifität konnte anhand der Suppression des frühen (CD154) und etwas später exprimierten (CD25) Aktivierungsmarker auf PSTeff gezeigt werden.

Ergebnisse: Die Pavian tolDC wiesen den bekannten tolerogenen Phänotyp auf, exprimierten CCR7 und produzierten hohe Mengen an IL-10, wohingegen IL-12p40 nicht produziert wurde. Pavian PSTreg konnten erfolgreich durch Cokultur von $CD4^+$ T-Zellen und PS ivtRNA-beladenen tolDC induziert werden. Auch sie wiesen den bekannten $CD4^+CD25^+CD127^{low/-}CD45RA^{low}FoxP3^+$ Phänotyp auf und zeichneten sich durch hohe Expression von IL-10 auf sowohl mRNA- als auch Protein-Ebene aus. Auch *TGFB1*, *EBI3* und *GARP* mRNA wurden hochreguliert. PSTreg zeigten eine hohe spezifische Suppressoraktivität gegenüber PSTeff und sezernierten eine hohe Menge an IL-10 nach Interaktion mit PSTeff.

Schlussfolgerung: Unsere schnelle 3-Tage-Methode ist auch anwendbar für die Generierung von Pavian tolDC und erlaubt die Induktion von PSTreg mit hoher Spezifität für Schweineantigen. Die Pavian Treg zeichnen sich ebenfalls durch die Expression hoher Mengen an IL-10 und TGF-β aus. Der adoptive Transfer

von Schweineantigen-spezifischen Pavian Treg könnte bei der Xenotransplantation von Organen und Zellen aus multitransgenen Schweinen in Paviane Anwendung finden.

Immunmonitoring von Pavianen vor und nach Transplantation von multitransgenen Schweineherzen

Hintergrund: Auch in der Xenotransplantation soll klinische Toleranz durch eine immunsuppressive Therapie erreicht werden. Für ein besseres Verständnis der Immunreaktionen vor und nach der Transplantation würde das Immunmonitoring ein geeignetes Verfahren darstellen. Hierzu wurde das Modell der Xenotransplantation von Schweineherzen in Paviane verwendet.

Methode: Drei Gruppen von Pavianen bekamen unterschiedliche immunsuppressive Behandlungen. Die Paviane der Gruppe 1 bekamen ein multitransgenes Schweineherz (α-1,3-galactosyltransferase (*GGTA1*) homozygous knockout/heterozygous *hCD46*; Pavian 49 *GGTA1* homozygous knockout/heterozygous *hCD46*/heterozygous *HLA-E*) und eine konventionelle immunsuppressive Therapie mit Bortezomib und Cyclophosphamid. Paviane der Gruppe 2 (*GGTA1* homozygous knockout/heterozygous *hCD46* Schweineherz) bekamen statt der konventionellen immunsuppressiven Therapie eine immunsuppressive Therapie mit anti-CD40 monoklonalem Antikörper. Paviane der Gruppe 3 (*GGTA1* homozygous knockout/heterozygous *hCD46*/homozygous *hTM*) bekamen entweder den anti-CD40 Antikörper oder einen anti-CD40L Antikörper. Die Antikörper sollten zur Blockade der Interaktionen zwischen Antigen-präsentierenden Zellen (APC) und T-Zellen dienen. In allen Pavianen wurden die B-Zellen durch die Gabe von anti-CD20 Antikörper depletiert. Für das Immunmonitoring sollte die Anzahl der unterschiedlichen T-Zellpopulationen, Monozyten, B-Zellen, der natürlichen Killer (NK) Zellen sowie der NKT-Zellen mittels Durchflusszytometrie analysiert werden.

Ergebnisse: In den Pavianen der Gruppe 1 konnten die T-Zellen vor der Transplantation durch die konventionelle immunsuppressive Therapie erfolgreich dezimiert werden. Nach der Transplantation nahm jedoch der Anteil aktivierter T-Zellen zu. In Pavian 49, der das HLA-E-transgenes Herz bekam, blieb die Zahl der NK-Zellen auf niedrigem Niveau. Dieser Pavian zeigte ein längeres Überleben im Vergleich zu den anderen Pavianen. In den Pavianen der Gruppe 2, resultierte die Blockade durch den CD40 Antikörper zu einer Herunterregulation der T-Zellen und auch der Monozyten. Hier zeigte Pavian 54

ein längeres Überleben. Ein signifikanter Unterschied zwischen der Blockade durch den CD40 oder den CD40L Antikörper zeigte sich in den Pavianen der Gruppe 3 nicht. Vergleicht man jedoch Gruppe 2 und Gruppe 3 mit der Gruppe 1 konnte eine etwas stärkere Herunterregulation der aktivierten T-Zellen gezeigt werden und die Anzahl der NK- und NKT-Zellen nahm mehrheitlich nach Transplantation ab. Pavian 64 von Gruppe 3 zeigte das längste Überleben. Der Hauptunterschied konnte in der ersten Woche nach Transplantation beobachtet werden. In dieser Zeit nahmen die aktivierten T-Zellen ab und die Treg Zellen nahmen zu.

Schlussfolgerung: Immunsuppression durch die Blockade des CD40:CD40L Signalwegs führte im Allgemeinen zu einer Verlängerung des Überlebens der Paviane. Die Suppression der NK/NKT-Zellen, der B-Zellen und ein höherer Prozentsatz an Treg Zellen scheinen außerdem eine Rolle zu spielen.

2. Introduction

Organ transplantation remains the most effective treatment for patients with late stage organ failure. However, the shortage of human organs and cells remains a major obstacle for human organ transplantation. Transgenic pigs provide an alternative organ donor source to counteract the limited availability of human organs (Yang and Sykes 2007, Ekser, Ezzelarab et al. 2012).

Although tissue reprogramming alleviates organ hyperacute rejection and acute humoral xenograft rejection, the subsequent cellular rejection still needs to be overcome (Yang and Sykes 2007, Klymiuk, Aigner et al. 2010). Several groups reported that immunosuppression therapy prolongs xeno-organ survival (Hering, Wijkstrom et al. 2006, Mohiuddin, Corcoran et al. 2012), but high dose administration of immune suppressive drugs is associated with severe side effects. Therefore, a better tolerated and effective means to alleviate xeno-reactions is urgently needed and is the key step to be resolved for clinical xenotransplantation applications in the future.

2.1 Xenotransplantation

2.1.1 Transgenic pigs

2.1.1.1 α-1,3-Galactosyltransferase-deficient pigs

Major progress in xenotransplantation was accomplished by utilizing α-1,3-galactosyltransferase (α-1,3-GalT, also known as *GGTA1*) deficient pigs as organ donors. The well-defined carbohydrate cell surface structure Galα1–3Galβ1–4GlcNAc (α-1,3-Gal) synthesized by α-1,3-GalT is expressed in most mammals except humans, Old World monkeys and apes. α-1,3-Gal mediates hyperacute rejection in pig to human organ transplantation.

In 1999, Cristina Costa and colleagues reported that transgenic pigs expressing human α-1,2-fucosyltransferase (H transferase) downregulated α-1,3-Gal expression (Costa, Zhao et al. 1999). In the next few years, Liangxue Lai, and

Carol J. Phelps both reported the successful generation of α-1,3-Gal-deficient pigs by mutation of the exon 9 in *GGTA1* (Lai, Kolber-Simonds et al. 2002, Phelps, Koike et al. 2003). In 2009, transgenic pigs expressing endo-β-galactosidase C, an enzyme that destroys α-1,3-Gal by cleaving the β-galactosidic linkage and expressing human decay accelerating factor (hDAF, CD55) were reported. The pigs only expressed 2-14% of α-1,3-Gal compared to wild type pigs, and upregulated 10-70-fold hDAF compared to that in human umbilical vein endothelial cells (Yazaki, Iwamoto et al. 2009).

Researches using α-1,3-Gal-deficient pigs as organ donor were carried out in xenotransplantation. In 2004, Cooper et al. reported that transplanted α-1,3-Gal-deficient pig bone marrow into baboon prolonged the cellular hyporesponsiveness (Tseng, Dor et al. 2004). In the following year, α-1,3-Gal-deficient pig heart transplantation to baboon was reported, survival of pig heart transplanted baboons was prolonged up to 2-6 month (Kuwaki, Tseng et al. 2005, Tseng, Kuwaki et al. 2005). Other studies of transplantation of α-1,3-Gal-deficient pig skin (Weiner, Yamada et al. 2010) and liver (Kim, Schuetz et al. 2012) to baboon was performed, extended survivals and reduced hyperacute rejections were observed.

2.1.1.2 Human decay-accelerating factor (hDAF) expressing pigs

DAF is a membrane protein which consists of 4 short consensus repeats (SCR1, SCR2, SCR3, SCR4). The SCR domain is attached to the cell surface by a glycophosphatidylinositol (GPI) anchor. The SCR2-SCR4 region is required for complement regulation.

Using human DAF-transgenic pigs as organ donor resulted in prolonged survival of the xenograft and inhibition of hyperacute rejection. Experiments showed that the survival and function of hDAF-transgenic α-1,3-Gal-deficient pig liver was prolonged within 13 and 24 h after transplantation into baboon (Ramirez, Montoya et al. 2005). hDAF-transgenic α-1,3-Gal-deficient pig kidney transplanted into baboon avoided hyperacute rejection (Yazaki, Iwamoto et al. 2009).

2.1.1.3 Human CD46-expressing pigs

CD46 (human membrane cofactor protein) is a type I membrane complement regulatory protein which protects the host cells from complement-mediated damage. CD46 inactivates complement components C3b and C4b as a cofactor.

In 2000, Lanteri reported that *in vivo* administration of soluble proteins with functional domains of CD46 in a hyperacute rejection model prevented complement-mediated rejection in mice (Lanteri, Powell et al. 2000). In 2001, Diamond and colleagues first reported of a transgenic human CD46 expressing pig and after transplantation into baboon the heart resisted to hyperacute rejection and survived for 23 days (Diamond, Quinn et al. 2001).

2.1.1.4 Human CD59 expressing transgenic pigs

CD59 (also known as membrane inhibitor of reactive lysis, MIRL) is a membrane glycoprotein inhibiting complement-mediated lysis. It is expressed on peripheral blood hematopoietic cells and endothelial cells in humans. CD59 is bound to the cell membrane via a GPI anchor. CD59 inhibits C9 from binding to C5b678 complex, thereby inhibits the formation of the membrane attack complex (MAC).

In 1995 experiments showed, that human CD59 expression in mouse hearts inhibited MAC formation following perfusion with human plasma *ex vivo* (Diamond, McCurry et al. 1995). In the next year, *in vivo* experiments revealed that using human CD59 expressing pig hearts reduced tissue damage through inhibition of MAC formation (Diamond, McCurry et al. 1996). Other reports demonstrated that CD59 expressing pig organs were resistant to hyperacute rejection. In an *ex vivo* profusion model, Pig hearts and kidneys expressing transgenic human CD59 at the same level as in human organs resisted hyperacute rejection in contrast to wild type pig organs (Kroshus, Bolman et al. 1996). Coexpression of human CD59 and H transferase (α-1,3-Gal-deficient) on pig aortic endothelial cells markedly increased the resistance to human serum-mediated lysis compared to human CD59 or H transferase alone expressing pig aortic endothelial cells (Costa, Zhao et al. 2002).

2.1.1.5 Human CD47 expressing pigs

CD47 is a membrane glycoprotein ubiquitously expressed on all cells. CD47 is a ''marker of self'' and is the inhibitory receptor of SIRP-α (signal regulatory protein α) on macrophages (Oldenborg, Zheleznyak et al. 2000) to prevent autologous phagocytosis. In allotransplantation, donor CD47 induced tolerance and controlled alloreactive T cell response (Zhang, Wang et al. 2016).

The interspecies incompatibility of CD47 causes macrophage mediated xenograft rejection (Ide, Wang et al. 2007, Wang, VerHalen et al. 2007, Yang 2010), and transgenic expression of human CD47 and mouse CD47 on porcine cells prevented human and mouse macrophage mediated rejection (Ide, Wang et al. 2007, Wang, VerHalen et al. 2007, Wang, Wang et al. 2011).

2.1.1.6 HLA-E expressing pigs

HLA-E is a non-classical MHC molecule that is not polymorphic and induces much lower T cell responses than other HLA molecules. HLA-E is considered to be a ligand for the NK cell inhibitory receptor CD94/NKG2A (Crew 2007).

Ex vivo experiments revealed that pig epithelial cells surface expressing correct folded HLA-E molecule protects porcine cells against NK cell-mediated lysis (Crew, Cannon et al. 2005). Expression of HLA-E on swine endothelial cells also protects the cell from macrophage-mediated cytotoxicity, and the suppression mediated by HLA-E transgene expression against macrophage was found to be equivalent to the suppression mediated by CD47 transgene expression (Maeda, Kawamura et al. 2013). Also another group shows that transgenic pigs generated by pronuclear microinjection of genomic fragments of HLA-E with an HLA leader sequence and of human β2-microglobulin into zygotes protect porcine tissues against human NK cell-mediated lysis (Weiss, Lilienfeld et al. 2009).

2.1.2 Immune regulation and xenotransplantation

Intensive immune suppressive therapy is used to alleviate humoral and cellular rejection in xenotransplantation. Blocking costimulation signaling of CD40 by anti-CD154 antibodies prolonged the survival of xenografts (Mohiuddin, Singh

et al. 2014, Choi, Lee et al. 2015, Higginbotham, Mathews et al. 2015). Inducing CTLA4 expression on transgenic mice islet significantly prolonged the graft survival (Londrigan, Sutherland et al. 2010). In 2009, a transgenic pig expressing CTLA4-lg was produced (Phelps, Ball et al. 2009). Mutation of major histocompatibility antigen class II (MHC-II) on porcine organs also reduces T cell immune response in host animals. When a dominant-negative mutant of the human class II transactivator (CIITA-DN) was specifically induced in endothelial cells, the expression of MHC-II on APC and aortic endothelial cells was significantly reduced. Human $CD4^+$ T cell response to CIITA-DN aortic endothelial cells was reduced by 60-80% (Hara, Witt et al. 2013).

2.2 Dendritic cells

In 1973, Ralph M. Steinman and Zanvil A. Cohn found a novel cell type in mice spleen single cell suspensions, that had multiple branches or dendrites, and was hence named dendritic cell (Steinman and Cohn 1973). In 1980s, DC were widely accepted to be the professional antigen-presenting cell (APC) (Steinman, Hawiger et al. 2003).

In vivo, immature DC originate in the bone marrow, express low levels of costimulation molecules, MHC molecules and adhesion molecules, and have a potent antigen uptake ability during migration in the body. After encounter with foreign antigens, DC take up the antigens and process them by an exogenous pathway to form MHC-I/peptide complexes, or by an endogenous pathway to form MHC-II/peptide complexes, and upregulate the expression of costimulatory molecules and adhesion molecules and migrate to the secondary lymphoid organs to activate $CD4^+$ and $CD8^+$ T cells and probably also to activate B cells and NK cells.

2.2.1 Tolerogenic DC

Besides promoting immune reactions, DC also play tolerogenic function to maintain homeostasis *in vivo*.

2.2.1.1 TolDC mediate homeostasis *in vivo*

DC in thymus majorly localized in medulla, and promote the induction of nTreg. DC in thymus was also found to maintain homeostasis by contributing to the negative selection of $CD4^+$ thymocytes (Oh and Shin 2015). Peripheral tolerance is also needed for homeostasis. At inflammatory sites, mature DC process and present both foreign antigens produced by virus or bacteria, and self-antigens from dying cells. Self-reactive T cells can escape negative selection because the lower affinity for self-antigens. In this case, tolDC can deplete self-reactive T cells in the periphery through induction of Treg (Maldonado and von Andrian 2010).

Oral tolerance is induced *in vivo* by the following way. DC in the intestinal mucosa transfer the intestinal tract samples to mesenteric lymph nodes in a CCR7 dependent manner and differentiate naïve T cells into Treg in the mucosal environment, which is rich in anti-inflammatory factors: TGF-β, retinoic acid, IL-10, vasoactive intestinal peptide, thymic stromal lymphoietin and hepatocyte growth factor (Maldonado and von Andrian 2010, Bekiaris, Persson et al. 2014). Plasmacytoid DC (pDC) in lung which exhibited immature phenotype: MHC-II^{low}PD-$L1^{high}$, was found to promote tolerance in airways, depletion of pDC in lung resulted in airway eosinophilia, and Th2 cytokine production. The pDC in lung suppressed the effector T cells generated by mature DC (de Heer, Hammad et al. 2004). IL-10 producing pulmonary DC induced tolerance by inducing $CD4^+$ T regulatory 1-like cell which also produce IL-10 (Akbari, DeKruyff et al. 2001).

2.2.1.2 TolDC generation *in vitro*

By mimicking the *in vivo* microenvironment where DC mediate immune tolerance, tolDC can be also induced *in vitro*.

2.2.1.2.1 IL-10 in tolDC generation

By administration of IL-10, monocyte-derived DC exhibit tolerogenic function and gain the ability to induce Treg (Rutella, Bonanno et al. 2004). By administration of IL-10, a $CD83^{high}CCR7^+$IL-10 DC population expressing high

levels of soluble and surface CD25 can be generated, and showed higher capacity to induce Treg (Kryczanowsky, Raker et al. 2016). Signaling through the IL-10 receptor maintains DC in an immature state triggered by Janus kinases (JAK)-mediated phosphorylation of STAT3 (signal transducer and activator of transcription 3). Phosphorylated STAT3 translocates into the nucleus where it suppresses genes related to DC maturation. IL-10 also inhibits the expression of MHC-II, and the expression of the costimulatory molecules CD80 and CD86 via a posttranscriptional mechanism involved in inhibiting the transport of peptide-loaded MHC-II to the cell surface (Maldonado and von Andrian 2010).

2.2.1.2.2 TGF-β in tolDC generation

Another cytokine widely used in DC generation *in vitro* is TGF-β. TGF-β prevents dendritic Langerhans cells maturation (Geissmann, Revy et al. 1999), and induces tolDC generation from $CD34^+$ progenitors in vitro (Strobl, Riedl et al. 1996). TGF-β binds to the TGF-β receptor leading to heterodimerization of SMAD2 and SMAD3, followed by complex formation with SMAD4, which shuttles the complex into the nucleus to regulate gene expression (Miyazono 2000, Zhu and Burgess 2001).

2.2.1.2.3 Other compounds in tolDC generation

There are some other compounds which are able to generate tolDC *in vitro*, for example vitamin D3. The phenotype of tolDC generated by vitamin D3 is characterized by high expression of MHC class II, intermediate expression of co-stimulatory molecules CD80 and CD86 and low expression of CD40 and CD83. A clinical trial has been carried out for the treatment of rheumatoid arthritis with vitamin D3-derived tolDC (Hilkens and Isaacs 2013). A tolerogenic phenotype of DC, differentiated from human embryonic stem cells, was induced by treatment with rapamycin. Rapamycin-derived tolDC were able to induce Treg (Silk, Leishman et al. 2012).

2.2.1.3 TolDC induce Treg

TolDC promote Treg induction by low antigen presentation and low expression of costimulatory signals (Maldonado and von Andrian 2010). TolDC upregulate

anti-inflammatory cytokines, such as IL-10 and TGF-β, and downregulate inflammatory cytokines, such as IL-12p70. Treg can also induce tolDC in turn. Treg create the microenvironment that is rich in tolDC favoring cytokines to induce DC to convert into tolerogenic DC. By depletion of Treg in mice, researchers found out that tolDC play tolerogenic function only in the presence of Treg cells (Luckey, Schmidt et al. 2012), and Treg generation relies on DC–T cell contact *in vivo* (Darrasse-Jeze, Deroubaix et al. 2009).

2.2.1.4 Adoptive transfer of tolDC

Adoptive transfer of tolDC prolonged organ survival in heart, skin, and islet murine transplantation models, and along with the administration of immunosuppressive drugs, tolDC prolonged organ survival more than using immunosuppressive drugs alone (Zhou, Shan et al. 2016).

2.3 Treg

In the immune system, immune regulatory cells protect hosts from misguided or excessive immune reactions. Treg are the most potent immune cell population to maintain T cell central and peripheral tolerance in the immune system.

Treg represent 5-10% of peripheral $CD4^+$ T cells in humans and 1.7 % in blood T cells in the baboon. The phenotype is $CD3^+CD4^+CD25^+CD127^{low/-}Foxp3^+$. Trig arise from the thymus and maintain self-tolerance and immune homeostasis, demonstrated by the depletion of Treg in mice (Fontenot and Rudensky 2005).

2.3.1 Forkhead box P3

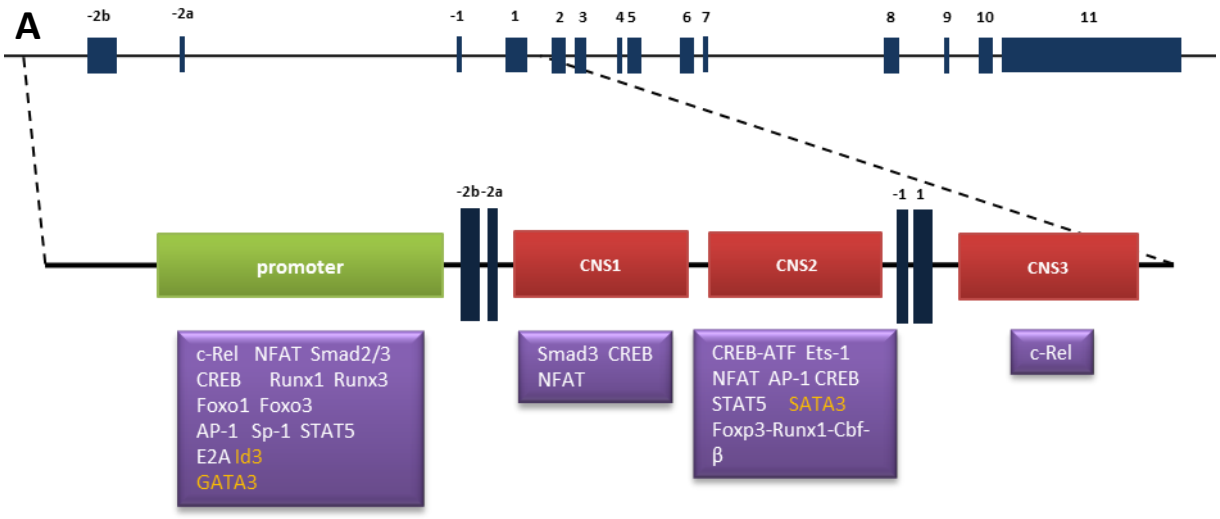
FOXP3 (forkhead box P3), also termed scurfin, is a member of the forkhead/winged-helix family of transcriptional regulators involved in immune regulation (Brunkow, Jeffery et al. 2001). FOXP3 is encoded by a gene located on the X chromosome. Research in mice and humans confirmed that only males but not heterozygous females with FOXP3 mutants were affected by autoimmune diseases (Chatila, Blaeser et al. 2000, Wildin, Ramsdell et al. 2001). FOXP3 is well accepted to be the key factor of Treg function and

development (Hori, Nomura et al. 2003). Foxp3 reporter mice revealed that Foxp3 is mainly expressed in the $CD4^+$ T cell subpopulation and exhibits suppressive function (Wan and Flavell 2005).

2.3.1.1 *Foxp3* gene

Foxp3 gene expression is regulated by conserved non-coding sequences (CNS) in the promoter and intron 2 region (Figure 2.1 A). Zheng and Josefowicz et al. found permissive histone modifications (H3K4me1, H3K4me2, H3K4me3 and H3K9/14Ac) located at CNS1 (enhancer 1), CNS2 (enhancer 2), CNS3 of the *Foxp3* locus, exclusively in Treg *in vivo* (Zheng, Josefowicz et al. 2010). Experiments with CNS1, CNS2, CNS3 mutations in mice suggested that CNS1 was critical for Treg differentiation in the periphery but not in the thymus, and CNS1 mutation disabled $Foxp3^+$ Treg in gut and mesenteric lymph nodes primarily indicating that CNS1 mediates TGF-β regulation in Treg (Zheng, Josefowicz et al. 2010). CNS2 mainly influences Foxp3 maintenance in mature Treg but not in newly generated Treg in CNS2 deficient mice. *In vitro* mutation of CNS2 resulted in severe loss of Foxp3 expression in mutant Treg in comparison to wild type Treg (Zheng, Josefowicz et al. 2010). CNS3 is a pioneer gene element, which facilitates Foxp3 induction during thymic and peripheral differentiation of Treg (Zheng, Josefowicz et al. 2010).

2.3.1.1.1 Transcriptional regulation of *Foxp3* gene expression



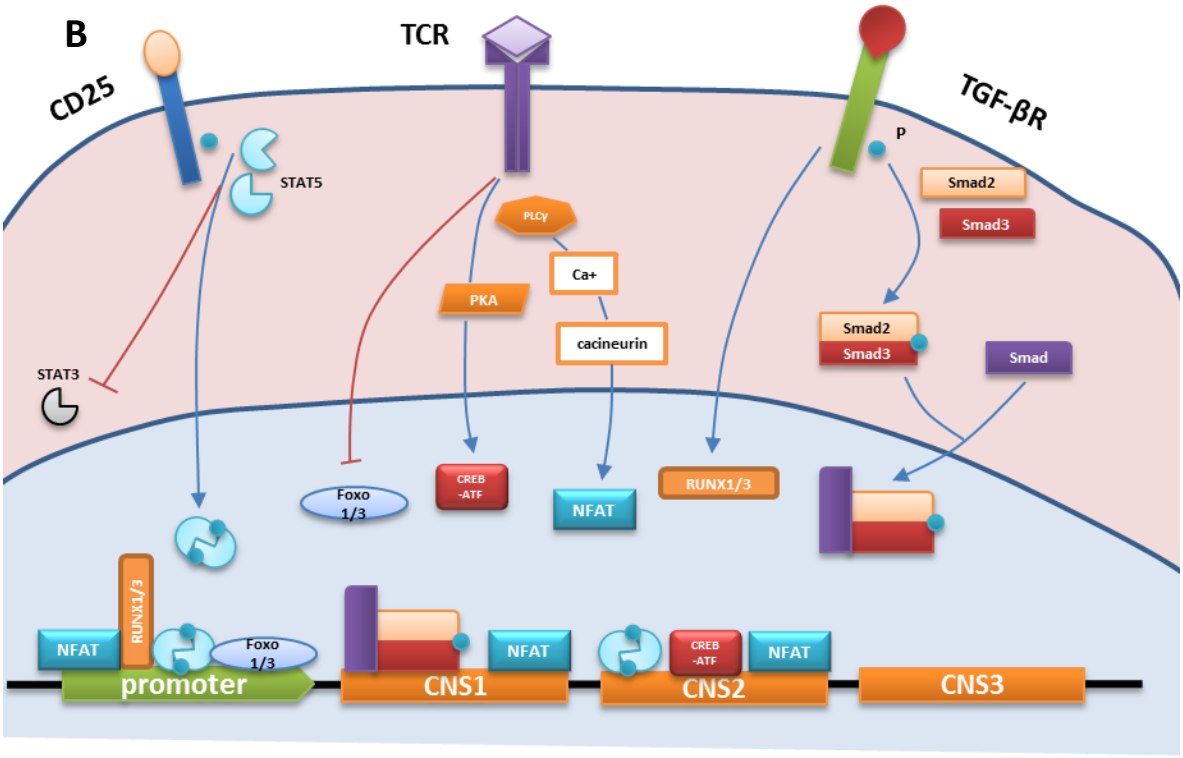


Figure 2.1 Transcriptional regulation of *Foxp3* gene expression. (A) Mice *Foxp3* gene structure and transcriptional regulation. Exons are shown as black numbered boxes. Negative numbers indicate elements upstream of the transcription start site. The region containing the promoter and CNS1 (enhancer 1), CNS2 (enhancer 2) and CNS3 with interacting transcription factors has been enlarged (modified by (Tone and Greene 2011)). (B) Simplified pathways that regulate *Foxp3* gene expression (modified by (Nie, Li et al. 2015)).

Upon TCR stimulation, *Foxp3* gene expression is regulated via nuclear factor (NF)-κB pathway. Rel is a member of the mammalian Rel/NF-κB family, preferentially expressed in lymphoid organs. Experiments with c-Rel deficient mice revealed that c-Rel deficiency results in the reduction of Treg cells, c-Rel acts as a pioneer transcription factor in initiating Foxp3 transcription in Treg precursors in the thymus (Hori 2010). *In silico* analysis of CNS3 demonstrated that c-Rel binds to a motif as CD28 response element in CNS3, suggesting that after stimulation of TCR and CD28 c-Rel binds to CNS3 to open the *Foxp3* locus (Zheng, Josefowicz et al. 2010). Luciferase-based promoter reporter assays revealed that c-Rel and p65, but not p50 or RelB, are involved in NF-κB signaling by activation of the *Foxp3* promoter. First, c-Rel, p65 and NFATc2 bind to the *Foxp3* promoter, Smad binds to enhancer-1, and pCREB binds to both enhancer 1 and 2. After 8 h activation, Smad and pCREB dissociate from the

enhancer and bind to the promoter with c-Rel, p65 and NFATc2 (Ruan, Kameswaran et al. 2009).

CD25 is the α-subunit of IL-2 receptor expressed on the Treg surface. High amounts of IL-2 are indispensable for Treg maintenance and Foxp3 expression. Experiments forcing expression of constitutively active STAT5b (STAT5b-CA) rescued IL-2Rβ ablated mice and reinduction of high levels of IL-2Rα suggested that IL-2Rα expression is driven by STAT5 (Chinen, Kannan et al. 2016). The well-established pathway of STAT5 in Treg is: IL-2 binds to the IL-2R on Treg which leads to tyrosine kinase JAK1 and JAK3 phosphorylation. The phosphorylated sequence motifs recruit the adaptor molecule SHC1 to activate STAT5 for binding to the *FOXP3* promoter (Malek and Bayer 2004, Zorn, Nelson et al. 2006, Burchill, Yang et al. 2007, Laurence, Tato et al. 2007). STAT5 targets at CNS2 where serves as a sensor for IL-2 to maintain stable inheritance of Foxp3 expression (Feng, Arvey et al. 2014). After CNS2 is opened by STAT5, AP-1 and Creb bind to CNS2 to maintain CNS2 activity, and this regulation is methylation sensitive (Ogawa, Tone et al. 2014).

TGF-β signaling cascade is also involved in Treg generation (Chen, Jin et al. 2003). TGF-β induces RUNX1 and RUNX3 binding to the *FOXP3* promoter (Bruno, Mazzarella et al. 2009, Klunker, Chong et al. 2009). Furthermore, transcription factors Smad3 in TGF-β signaling cascade is essential for histone acetylation in CNS1, and Smad3 binding to *Foxp3* promoter is required for Foxp3 expression (Tone, Furuuchi et al. 2008). TGF-β decreases methylation in the CpG islands in CNS2 of *Foxp3* to facilitate Foxp3 expression (Kim and Leonard 2007).

The NFAT signaling cascade is activated in the presence of TGF-β, IL-2, and low affinity antigen but without activation of costimulatory factors in Treg. NFAT binds to the promoter of *Foxp3* together with AP-1, Sp1, Smad2/3 and STAT5, also binds to enhancer 1 together with phospho-Smad3 to facilitate *Foxp3* expression. NFAT together with Foxp3 binds to the promoters of genes like *Ctla4*, *Cd25* and *Gitr* to upregulate their expression (Hermann-Kleiter and Baier 2010). In activated Treg, NFAT in response to TCR activation interacts with CNS2 to stabilize *Foxp3* expression (Li, Liang et al. 2014).

Foxo1 and Foxo3, inhibited by TCR engagement, directly binds to the *Foxp3* promoter region, Foxo1 also binds to the *Ctla4* gene transcription initiation site to start CTLA-4 expression (Ohkura and Sakaguchi 2010).

CREB-ATF facilitates Foxp3 expression by binding to the demethylation region in CNS2 (Kim and Leonard 2007).

Ets-1 depleted mice resulted in low expression of Foxp3 in Treg (Mouly, Chemin et al. 2010). Ets-1 binds to demethylated CpG in CNS2 to facilitate Foxp3 expression (Polansky, Schreiber et al. 2010).

Foxp3 also regulates its own expression by binding at CNS2 as a Foxp3-Runx1-Cbf-β complex (Zheng, Josefowicz et al. 2010).

Tcf3 is a transcription factor that binds to the promoter of *Foxp3* to positively regulate the expression. Id3 contains a helix-loop-helix domain which can dimerize with E2A to inhibit DNA transcription. However, Id3 knockout mice resulted in low expression of Foxp3 because GATA3 expression was upregulated by E2A. Therefore, the Foxp3 expression is regulated by an intricate balance of E2A-Id3 (Tone and Greene 2011).

STAT3 is a critical transcriptional factor in Th17 cell differentiation, which was found to act as a negative modulator of Foxp3 expression (Chaudhry, Rudra et al. 2009). STAT3 inhibits Foxp3 expression by two ways: first, by induction of nTreg instability; and secondly, by inhibition of iTreg polarization from $CD4^+$ naïve T cells (Laurence, Amarnath et al. 2012).

2.3.1.2 Foxp3 function in immune tolerance

Experiments with diphtheria toxin receptor (DTR) knock-in mice at the *Foxp3* locus suggested that ablation of Foxp3 expressing cells by administration of diphtheria toxin at birth resulted in death within 4 weeks. Further research with adult mice suggested that after ablation Foxp3 expressing cells the mice developed a fatal immune disease faster than in newborn mice (Kim, Rasmussen et al. 2007). This result demonstrates that Foxp3 expressing cells are critical for the immune system of newborn mice as well as of adult mice.

2.3.1.3 Foxp3 in Treg

The association of Foxp3 with the suppressive function of Treg was demonstrated by Wen Lin and colleagues. Treg with a nonfunctional Foxp3 fusion protein maintain their Treg signature but lose the suppressive function (Lin, Haribhai et al. 2007). By transduction of Foxp3 into naïve T cells, the expression of CD25 and other Treg-associated cell surface molecules as CTLA-4 and GITR, were upregulated while the production of IL-2, IFN-γ, and IL-4 was suppressed (Sakaguchi, Yamaguchi et al. 2008).

2.3.2 Treg suppressive function

In general, Treg inhibit Tcon in several ways: a) by secretion of suppressor cytokines, such as IL-10 and TGF-β, which inhibit Tcon directly, b) by expression of high levels of CD25, leading to competition for IL-2 with Tcon, c) by acting as cytotoxic cells that directly kill responder T cells, and d) by inducing expression of galectin-1 or other unknown molecules on the cell surface leading to Tcon cell cycle arrest (Shevach 2009).

2.3.2.1 Treg cytokines

2.3.2.1.1 TGF-β

As a major cytokine of Treg TGF-β is highly produced as a membrane-bound molecule and is complexed with latency-associated peptide (LAP) on activated Treg. Treg produced LAP-TGF-β is able to suppress the proliferation of activated T cells in infectious tolerance (Andersson, Tran et al. 2008). TGF-β converts $CD4^+CD25^-$ naïve T cell into $CD4^+CD25^+Foxp3^+$ cells (Chen, Jin et al. 2003). T cell-specific TGF-β1 blocks Th1 and Th2 responses: TGF-β inhibits Th1 cell differentiation by interfering the production of T-bet (Gorelik, Constant et al. 2002); in aspect of Th2, TGF-β was firstly found to inhibit Th2 proliferation by downregulating GATA3 expression (Heath, Murphy et al. 2000), and TGF-β inhibits IL-5 expression by upregulation of SOX4 binding to GATA3 and to the *IL-5* promoter (Kuwahara, Yamashita et al. 2012). Ex vivo expanded Treg by TGF-β showed inhibition of RORγt to promote Foxp3 expression and suppress Th17 production (Zhou, Lopes et al. 2008). Besides acting as an inhibitor of Tcon,

TGF-β also promotes Tcon proliferation: TGF-β together with IFN-γ and IL-4 facilitates $CD103^+$ Th1 cell proliferation (Tofukuji, Kuwahara et al. 2012); TGF-β together with IL-6, IL-1β, and IL-21 induces Th17 (Hu, Troutman et al. 2011). Furthermore, TGF-β signaling in $CD8^+$ T cells prevents over-proliferation by mediating apoptosis in short-lived effector cells during bacterial infection (Sanjabi, Mosaheb et al. 2009, Tinoco, Alcalde et al. 2009), but complete loss of TGF-β signaling leads to $CD8^+$ T cell reduction in the thymus (Travis and Sheppard 2014).

2.3.2.1.2 IL-10

IL-10 protein is a homodimer consisting of two subunits, each 178 amino acids long (Zdanov, Schalk-Hihi et al. 1995). The IL-10 receptor is composed of two IL-10R1 chains, which are ubiquitously expressed, and two IL-10R2 subunits, which are expressed exclusively on T cells, B cells, NK cells, monocytes, mast cells and DC (Tan, Braun et al. 1995, Nagalakshmi, Murphy et al. 2004). IL-10 suppresses the expression of MHC-II and of the costimulatory molecules CD80 and CD86 on monocytes, macrophages and DC. Furthermore, IL-10 blocks expression of CD28, inducible costimulator (ICOS), and CD2 to inhibit T cell proliferation (Palomares, Martin-Fontecha et al. 2014). IL-10 as an immunosuppressive cytokine is involved in Treg-mediated suppression. Experiments of transferring IL-10 deficient $CD4^+CD45RB^{low}$ cells into SCID mice failed to rescue colitis, while transferring wild type $CD4^+CD45RB^{low}$ cells inhibited colitis. This indicates that IL-10 secreted by Treg is a major factor for Treg-mediated suppression of intestinal inflammation (Asseman, Mauze et al. 1999). During cure of experimental colitis, $CD4^+CD25^+Foxp3^+$ cells were found to accumulate in the colon and secondary lymphoid organs. The similar situation was found in human colitis. IL-10-producing $CD4^+CD25^+$ T cells are the major population to cure colitis (Uhlig, Coombes et al. 2006). In addition, IL-10 produced by Treg mediates experimental autoimmune encephalomyelitis recovery (McGeachy and Anderton 2005). Ablation of the *Il-10* gene in Foxp3 specific cells showed that IL-10 was essential for keeping immune responses in check at the environmental interface like in lung and colon, although no systemic autoimmunity was observed (Rubtsov, Rasmussen et al. 2008).

2.3.2.1.3 IL-35

IL-35 is a novel IL-12 family cytokine, which drew much attention, because of its regulatory function. IL-35 consists of EBV-induced gene 3 (EBI3) and IL-12p35 subunits. First, IL-35 was found to be highly expressed in mouse $Foxp3^+$ Treg cells, and can directly suppress Tcon proliferation (Collison, Workman et al. 2007). IL-35 promotes regulatory B cells (Breg) and Treg proliferation and converts naïve T cells into IL-35-producing induced regulatory T cells (iTR35) in the absence of Foxp3 and mediates suppression via IL-35 and not via IL-10 and TGF-β. (Collison, Workman et al. 2007, Collison, Chaturvedi et al. 2010, Wang, Yu et al. 2014). However, in human Treg IL-35 does not seem to be constitutively expressed, only activated Treg produce IL-35 (Bardel, Larousserie et al. 2008).

IL-27 shares the β-chain (EBI3) with IL-35, the α-chain of IL-27 is IL-27p28 (IL-27A). Generally, IL-27 is an immune regulatory cytokine, which induces Th17 cells to produce IL-10 (Murugaiyan, Mittal et al. 2009, Hirahara, Ghoreschi et al. 2012), but it also exerts anti-tumor effect (Liu, Liu et al. 2013) and suppresses the expression of Foxp3 via STAT1 and STAT3 (Neufert, Becker et al. 2007, Huber, Steinwald et al. 2008).

2.3.2.2 Treg suppressive molecules

2.3.2.2.1 CD25

CD25 is the IL-2 receptor α chain with high affinity for IL-2. IL-2 together with TGF-β is required for Foxp3 expression. However, Treg produce no IL-2. IL-2 promotor in Treg does not undergo chromatin remodeling upon TCR activation. Foxp3 together with NFAT binds to the IL-2 promoter to suppress IL-2. Furthermore, Blimp1 which negatively regulates IL-2 expression is highly expressed in Treg (Malek 2008). Therefore, Treg highly express CD25 to deprive IL-2 from Tcon. It was found that Treg-mediated Tcon apoptosis was Bim associated, as Bim-depleted Tcon were completely protected from apoptosis in Treg coculture (Pandiyan, Zheng et al. 2007). However, others argued that apoptosis in human Tcon and Treg cocultures was not observed (Vallabhapurapu and Karin 2009). Administration of exogenous IL-2 could

abrogate the Treg-mediated proliferation inhibition (de la Rosa, Rutz et al. 2004). However, another group showed that supplementation of IL-2 can partly abrogate Treg-mediated suppression, but a rapid suppression of IL-2 transcription in Tcon by Treg was not interfered by adding exogenous IL-2 in culture (Oberle, Eberhardt et al. 2007).

2.3.2.2.2 T-cell receptor (TCR)

Treg can inhibit TCR induced proliferation of Tcon and induce Tcon anergy by upregulating GRAIL expression, GRAIL is related to anergy in lymphocytes (Ermann, Szanya et al. 2001). Treg can suppress TCR-mediated Tcon activation (Thornton and Shevach 2000, Karim, Feng et al. 2005). Additionally, Treg impede Tcon function directly by impacting TCR signal components. Researchers found out that following interaction with Treg, Ca^{2+} influx was blocked in Tcon which resulted in decreased NFAT1 dephosphorylation, and phosphorylation of IKK, IκBα, and p65 which inhibit NFκB activation (Schmidt, Oberle et al. 2011). The protein kinase c-theta (PKCθ) is an enzyme involved in mobilization of the transcription factors AP-1 and NFκB. Treg cells inhibit the recruitment of PKCθ to the immune synapse of naïve T cells if both recognize the same antigen and contact the same APC (Sumoza-Toledo, Eaton et al. 2006).

2.3.2.2.3 CTLA-4

CTLA-4 is located primarily intracellular. Upon TCR and CD28:B7 engagement, CTLA-4 is also expressed on the cell surface. Strong TCR engagement results in more CTLA-4 expression on the surface (Linsley, Bradshaw et al. 1996, Walker and Sansom 2015). CTLA-4 raises the threshold of T cell activation to prevent over-activation of T cells (Alegre, Frauwirth et al. 2001). Depleting CTLA-4 resulted in spontaneous autoimmunity in mice (Tivol, Borriello et al. 1995). CTLA-4 is expressed on the Treg surface, depleting CTLA-4 on Treg resulted in lymphoproliferation and T cell-mediated autoimmune disease (Wing, Onishi et al. 2008). *In vitro* experiments revealed that Treg-mediated Tcon suppression can be inhibited by blocking CTLA-4. However, Treg of CTLA-4 deficient mice mediated uncompromised suppression by upregulating secretion of IL-10 and TGF-β, which indicates that Treg developed into a compensatory suppressive

mechanism to override CTLA-4 deficiency (Tang, Boden et al. 2004). Rather than suppressing Tcon directly, CTLA-4 influences Tcon activation by downregulating CD80 and CD86 expression on APC (Wing, Onishi et al. 2008). Moreover, CTLA-4 can induce DC to express indoleamine-2,3-dioxygenase (IDO), which mediated tryptophan degradation in Tcon (Meisel, Zibert et al. 2004, Curti, Pandolfi et al. 2007). In addition, Treg inhibits the synthesis of glutathione in DC by interfering the expression of γ-glutamylcysteine synthetase, which is mediated by the CTLA-4 dependent extracellular redox remodeling (Yan, Garg et al. 2009, Yan, Garg et al. 2010).

2.3.2.2.4 Glycoprotein A repetitions predominant (GARP)

GARP is a transmembrane protein highly expressed on activated Treg. The extracellular domain of GARP contains LRRC32 (leucine rich repeats containing 32) (Probst-Kepper, Geffers et al. 2009), which is highly homologous to the extracellular domain of Toll-like receptor 3 (TLR3) (Bell, Botos et al. 2005, Sun, Jin et al. 2016). GARP is associated with Treg suppressive function (Wang, Wan et al. 2008). In recent years, GARP was used as a marker to isolate highly suppressive Treg (Wang, Kozhaya et al. 2009, Noyan, Lee et al. 2014, Abd Al Samid, Chaudhary et al. 2016). Downregulation of GARP in Treg is associated with the downregulation of Foxp3, CD27, and CD83 (Probst-Kepper, Geffers et al. 2009). Overexpression of GARP on non-Treg upregulated Foxp3 expression and other Treg-associated molecules: CD25, CTLA-4, LGALS3, LGMN and CD27 (Wang, Wan et al. 2008). In return, Foxp3 depletion results in GARP decrease (Probst-Kepper, Geffers et al. 2009). GARP is essential for the surface expression of latent TGF-β on Treg: latent TGF-β binds to GARP as a LAP-GARP complex through disulfide linkage and non-covalent association as membrane bound TGF-β, after recognition by integrin $\alpha_v\beta_6$ and $\alpha_v\beta_8$, active TGF-β was released from the GARP-LAP complex on the Treg surface (Wang, Zhu et al. 2012).

2.3.2.2.5 Other molecules on Treg correlated with suppressive function

ICOS is expressed abundantly on T follicular regulatory (Tfr) cells, and inhibits the germinal center reaction (Baumjohann, Preite et al. 2013). ICOS is

associated with IL-10 production: $ICOS^{high}$ T cells express IL-10, and ICOS control Treg cells producing IL-10 (Greenwald, Freeman et al. 2005).

LAG3 (lymphocyte activation gene 3) is an adhesion molecule expressed on Treg. It binds to MHC-II molecules. Early growth response gene 2 (Egr-2), characteristically expressed by $LAG3^+$ Treg, forces naïve $CD4^+$ cell to express Egr-2 and convents naïve T cells into $LAG3^+$ Treg (Okamura, Fujio et al. 2009). $LAG3^+$ Treg express TGF-β3 and suppress B cell responses in mice lupus model (Okamura, Sumitomo et al. 2015). Block of LAG reduced Treg suppressive function (Huang, Workman et al. 2004).

Neuropilin 1 (NRP1) is a transmembrane glycoprotein, is thought selectively expressed on thymic-derived Treg in mice and on a subset of Treg isolated from secondary lymph nodes and on pDC in humans (Chaudhary, Khaled et al. 2014). NRP1 is involved in TGF-β signaling in Treg by acting as a high-affinity receptor for LAP (Solomon, Mueller et al. 2011). $NRP1^+$ Treg exhibits higher suppressive function than $NRP1^-$ Treg, and the suppressive function is mediated mainly by TGF-β (Lin, Chen et al. 2013).

cAMP is highly produced by Treg and inhibits the transcription of IL-2 and proliferation of Tcon (Bopp, Becker et al. 2007). Inducible cAMP early repressor (ICER) was considered to be involved in cAMP mediated suppression (Bodor, Bodorova et al. 2000). Through direct contacting nTreg, $CD4^+$ Tcon accumulate ICER which suppresses IL-2 synthesis, and suppresses the nuclear factor of activated T cell c1 (NFATC1) because the promoter of NFATC1 contains two cAMP-responsive elements (Vaeth, Gogishvili et al. 2011).

2.3.3 Treg in vitro expansion

Many studies were focused on Treg *ex vivo* expansion. nTreg can be isolated and expanded using IL-2, anti-CD3 mAb, and anti-CD28 mAb *ex vivo* (Hoffmann, Eder et al. 2004, Earle, Tang et al. 2005).

To convent $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ Treg cells *ex vivo*, TGF-β was initially widely used in Treg *ex vivo* expansion. Administration of TGF-β was found to be functional in induction of FOXP3 expression from $CD4^+CD25^-$ precursors (Chen, Jin et al. 2003, Fantini, Becker et al. 2004, Fu, Zhang et al. 2004).

In recent years, rapamycin gained much attention in Treg expansion. Rapamycin is an immunosuppressive drug that has been used to prevent GvHD in the clinic. Rapamycin inhibits mTOR, which regulates T cell early stage development. Deletion of RICTOR (rapamycin-insensitive companion of mTOR) results in systemically impaired thymocyte development, and lymphocytes were found to be defective in cell-cycle progression upon activation and went into apoptosis (Hoshii, Kasada et al. 2014). However, depletion of mTOR in T cells results in Treg development in the absence of IL-2 and TGF-β (Delgoffe, Kole et al. 2009). Now rapamycin is being used in expansion of Treg in humans (Strauss, Whiteside et al. 2007, Battaglia, Stabilini et al. 2012) and in baboon (Singh, Horvath et al. 2009, Singh, Seavey et al. 2012).

Other compounds also contribute to Treg expansion. Anti-thymocyte globulin (ATG) promotes Treg generation in mice by depleting T cells (Lopez, Clarkson et al. 2006). Low-dose cyclosporine favors Treg in the skin of atopic dermatitis patients (Brandt, Pavlovic et al. 2009). Bortezomib reduces GvHD via expansion of Treg *in vivo* (Weng, Lai et al. 2013).

2.3.4 Treg in immunotherapy

Adoptive transfer of Treg is used to achieve immune tolerance *in vivo*.

2.3.4.1 Adoptive transfer of Treg to prevent GvHD

Several studies supported that Treg reverse GvHD in allograft transplantation. In 1995, Sakaguchi and colleagues showed that transfer of $CD4^+CD25^+$ cell can prevent autoimmune diseases within a limited period following co-transplantation of allogeneic skin and $CD4^+CD25^-$ T cells (Sakaguchi, Sakaguchi et al. 1995). Further experiment demonstrated that $CD4^+CD25^+$ T cells prevent GvHD in mouse bone marrow transplantation model (Taylor, Lees et al. 2002, Xia, Kovochich et al. 2004, Hanash and Levy 2005, Trenado, Sudres et al. 2006, Cao, Soto et al. 2009). Also, in clinical trials Treg alleviate GvHD. Hellmann et al. reported the first clinical trial in which GvHD could be alleviated by an adoptive transfer of *ex vivo* expanded Treg (Trzonkowski, Bieniaszewska et al. 2009). Adoptive transfer of Treg into HLA-haploidentical hematopoietic stem cells-transplanted patients rebuilt immune balance and prevented GvHD in the

absence of post-transplantation immunosuppression (Di Ianni, Falzetti et al. 2011). Also, in a small clinical phase I trial, isolated donor Treg were transferred into patients with high risk of leukemic relapse after stem cell transplantation. Neither GvHD nor opportunistic infections or early disease relapse occurred (Edinger and Hoffmann 2011). Another study showed that umbilical cord blood-derived Treg can prevent GvHD and no toxicity was observed: the clinical trial using umbilical cord blood-derived Treg expanded *ex vivo* with an artificial APC (K562 modified to express CD64 and CD86) (Brunstein, Miller et al. 2016).

2.3.4.2 Adoptive transfer of Treg in type 1 diabetes

Type 1 diabetes is caused by infiltration of self-reactive $CD4^+$ and $CD8^+$ T cells into the pancreatic islets, which leads to a destruction of insulin-producing β-cells. Adoptive transfer of antigen-specific $CD4^+CD25^+$ Treg, expended with anti-CD3/anti-CD28 beads and high amounts of IL-2, into NOD mice reversed diabetes and required fewer cells (Tang, Henriksen et al. 2004). $CD4^+CD25^+CD62L^+$ islet antigen-specific Treg expanded with antigen-pulsed DC and IL-2 restored immune balance in non-obese diabetic (NOD) mice (Tarbell, Petit et al. 2007). Using a humanized mouse model transplanted with neonatal porcine islets, Shounan Yi and colleagues elongated survival of porcine islets by adoptive transfer of *in vitro* expanded autologous Treg (Yi, Ji et al. 2012). By transferring Treg to diabetic children, daily administration dose of insulin was reduced and 2 out of 10 children did no longer need insulin substitution. No toxicity was observed in this study (Marek-Trzonkowska, Mysliwiec et al. 2012).

2.3.4.3 Adoptive transfer of Treg in rheumatoid arthritis (RA)

Treg function also drew attention in RA. By injection of tolDC, the functionality of Treg was evaluated in mice with collagen-induced arthritis. It was found that this treatment alleviated RA (Carranza, Falcon et al. 2012). Following adoptive transfer of TCR gene-transduced Treg into recipient mice, Treg accumulated at the site of joint inflammation, resulting in a local reduction of Th17 cells. A significant decrease in arthritic bone destruction was observed (Wright, Notley et al. 2009).

2.4 Aim of the thesis

Cellular rejection remains to be the obstacle for xenotransplantation. The adoptive transfer of Treg could be helpful for modulating the xenogeneic cellular immune responses. However, nTreg generation in vitro is laborious and expensive. Antigen-specific Treg would be more effective and would require lower cell numbers. The baboon, as a non-human primate experimental animal model, is widely used in xenotransplantation research. An effective method to generate baboon xeno-specific Treg would benefit research on immune tolerance in xenotransplantation. Therefore, the aim of this thesis was to establish a method for generating xeno-antigen specific Treg in both human and baboon system, which can be used in the future to inhibit cellular rejection in xenotransplantation of porcine organs or cells into host animals or into human recipients in the clinic.

The second aim was to monitor subpopulations of peripheral blood mononuclear cells in baboons pre and post transplantation of pig hearts into baboons under different immunosuppressive therapies. This should provide a guideline for immunosuppressive treatment regime in xenotransplantation.

3. Materials and methods

3.1 Materials

3.1.1 Cells and tissues

Human peripheral blood samples were collected from 10 healthy donors to isolate PBMC. They were used to generate human tolDC and non-tolerogenic C5-DC, human PSTreg, non-specific Treg (NTreg), PSTeff cells, and non-specific Teff cells (NTeff). Olive baboon peripheral blood samples (provide by Dr. Jan-Michael Abicht and Tanja Mayr) were collected from 26 olive baboons to isolate PBMC that were used to generate baboon tolDC and non-tolerogenic C5-DC, and baboon PSTreg, NTreg, PSTeff cells, and NTeff cells, or were used as samples for immune monitoring pre- or post-transplantation. Wild type porcine aorta, heart and PBMC (provide by Dr. Jan-Michael Abicht and Tanja Mayr) were collected to isolate RNA for ivtRNA generation.

3.1.2 Cell isolation and cell culture

Table 3.1 Materials for cell isolation and cell culture

	Company
15 ml Centrifuge tubes	BD
50 ml Centrifuge tubes	BD
autoMACS Rinsing Solution	Miltenyi Biotec
autoMACS Running Solution	Miltenyi Biotec
Benzonase Nuclease HC	Novagen
CD14 MicroBeads, human	Miltenyi Biotec
CD14 MicroBeads, non-human primate	Miltenyi Biotec
$CD4^+$ T Cell Isolation Kit, human	Miltenyi Biotec
$CD4^+$ T Cell Isolation Kit non-human primate	Miltenyi Biotec
$CD4^+CD25^+$ Regulatory T Cell Isolation Kit	Miltenyi Biotec
CountessTM Automated Cell Counter	Invitrogen
Countess cell counting chamber slides	Invitrogen
CryoTube	Nunc
CTL-Wash	CTL
CTS™ OpTmizer™ T Cell Expansion SFM	Life Technologies

Erylyse-Puffer	Klinikum Großhadern
Ficoll-Paque	GE Health Care Life Sciences
Freezing Medium serum-free	Ibidi
GM-CSF, recombinant, human	Leukine sargramostim
human serum	self made
IFN-gamma, recombinant, human	Boehringer Ingelheim
IFN-gamma, recombinant, Rhesus Macaque	R&D
IL-1β, recombinant, human	R&D
IL-1β, recombinant, Rhesus Macaque	R&D
IL-2, (Proleukin)	Novartis
IL-4, recombinant, Rhesus Macaque	R&D
IL-4, recombinant, human	R&D
IL-6, recombinant, human	R&D
IL-10, recombinant, human	PEPRO-TECH
LEUCOSEP TUBE	Greiner bio-one
L-Glutamin	Invitrogen
MACS Separation Columns	Miltenyi Biotec
Mr. Frosty	Immatics
Nunclon™flasks	Nunc
PBS	Life Technologies
penicillin/streptomycin	Invitrogen
PGE2 (prostaglandin E2), recombinant, human	Sigma
R848 (Resiquimod)	InvivoGen
rapamycin	Sigma
RPMI 1640 medium	Invitrogen
T Cell Activation/Expansion Kit, human	Miltenyi Biotec
T Cell Activation/Expansion Kit, non-human primate	Miltenyi Biotec
TGF-β, 1recombinant, human	PEPRO-TECH
TNF-α, recombinant, Rhesus Macaque	R&D
Trypan Blue stain 0.4%	Life Technologies
TNF-α, recombinant, human	R&D
U-bottom 96 well plate	TPP
VLE-RPMI 1640	Biochrom AG

3.1.3 Antibodies

Table 3.2 Antibodies

Antibody	Fluorescence color	Clone	Isotype	Company
CCR4	PE-Cy7	1G1	Mouse IgG1, κ	Pharmingen
CCR7	BrilliantViolet421	G043H7	Mouse IgG2a	Biolegend
CD3	PerCP	SP34-2	Mouse IgG1, λ	BD
CD3	PE-Cy7	SP34-2	Mouse IgG1, λ	BD
CD4	APC	L200	Mouse IgG1, κ	BD

CD14	FITC	M5E2	Mouse IgG2a, κ	Biolegend
CD14	PE-Cy7	M5E2	Mouse IgG2a, κ	Pharmingen
CD16	APC	3G8	Mouse IgG1, κ	BD
CD19	PacificBlue	J3.119	Mouse IgG1	Coulter
CD25	PE	M-A251	Mouse IgG1, κ	BD
CD25	APC	M-A 251	IgG1k	Pharmingen
CD25	PE-Cy7	BC96	Mouse IgG1, κ	eBiosciences
CD45	PerCP	D058-1283	Mouse IgG1, κ	BD
CD45RA	BV421	5H9	Mouse IgG1, κ	BD
CD80	Alexa F 700	L307.4	Mouse IgG1, κ	BD
CD83	BrilliantViolet421	HB15e	IgG1, κ	BD
CD86	PE	2331	IgG1, κ	Pharmingen
CD127	PE	MB15-18C9	IgG2a	Miltenyi
CD154 (CD40L)	APC	TRAP1	Mouse IgG1, κ	BD
CD154 (CD40L)	BV421	TRAP1	Mouse IgG1, κ	BD
CD273 (B7-DC)	APC	MIH18	Mouse IgG1, κ	BD
CD274 (B7-H1)	FITC	MIH1	Mouse IgG1, κ	BD
CD274 (B7-H1)	29E.2A3	PE	Mouse IgG2b, κ	Biolegend
FoxP3	eFluor450	PCH101	Rat IgG2a, κ	eBiosciences
Isotype	PE-Cy7	G155-178	Mouse, IgG2a	Pharmingen
Isotype	FITC	MOPC-21	IgG1, κ	Pharmingen
Anti-human porcine MHC-class I				gift from Dr.Robert Kammerer

3.1.4 Flow cytometry

Table 3.3 Materials for flow cytometry

	Company
ArC Amine Reactive Compensation Beads Kit	Invitrogen
Anti-Mouse lg, κ/Negative Control Compensation Particles Set	BD
Anti-Rat lg, κ/Negative Control Compensation Particles Set	BD
Foxp3 Staining Buffer Set	eBioscience
LIVE/DEAD Fixable Blue Dye Cell Stain Kit	Life Technologies

3.1.5 Primers

Primers for quantification of baboon *GARP, EBI3, IL-12A, IL-12B, IL-27A, IL-10, TGFB1, GAPDH* cDNA, and primers for quantification of human *Foxp3, STAB1, GARP, EBI3, IL-12A, IL-27A* cDNA were designed with online tool primer-blast

(http://www.ncbi.nlm.nih.gov/tools/primer-blast/), Primers were supplied by Eurofins (Ebersberg, Germany), sequence of primers were listed in table 3.4.

Primers for quantification of human *IL-12B, IL-10, TGFB1* and housekeeping genes *ACTB, G6PDH and cyclophilin B (CYPB)* cDNA were purchased from Search LC (Germany). The quality of the PCR primers was confirmed by melting curve analysis and agarose gel electrophoresis.

Table 3.4 Primers

	Forward primer	Reverse primer
cDNA amplify primers for ivtRNA generation	TAATACGACTCACTATAGGGA GGAAGCAGTGGTAACAACGCA	AAGCAGTGGTATCAACGCAGAGT
Baboon *EBI3*	CTGCACCATCGCGGATGTC	ACTGGAGGACAGGTGGGAAGT
Baboon *GAPDH*	CAGCGCATCTCTGAGACACCA	ACTTGCCATGGGTGGAATCA
Baboon *IL-10*	GCCGGGAAACCTGTGATTGT	TCTCGAAGCATGTGAGGCAG
Baboon *IL-12A*	CAAAACTTGCTGAAGGCCGC	GCCAGGCAACTCCCTTTAGT
Baboon *IL-12B*	ACCAGGGGTGCATTTCTTCG	CATGGCTGCACCAGGTTAGA
Baboon *IL-27A*	CATCAGCGTTGGACAAGGGA	TGTAGGAGCAGAGAGGGGTT
Baboon *TGFB1*	CTGGCGATACCTCAGCAACC	CCACTTGCAGTGTGTTATCTTTGC
Human *ACTB*	Search LC	
Human /baboon *GARP*	GCTTGACCTGCATAGCAACG	CCGGATGAGGTTGTTGGACA
Human *CYPB*	Search LC	
Human *EBI3*	GCTCCCTACGTGCTCAATGT	CCCTGACGCTTGTAACGGAT
Human *Foxp3*	AGCCATGATCAGCCTCACAC	GACACCATTTGCCAGCAGTG
Human *G6PDH*	Search LC	
Human *IL-10*	Search LC	
Human *IL-12A*	TGGCCCTGTGCCTTAGTAGT	GTTTGGAGGGACCTCGCTTT
Human *IL-12B*	Search LC	
Human *IL-27A*	GCCAGGAGTGAACCTGTACC	CACAGCTGCATCCTCTCCAT
Human *SATB1*	CACTCGGGCCATCTGATGAA	GGGCAGCAGAGCTATGTGAAT
Human *TGFB1*	Search LC	

3.1.6 RNA and DNA isolation, cDNA synthesis, ivtRNA generation, electroporation of DC, and RT-PCR

Table 3.5 Materials for RNA and DNA isolation, cDNA synthesis, ivtRNA generation, electroporation of DC, and RT-PCR

	Company
6× DNA Load Dye	Fermentas
Advantage 2 PCR Kit	Clontech

DNeasy Blood & Tissue Kit	Qiagen
FastStart Essential DNA Green Master	Roche
Gene Pulser Cuvettes	BIO-RAD
GeneRuler 1kb DANN Ladder	Fermentas
LightCycler® 8-Tube Strips	Roche
MEGAclear Kit	Ambion
mMESSAGE mMACHINE T7 Ultra	Ambion
OPTI-MEM	Life Technologies
Reverse Transcription System	Progema
RNAlater Solution	Ambion
RNeasy Mini Kit	Qiagen
SMARTer PCR cDNA Synthesis Kit	Clontech
UltraPure 10×TAE Buffer	Invitrogen

3.1.7 Treg functional assays

Table 3.6 Materials for Treg functional assays

	Company
CellTrace™ CFSE Cell Proliferation Kit	Moleculer Probes
T Cell Activation/Expansion Kit, human	Miltenyi Biotec
T Cell Activation/Expansion Kit, non-human primate	Miltenyi Biotec
Vybrant® DiI Cell-Labeling Solution	Life Technologies

3.1.8 Cytokine quantification

Table 3.7 Materials for cytokine quantification

	Company
BD Cytometric Bead Array (CBA) Human Soluble Protein Master Buffer Kit	BD
Human IFN-γ Flex Set	BD
Human IL-10 Flex Set	BD
Human IL-10 Quantikine ELISA Kit	R&D
Human IL-12p70 Quantikine ELISA Kit	R&D
Human IL-12p70 Flex Set	BD
Human IL-17A Flex Set	BD
Human TGF-β1 Quantikine ELISA Kit	R&D
Human TGF-β1 Single Plex Flex Set	BD
Monkey IL-10 ELISA kit	U-CyTech
Monkey IL-12/23p40 ELISA kit	U-CyTech

3.1.9 Software and technical equipment

Table 3.8 Software and technical equipment

	Company
autoMACS	Miltenyi Biotec
AxioCam microscop	ZEISS
Bioanalyzer 2100	Agilent
Cell incubator	HERAEUS
Centrifuge 5417R	Eppendorf
Countess automated cell counter	Invitrogen
E max precision microplate reader	MWG-BIOTECH
Electrophoresis Power Supply PS304	LIFE TECHNOLOGIES
FlowJo	Tree Star Inc.
Fluor-S™ MultiImager	Bio-Rad
Freezer -80°C	Heraeus
GenePulser Xcell	BIO-RAD
graphpad prism 6	GraphPad Software,
HERAsafe clean bench	HERAEUS
LEICA DMIL microscope	LEICA
Light Cycler 96	Roche
LSRII flow cytometer	BD Biosciences
Megafuge 2.0	HERAEUS
NanoDrop 2000	Thermo Fisher Scientific
Peltier Thermal Cycler PTC-200	MJ RESEARCH

3.2 Methods

3.2.1 Generation of human and baboon tolDC and PSTreg

3.2.1.1 RNA isolation and ivtRNA generation

RNA of wild type porcine PBMC, porcine aorta and porcine heart was isolated using the RNeasy Mini Kit according to the manufacturer's instructions. RNA quality and quantity was controlled by Agilent capillary electrophoresis and Nanodrop, respectively. Reverse transcription of porcine RNA was accomplished with SMARTer™ PCR cDNA Synthesis Kit. cDNA was amplified by Advantage® 2 PCR Enzyme System. The cDNA was analyzed using agarose gel electrophoresis and quantified by spectrophotometry. ivtRNA (ivtRNA) of

porcine PBMC was generated by mMESSAGE mMachine T7 Ultra Kit and purified by MEGAclear™ Transcription Clean-Up Kit. The full length capped mRNA was analyzed and quantified using the Agilent system.

3.2.1.2 Generation of human and baboon tolDC and C5-DC

Human and baboon DC were generated from monocytes which were isolated from PBMC of healthy human donors and baboon donors, respectively. Briefly, after isolation of monocytes with CD14 microbeads, human and baboon monocytes were resuspended in VLE-RPMI 1640 with 1.5% human serum and a total of 5×10^6 cells were seeded in Nunclon™ flasks at the concentration of 1×10^6/ml. On day 0, 100 ng/ml GM-CSF and 20 ng/ml IL-4 were added to the cultures. At day1, 1 ng/ml TGF-β1 and 20 ng/ml IL-10 were added only to tolDC. On the following day, maturation cocktails (Burdek, Spranger et al. 2010) for human (Table 3.9) and for baboon (Table 3.10) were added to tolDC and C5-DC to induce maturation. For immunophenotyping of tolDC and C5-DC, the following antibodies were used: anti-CD14, anti-CD80, anti-CD83, anti-CD86, anti-B7-H1, anti-B7-DC for human, and anti-CD80, anti-CD83, anti-B7-H1, anti-B7-DC, anti-CCR7 for baboon.

Table 3.9 Maturation cocktail for human tolDC and C5-DC

	tolDC	C5-DC
GM-CSF, recombinant, human	100 ng/ml	100 ng/ml
IL-4, recombinant, human	20 ng/ml	20 ng/ml
IL-1β, recombinant, human	10 ng/ml	10 ng/ml
TNF-α, recombinant, human	20 ng/ml	20 ng/ml
PGE_2, recombinant, human	250 ng/ml	1 µg/ml
R848	--	1 µg/ml
IFN-γ, recombinant human	--	5000 U/ml
IL-6, recombinant, human	15 ng/ml	--
TGF-β1, recombinant, human	1 ng/ml	--
IL-10, recombinant, human	20 ng/ml	--

Table 3.10 Maturation cocktail for baboon tolDC and C5-DC

	tolDC	C5-DC
GM-CSF, recombinant, human	100 ng/ml	100 ng/ml
IL-4, recombinant, Rhesus Macaque	20 ng/ml	20 ng/ml
IL-1β, recombinant, Rhesus Macaque	10 ng/ml	10 ng/ml

TNF-α, recombinant, Rhesus Macaque	20 ng/ml	20 ng/ml
PGE_2, recombinant, human	250 ng/ml	1 µg/ml
R848	--	1 µg/ml
IFN-γ, recombinant, Rhesus Macaque	--	5000 U/ml
IL-6, recombinant, human	15 ng/ml	--
TGF-β1, recombinant, human	1 ng/ml	--
IL-10, recombinant, human	20 ng/ml	--

3.2.1.3 Loading of human and baboon DC with PS ivtRNA

Gene Pulser Xcell™ from Biorad was used to perform electroporation of DC in 0.4 cm electroporation cuvettes. The human and baboon DC were harvested and washed twice with Opti-MEM medium. 1.5×10^6 DC were resuspended in 200 µl Opti-MEM and incubated with 10 µg ivtRNA for 5 minutes on ice. DC were electroporated using the following conditions: exponential protocol, 150 µF, 300 V. DC electroporated in the presence of PBS served as controls. Immediately after electroporation, the cuvettes were placed on ice for 5 minutes and then cultured with VLE-RPMI 1640 plus 1.5% human serum at 37°C and 5% CO_2 for 24 h. The expression of porcine PBMC ivtRNA on human DC and baboon DC was assessed by detection of porcine MHC-class I protein.

3.2.1.4 Generation of human and baboon PSTreg and PSTeff

PSTreg were generated from human and baboon $CD4^+$ T cells cocultured with PS ivtRNA-loaded human and baboon tolDC. Briefly, human and baboon $CD4^+$ cells were isolated using the $CD4^+$ T Cell Isolation Kit, cocultured with PS ivtRNA-loaded human and baboon tolDC at the ratio of 1:10. To generate human and baboon PSTeff, human and baboon $CD4^+$ cells were cocultured with PS ivtRNA-loaded human and baboon C5-DC at the ratio of 1:10. (Spranger, Javorovic et al. 2010). $CD4^+$ T cells cocultured with mock PBS-loaded tolDC and C5-DC were used for NTreg and NTeff controls. Human T cells were incubated for 10 days in medium supplemented with IL-2 (Treg: 500 U/ml Teff: 50 U/ml) and with or without rapamycin (Treg: 1 nM). In the human system, nTreg were enriched or depleted using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit. The remaining $CD4^+$ cell were used as precursor cells to generate PSTreg and NTreg using tolDC loaded with PS-antigen- or mock-loaded tolDC as mentioned above.

To restimulate Treg, PSTreg and NTreg were purified with the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, and cocultured with DC.

Baboon T cells were incubated for 10 days in medium supplemented with IL-2 (Treg: 2000 U/ml Teff: 200 U/ml) and with or without rapamycin (Treg: 1 nM). Treg phenotype was characterized by flow cytometry (LSRII).

The following monoclonal antibodies were used: anti-CD3, anti-CD4, anti-CD25, anti-CD127, anti-Foxp3, anti-CD45RA, anti-CCR7, anti-CCR4. Intracellular staining for Foxp3 was performed by Foxp3 staining buffer set. LIVE/DEAD® Fixable Blue Dead Cell Stain Kit was used to determine viable cells and to exclude dead cells. Evaluation of Treg was done using FlowJo software.

3.2.1.5 Human and baboon PSTreg functional assays

Two assays were used to evaluate the suppressive function of human and baboon PSTreg: inhibition of Teff early activation marker (CD154) expression (Ruitenberg, Boyce et al. 2011), and inhibition of Teff intermediate activation marker (CD25) expression. In addition, a Teff proliferation assay was used to test human PSTreg suppressive function.

Cells were harvested and washed twice with culture medium. Then, the Treg and Teff were stained separately with the Vybrant® DiI Cell-Labeling Solution and with CFSE, respectively, according to the manufacturer's instructions. 1×10^5 PSTeff and PSTreg (PP) each were seeded in 96 U-bottom wells. CD3/CD28 beads were then added at the ratio of 1:4 according to cell numbers. PSTeff+NTreg (PN), NTeff+PSTreg (NP), NTeff+NTreg (NN), PSTeff+nTreg (PSTeff+nTreg), NTeff+nTreg (NTeff+nTreg) were set up as Treg and Teff controls, PSTeff (P) and NTeff (N) in humans as Teff controls, PSTeff with/or NTeff in baboons as Teff controls (C). In the human system, Treg and Teff were seeded at different ratios: Treg:Teff 1:1, 1:2, 1:4, 1:8, 1:16, 1:32.

After 7 h incubation, human and baboon cells were harvested and stained for CD154. After 96 h incubation, a monoclonal antibody against CD25 was used to measure the human and baboon Teff activation. Human Teff proliferation was tested after 96 h. In all assays, LIVE/DEAD® Fixable Blue Dead Cell Stain Kit was used to determine the viability and exclude the dead cells during analysis.

Stained cells were analyzed by using the LSRII. Data were processed by using FlowJo software.

3.2.1.6 Human and baboon cytokine production

In the human system, supernatants of DC, PS/NTreg and PS/NTeff cultures, and supernatants of PP, PN, NP, NN, and P, N were harvested to determine cytokine production. Secreted IL-12p70, IL-10 and TGF-β1 levels were measured with enzyme-linked immunosorbent assays (ELISA) and BD™ CBA Flex Set system according to the manufacturers' instructions. IL-17A and IFN-γ were quantified with BD™ CBA Flex Set system according to the manufacturer's instructions. DC culture medium without cells served as control for DC supernatants.

Similarly, in the baboon system, supernatants of DC, PS/NTreg and PS/NTeff cultures, supernatants of PP, PN, NP, NN, and C were harvested to determine cytokine production. Secreted IL-12p40, IL-10 levels were measured with ELISA according to the manufacturers' instructions. DC culture medium without cells served as control for DC supernatants.

3.2.1.7 Reverse transcription polymerase chain reaction (RT-PCR)

In the human system, tol/C5-DC, PS/N Treg/Teff and PP, PN, NP, NN were harvested and washed twice with PBS. RNA was isolated as described above. Reverse transcription was accomplished using the Reverse Transcription System, according to the manufacturer's instructions. Quantification of *Foxp3*, *STAB1*, *GARP*, *EBI3*, *IL-12A*, *IL-12B*, *IL-27A, IL-10*, *TGFB1* mRNA and *ACTB*, *G6PDH* and *CYPB* (for housekeeping genes) was performed with RT-PCR using FastStart Essential DNA Green Master and Light Cycler 96. The quality of the PCR primers was confirmed by melting curve analysis.

In the baboon system, tol/C5-DC and PS/N Treg/Teff were harvested and washed twice with PBS. RNA isolation and reverse transcription were accomplished as described above in the human system. Quantification of *GARP*, *EBI3*, *IL-12A*, *IL-12B*, *IL-27A, IL-10*, *TGFB1* mRNA and housekeeping gene *GAPDH* mRNA was performed as described above in the human system.

All relative amounts of the cDNA content of interest in the unknown samples were calculated with the Livak method: relative expression of cDNA of interest

was normalized with the control sample, and target gene relative expression was normalized by the reference gene.

$$\text{relative normalized expression ratio} = 2^{(-((\text{Cq (target,unknow)} - \text{Cq (reference,unknow)}) - (\text{Cq (target,control)} - \text{Cq (reference,control)})))}$$

, Cq represents the crossing point where a fluorescence value of one is reached.

3.2.1.8 Statistical analysis

Data are displayed as mean ± s.e.m. (standard error of the mean). Significance of data was analyzed by ONE-Way Analysis Of Variance (ANOVA) with Bonferroni's Multiple Comparison Test, or turkey test, or Wilcoxon signed-rank test in Graphpad Prism 5.00 software.

3.2.2 Immune monitoring for porcine heart transplanted baboons

3.2.2.1 PBMC isolation from peripheral blood and leucocyte removal filter

For PBMC isolation from baboon peripheral blood, blood was diluted 4 times with RPMI 1640 medium and carefully overlaid on Ficoll, then centrifuged at 2000 rpm without break for 20 min at RT. The PBMC layer was transferred into a new tube and filled up with RPMI 1640 medium, and centrifuged at 1200 rpm for 10 min. Cell numbers and viability was determined using Countess™ Automated Cell Counter.

For baboon PBMC flashed from leucocyte removal filter, the filters were rinsed with 100 ml 0.9% NaCl in flow direction, and then rinsed against the flow direction. The flow was collected and aliquoted into four 50 ml falcon tubes. Ery-Lysis buffer was added up to 50 ml and the cells were incubated at 37˚C for 15 min. Following centrifugation at 1200 rpm for 10 min, cells were resuspended again in Ery-Lysis buffer up to 50 ml and incubated at 37˚C for 15 min. After the last centrifugation step at 1200 rpm for 10min, cells were counted and the viability was determined.

3.2.2.2 Freezing and thawing of cells

Cells were centrifuged at 1200 rpm for 10 min, and 1 ml Ibidi freezing medium was added per 1×10^9 cells/CryoTube. Cells were first stored in Mr. Frosty and put into -80˚C freezer for at least 24 h, and then transferred in the gaseous phase of liquid nitrogen.

For thawing, cells were immediately transferred in 37˚C water bath and thawed until a little bit ice was left, and then put into 10 ml pre-warmed CTL-Wash solution with 50 U/ml Benzonase. Cells were centrifuged at 1200 rpm for 10 min and resuspended again in 10 ml pre-warmed CTL-Wash with 50 U/ml Benzonase and centrifuged.

3.2.2.3 Immunosuppressive therapy for baboon pre- and post-transplantation

The xenotransplantation study was approved by the local authorities and the Government of Upper Bavaria. All animals received treatment in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (2011) and National Law.

In group 1, all baboons received a pre-treatment in the last week before transplantation. Donor pigs of group 1 had the genotype *GGTA1* homozygous knockout/heterozygous *hCD46*. The donor pig for baboon 49 had the genotype *GGTA1* homozygous knockout/heterozygous *hCD46*/heterozygous *HLA-E*.

The immunosuppression of group 1 is shown in table 3.11.

Table 3.11 Immunosuppressive regimen in group 1

Drugs	Dose	Timing
Induction		
Anti-CD20	19 mg/kg	Pre-trans day -7, 0
bortezomib	0.05 mg/kg	Pre-trans day -7 & -2
cyclophosphamide	10 mg/kg	Pre-trans day -7
Maintenance		
Anti-CD20	19 mg/kg	Post-trans weekly
anti-thymocyte globuline (ATG)	1.5 mg/kg	post-trans 0, 1, 2, 3, 4
Tacrolimus	0.01 mg/kg	daily
mycophenolate mofetil (MMF)	20 mg/kg	daily
Methylprednisolone	10 mg/kg	daily
cyclophosphamide	1 mg/kg	post-trans 4, 7

bortezomib	0.05 mg/kg	post-trans 4, 7
Heparin	ACT 2x Baseline	Continuous infusion

All baboons in group 1 received a computer tomography-based total thoracic and abdominal lymphoid irradiation (TLI; single dose of cGY on day 5 post transplantation).

In group 2, all baboons received a pre-treatment with anti-CD40 mAb. All the donor pigs of group 2 had the genotype *GGTA1* homozygous knockout/heterozygous *hCD46*.

The immunosuppression of group 2 is shown in table 3.12.

Table 3.12 Immunosuppressive regimen in group 2

Drugs	Dose	Timing
Induction		
Anti-CD20	19 mg/kg	Pre-trans day -7, 0, 7 & 14, then weekly
ATG	5 mg/kg	Pre-trans day -2 & -1
Anti-CD40	50 mg/kg	Pre-trans day -1 & 0
Maintenance		
Anti-CD20	19 mg/kg	Post-trans weekly
Anti-CD40	50 mg/kg	Post-trans days 3, 7, 10, 14, 19, then weekly
MMF	20 mg/kg	daily
Tacrolimus	0.01 mg/kg	daily
Steroids	10 mg/kg	2 times daily, tapered off in 7 weeks
Aspirin	25 mg	daily
Heparin	ACT 2x Baseline	Continuous infusion

In group 3, all baboons received a pre-treatment with anti-CD40 mAb or anti-CD40L mAb. All donor pigs of group 3 had the genotype *GGTA1* homozygous knockout/heterozygous *hCD46*/homozygous *hTM*. Group A included the baboons 55, 57, 63 and they received anti-CD40 mAb. Group B included the baboons 60, 64 and they received anti-CD40L mAb.

The immunosuppression of group 2 is shown in table 3.13.

Table 3.13 Immunosuppression regimen in group 3

Drugs	Dose	Timing
Induction		
Anti-CD20	19 mg/kg	Pre-trans day -7, 0, 7 & 14

ATG	5 mg/kg	Pre-trans day -2 & -1
Anti-CD40 (Group A)	50 mg/kg	Pre -trans day -1 & 0
Anti-CD40L (Group B)	20 mg/kg	Pre -trans day -1 & 0
Maintenance		
Anti-CD20	19 mg/kg	Post-trans weekly
Anti-CD40 (Group A)	50 mg/kg	Post-trans days 3, 7, 10, 14, 19, then weekly
Anti-CD40L (Group B)	20 mg/kg	Post-trans days 3, 7, 10, 14, 19, then weekly
MMF	40 mg/kg	daily
Steroids	10 mg/kg	2 times daily, tapered off in 7 weeks
Aspirin	25 mg	daily
Heparin	ACT 2x Baseline	Continuous infusion

3.2.2.4 Flow cytometric analysis of baboons pre and post porcine heart transplantation

3.2.2.4.1 Overall survey analysis

$1x10^6$ cells were aliquoted into flow cytometry tubes. 1 µl Blue Dye in 50 µl PBS was added and the cells were incubated for 30 min at RT. Cells were washed with PBS and then the antibodies were added according to table 3.14.

Table 3.14 Staining for overall survey analysis

	Tube 1	Tube 2 (FMO CD16)	Tube 3 (FMO CD19)
CD3-PE-cy7	√	√	√
CD14-FITC	√	√	√
CD16-APC	√	--	√
CD19-PacificBlue	√	√	--
CD45-PerCP	√	√	√

FMO: fluorescence minus one

Gating strategy for overall survey analysis by FlowJo was shown in figure 3.1.

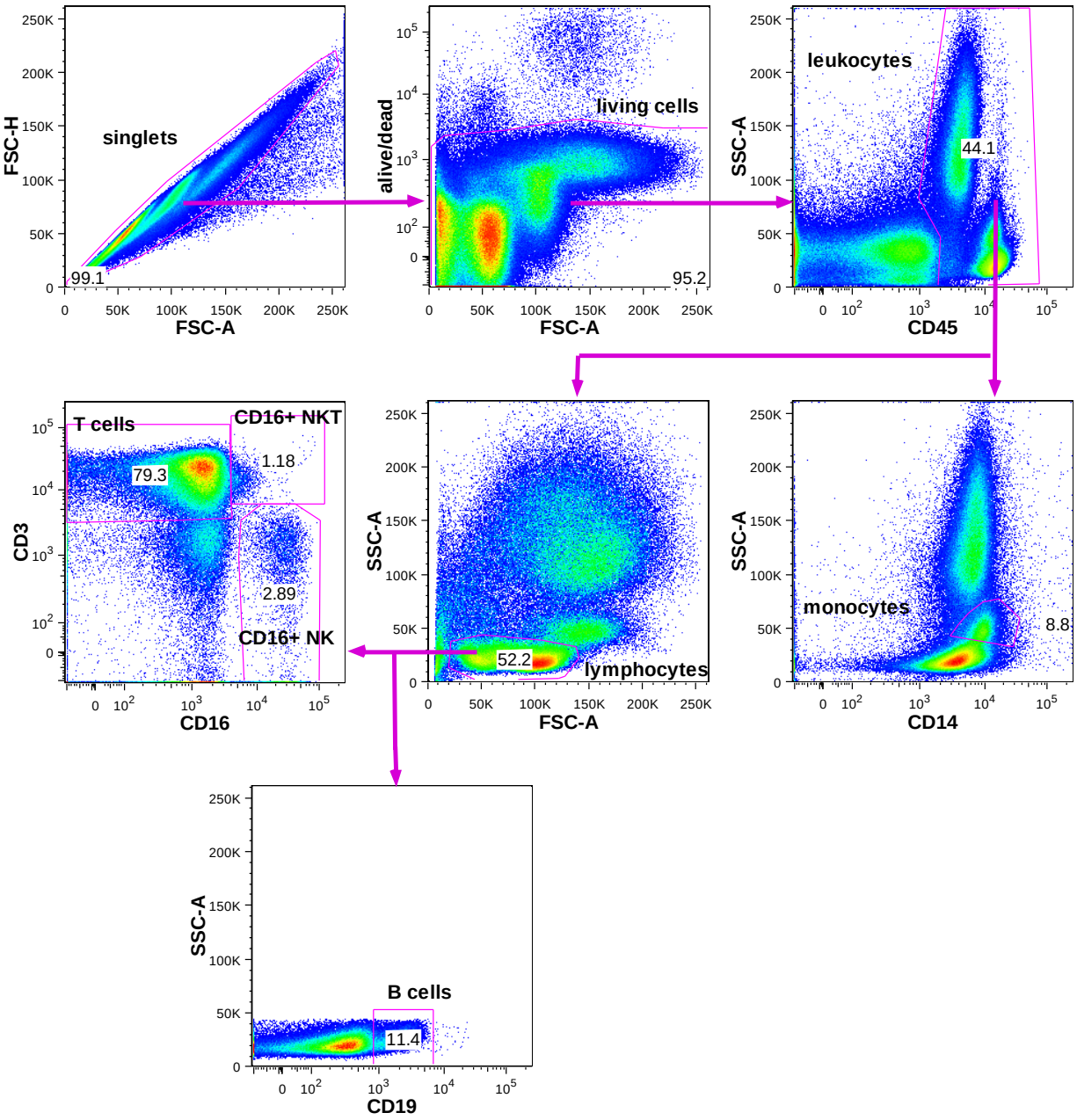


Figure 3.1 Gating strategy for overall survey analysis. For immune monitoring of leukocytes, T cells, monocytes, NK cells, NKT cells and B cells, the following antibodies are used: anti-NHP CD45 was used to determine the leukocytes, anti-human CD3 (cross-reactive with baboon) was used to determine the T cells, anti-human CD14 (cross-reactive with baboon) and side scatter (SSC) were used to determine monocytes, anti-human CD16 (cross-reactive with baboon) was used to determine NK cells, anti-human CD3 and anti-human CD16 were used to determine the NKT cells, anti-human CD19 (cross-reactive with baboon) was used to determine the B cells.

3.2.2.4.2 Treg analysis

1x10^6 cells were aliquoted into flow cytometry tubes. 1 µl Blue Dye in 50 µl PBS was added and the cells were incubated for 30 min at RT. Cells were washed with PBS. Antibodies for CD3, CD4, CD25 and CD127 were added according to Table 3.15. Intracellular staining for Foxp3 was performed using Foxp3 staining buffer set according to manufacturer's instruction. Foxp3 antibody was added to the samples afterwards.

Table 3.15 Treg staining

	Tube 1	Tube 2 (FMO CD25)	Tube 3 (FMO CD127)	Tube 4 (FMO Foxp3)
CD3-PerCP	√	√	√	√
CD4-APC	√	√	√	√
CD25-PE-Cy7	√	--	√	√
CD127-PE	√	√	--	√
Foxp3-ef450	√	√	√	--

FMO: fluorescence minus one

Gating strategy for Treg analysis by FlowJo is shown in figure 3.2.

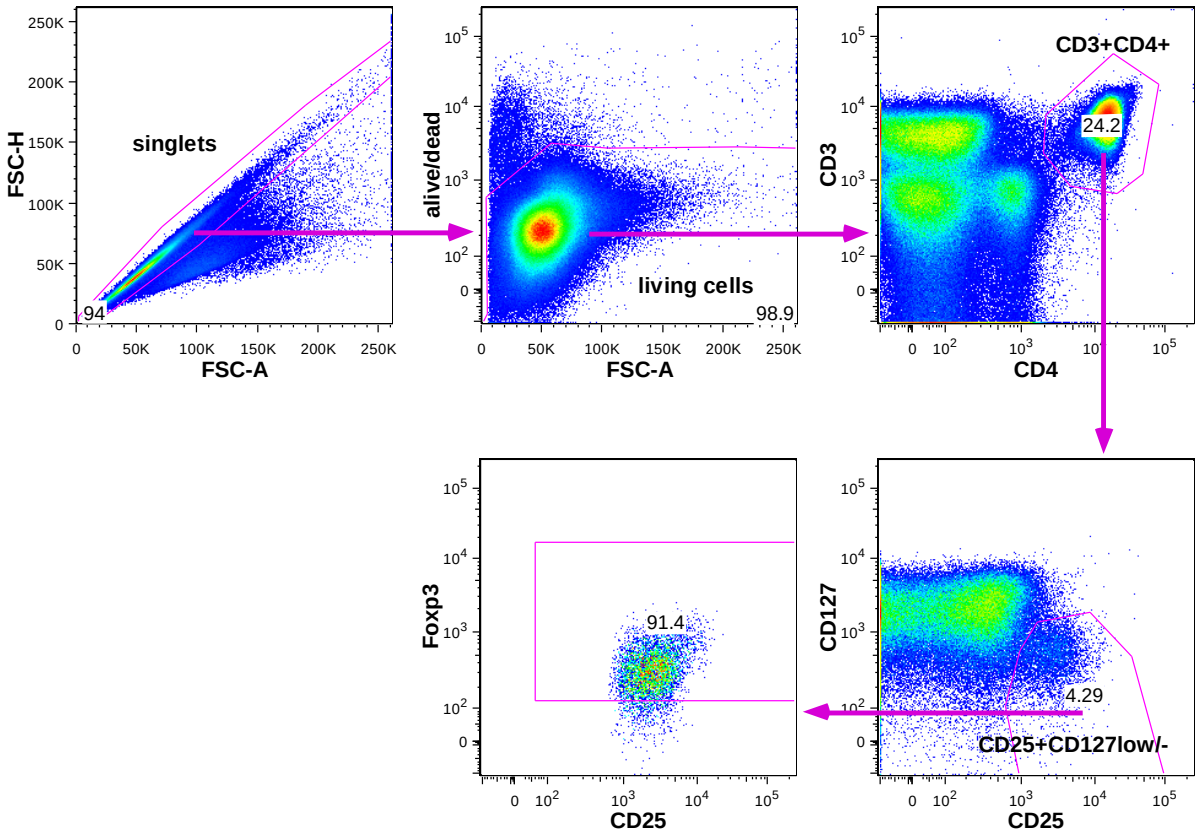


Figure 3.2 Gating strategy for Treg analysis. For immune monitoring of $CD3^+CD4^+CD25^+CD127^{low/-}$ $Foxp3^+$ Treg, the following antibodies were used: anti-human CD3, anti-human CD4, anti-human CD25, anti-human CD127, and anti-human Foxp3. All these antibodies are cross-reactive with baboon.

3.2.2.4.3 Analysis of activated T cells

$1x10^6$ cells were aliquoted into flow cytometry tubes. 1 µl Blue Dye in 50 µl PBS was added and cells were incubated for 30 min at RT. Cells were washed with PBS and then antibodies were added according to table 3.16.

Table 3.16 Staining for activated T cells

	Tube 1	Tube 2 (FMO CD25)	Tube 3 (FMO CD69)
CD3-PE-Cy7	√	√	√
CD4-APC	√	√	√
CD8-V450	√	√	√
CD25-PE	√	--	√
CD69-FITC	√	√	--

FMO: fluorescence minus one

Gating strategy for activated T cells analysis by FlowJo is shown in figure 3.3.

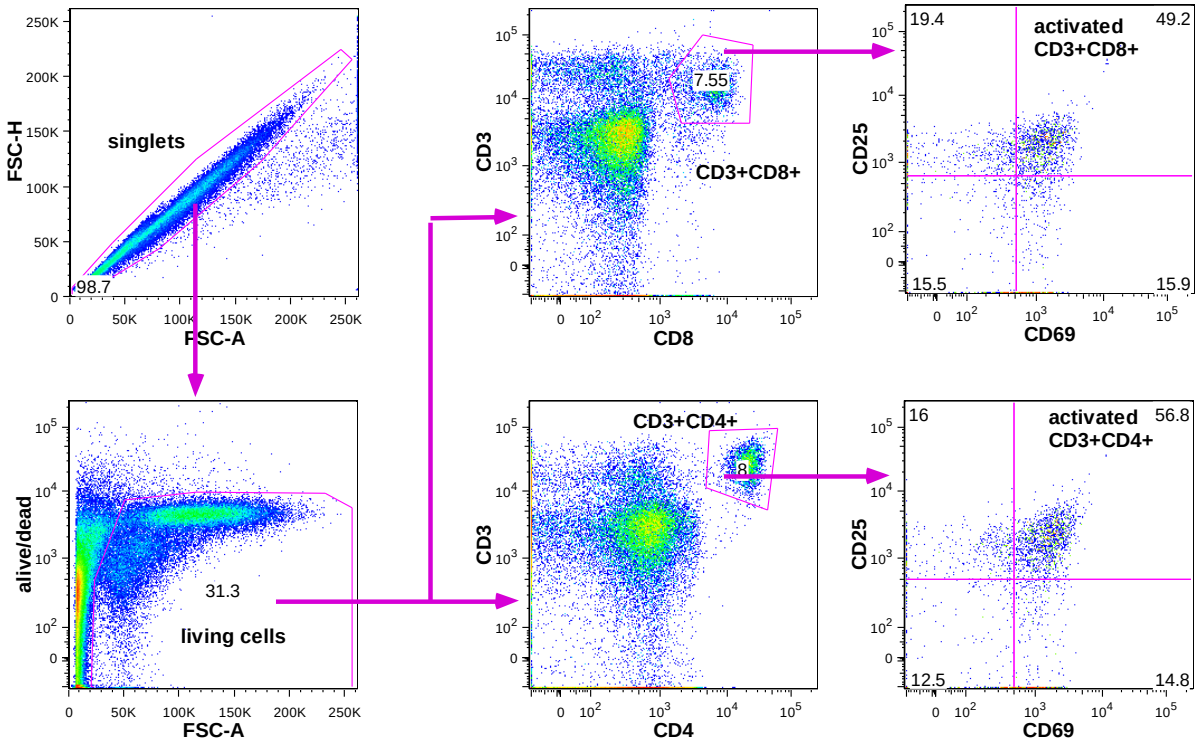


Figure 3.3 Gating strategy for T cell activation analysis. For immune monitoring of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, and activated T cells, the following antibodies are used: anti-human CD3 (cross-

reactive with baboon) and anti-human CD4 (cross-reactive with baboon) were used to determine the $CD3^+CD4^+$ T cells, anti-human CD3 and anti-human CD8 (cross-reactive with baboon) were used to determine the $CD3^+CD8^+$ T cells, anti-human CD25 (cross-reactive with baboon) and anti-human CD69 (cross-reactive with baboon) were used to determine the activated T cells.

All results were analyzed by FlowJo 8.8.7 or FlowJo 10.2.

4 Results

4.1 Generation of tolDC and PSTreg in the human system

4.1.1 TolDC express IL-10, TGF-β1, B7-H1, and B7-DC

To generate tolDC and non-tolerogenic C5-DC, fresh $CD14^+$ monocytes were isolated from healthy donor PBMC (Figure 4.1.1) and anti-inflammatory or inflammatory cytokines were added to induce tolerogenic or non-tolerogenic phenotypes, respectively.

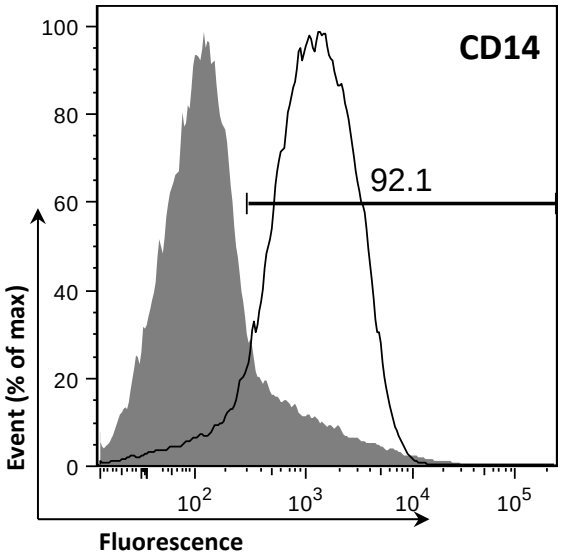
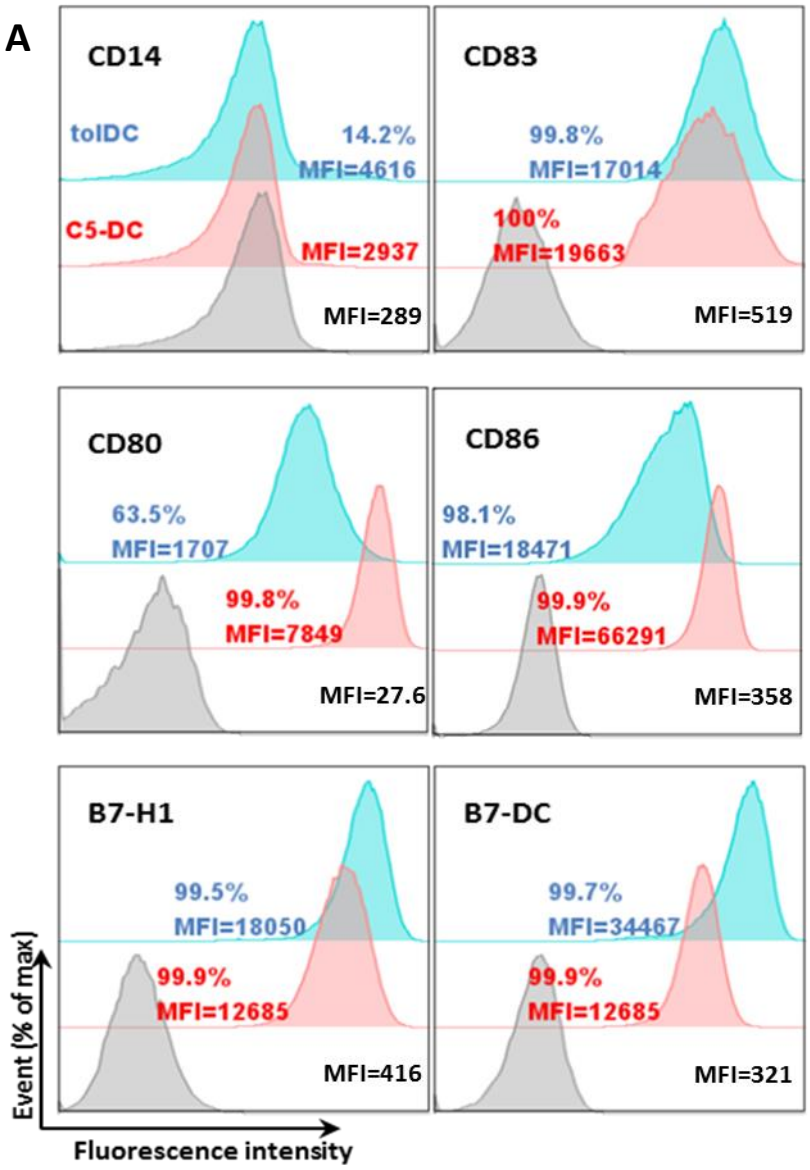


Figure 4.1.1 Isolation of monocytes from human PBMC. Monocytes were isolated from human PBMC using CD14 microbeads by positive selection. The purity of $CD14^+$ cell was analyzed by flow cytometry.

TolDC retained some CD14 expression compared with C5-DC, which totally lost CD14 expression after maturation. On the other hand, tolDC expressed significantly lower levels of CD83 compared with C5-DC. These results support the conclusion that tolDC are semi-mature cells (Figure 4.1.2 A, B) (Maldonado and von Andrian 2010). CD80 and CD86, as costimulation molecules of the B7

family, were expressed at significantly lower levels on tolDC compared to C5-DC, showing 4-fold and 3.5-fold decrease compared to C5-DC, respectively. In contrast, the negative costimulators B7-H1 and B7-DC were significant highly expressed on tolDC compared to C5-DC. We detected 1.5- and 4-fold higher levels of expression of B7-H1 and B7-DC, respectively, on tolDC in comparison to C5-DC (Figure 4.1.2 B).



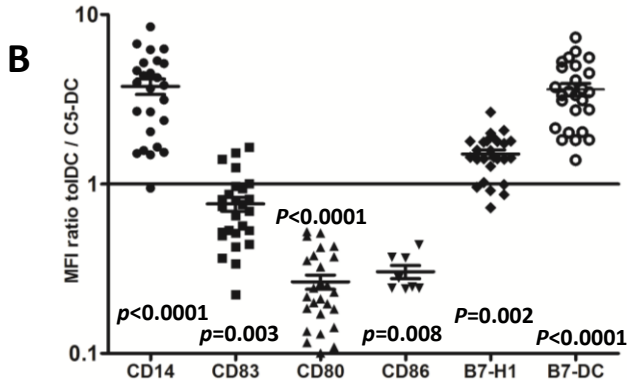


Figure 4.1.2 DC generated from monocyte using anti-inflammatory cytokines exhibit a tolerogenic phenotype. TolDC and C5-DC were harvested after maturation and characterized by flow cytometry. Phenotype analysis (A) and dot chart of MFI ratio (B) of tolDC and C5-DC are shown. Grey histograms represent isotype control or unstained control in (A). Data represent 26 independent experiments from 9 healthy donors. (CD86: n=8). Wilcoxon signed-rank test was used to determine p values.

To evaluate tolDC function, IL-10, TGF-β1, IL-12p70 and IFN-γ cytokine production was quantified using the BD™ CBA Flex Set system and ELISA. As expected, at the protein level, tolDC produced a significant higher amount of IL-10 and TGF-β1, and no IL-12p70 and IFN-γ when compared with C5-DC, while C5-DC produced greater amounts of IL-12p70 and IFN-γ, less TGF-β1, and no IL-10 (Figure 4.1.3).

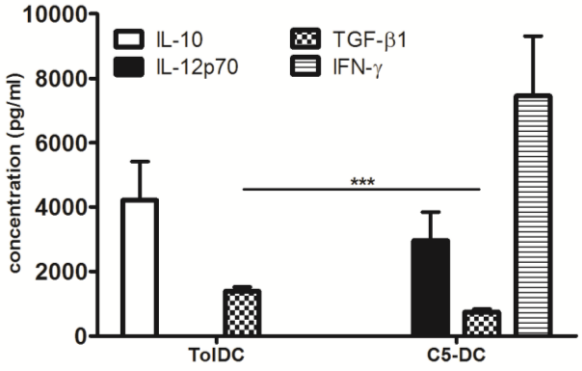


Figure 4.1.3 TolDC produce anti-inflammatory cytokines. IL-10, IL-12p70, TGF-β1 and IFN-γ were quantified in DC supernatants using BD CBA Flex Set and ELISA. Culture medium plus maturation cocktail without cells were used as control. These data represent 10-19 independent experiments with cells from 9 healthy donors. ***$p < 0.001$, error bars: s.e.m. Turkey test was used to determine the p value.

Consistently, *IL-10* mRNA levels were also upregulated in most tolDC of different donors, although the data did not meet statistical significance. Half of the samples showed elevated *TGFB1* RNA levels in tolDC compared to C5-DC (Figure. 4.1.4). Generally, *EBI3* and *IL-12A* mRNA expression showed no significant difference in tolDC compared with C5-DC. *IL-27A* expression was downregulated in tolDC compared with C5-DC, however with a p-value of only 0.078. *IL-12B* was significantly downregulated in tolDC, which was consistent with the protein level results showing that C5-DC expressed high amounts of IL-12p70 while tolDC produced no IL-12p70 (Figure 4.1.4).

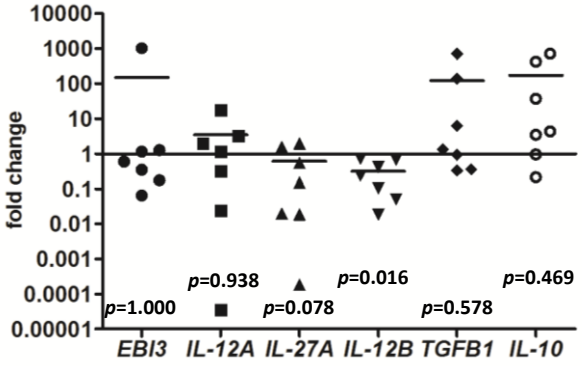


Figure 4.1.4 TolDC upregulate anti-inflammatory cytokine RNA. RNA was isolated from tolDC and C5-DC. Relative cDNA levels of *EBI3, IL-12A, IL-27A, IL-12B*, *IL-10*, *TGFB1* in tolDC were quantified by RT-PCR, C5-DC served as control. *ACTB, G6PDH or CYPB* cDNA was used as endogenous reference gene for each sample. These data represent 7 independent experiments from 6 donors. Wilcoxon signed-rank test was used to determine p values.

In summary, our tolDC express high levels of IL-10, TGF-β1, and no IL-12p70 and IL-27.

4.1.2 DC express porcine antigen following electroporation with porcine ivtRNA

Electroporation of PS ivtRNA into DC is a rapid way to induce foreign antigen production in DC. However, unlike transfection of a specific mRNA, the efficiency of transfection of total cellular mRNA is difficult to assess in host cells. In these studies we used detection of porcine MHC-I molecule as a surrogate marker of PS antigen expression in DC. The quality of porcine PBMC

RNA, cDNA and ivtRNA was controlled with capillary or agarose gel electrophoresis (Figure 4.1.5).

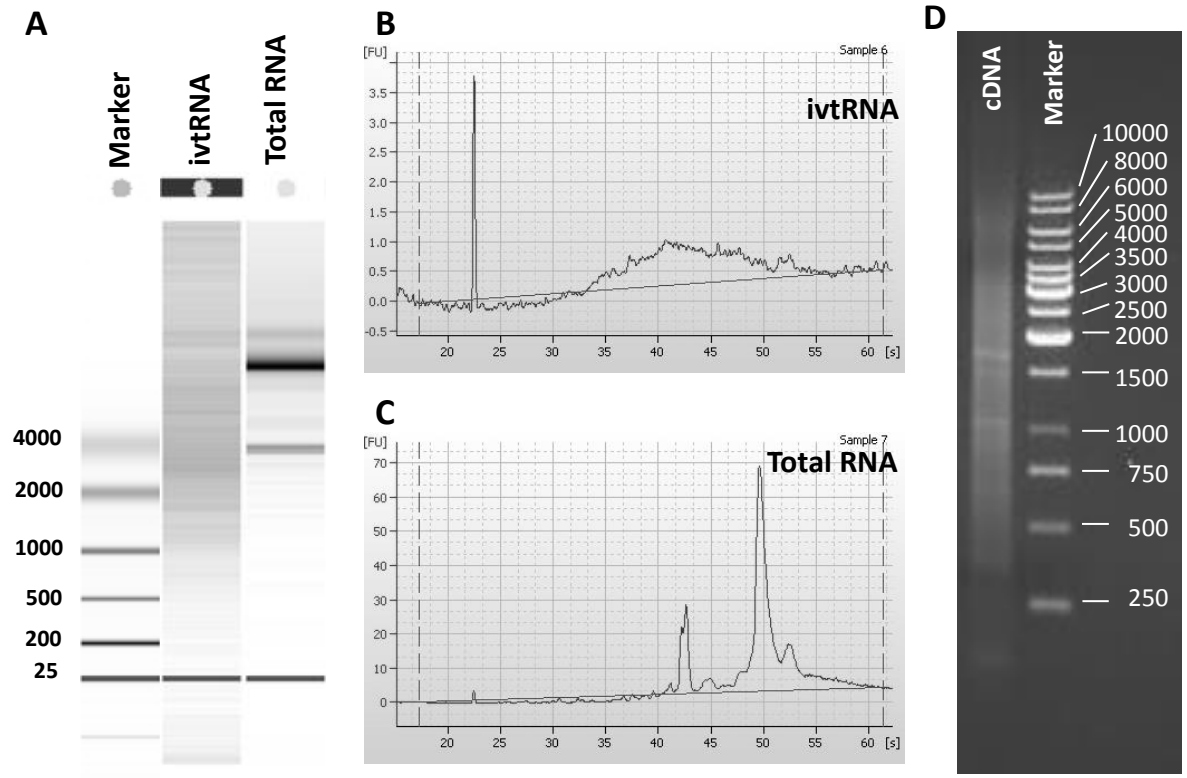


Figure 4.1.5 Generation of PS ivtRNA. Porcine PBMC RNA was isolated using the RNeasy Mini Kit, cDNA was synthesized using reverse transcription with SMARTer™ PCR cDNA Synthesis Kit and the Advantage® 2 PCR Enzyme System. The following primers were used: 5' primer/T7: 5'-TAATACGACTCACTATAGGGAGGAAGCAGTGGTAACAACGCA-3' 3' CDS primer: 5'-AAGCAGTGGTATCAACGCAGAGT-3'. Then ivtRNA was generated by mMESSAGE mMachine T7 Ultra Kit. The quality of porcine PBMC RNA (A, C) and ivtRNA (A, B) was tested by Agilent. The cDNA (D) quality was tested by agarose gel electrophoresis.

Detection of porcine MHC-I expression by flow cytometry indicated that PS ivtRNA was successfully transfected and expressed in tolDC and C5-DC. As expected, PS antigen expression from ivtRNA was transient: MHC-I antigen became detectable on both tolDC and C5-DC after 24 h and peaked at 72 h (Figure 4.1.6 A, B, C). The viability was shown in the Figure 4.1.6 D.

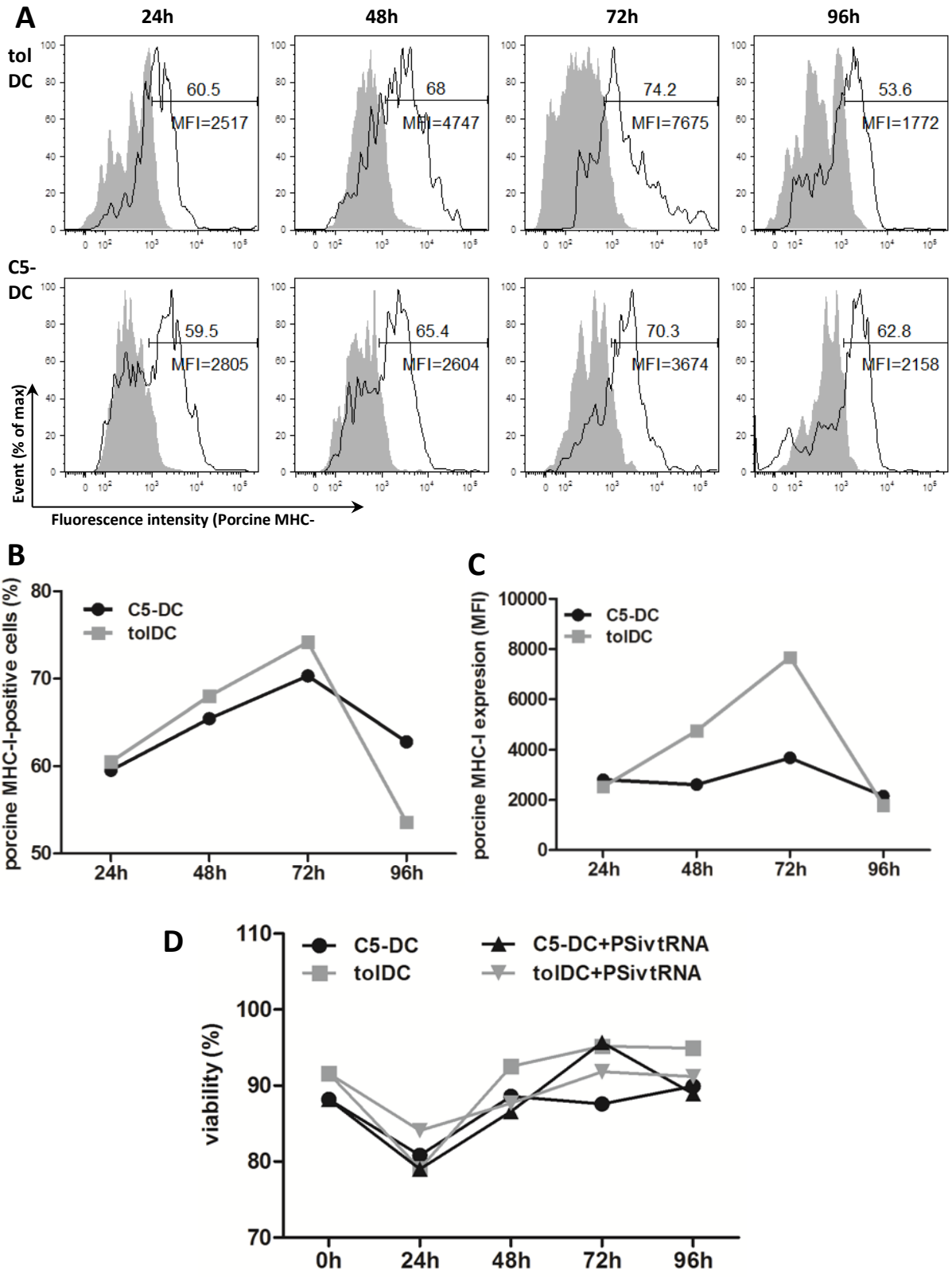


Figure 4.1.6 TolDC and C5-DC successfully express porcine antigen following electroporation with porcine-specific ivtRNA. (A) PS MHC-I flow cytometry result, mock-electroporated tolDC and C5-DC were used as controls (grey peak). (B-C) Line charts of PS MHC-I in % cells and MFI. (D) Viability of tolDC and C5-DC after electroporation.

4.1.3 PSTreg can be generated with PS ivtRNA loaded tolDC

PSTreg were induced from purified $CD4^+$ naïve T cells (Figure 4.1.7) by coculture with PS ivtRNA loaded tolDC in medium supplemented with high concentration of IL-2 and addition of rapamycin. In parallel, PSTeff were generated from $CD4^+$ naïve T cells by coculture with PS ivtRNA loaded C5-DC in medium supplemented with a lower concentration of IL-2 and without rapamycin. In parallel, NTreg and NTeff were generated by coculture with mock-electroporated tolDC and C5-DC, respectively.

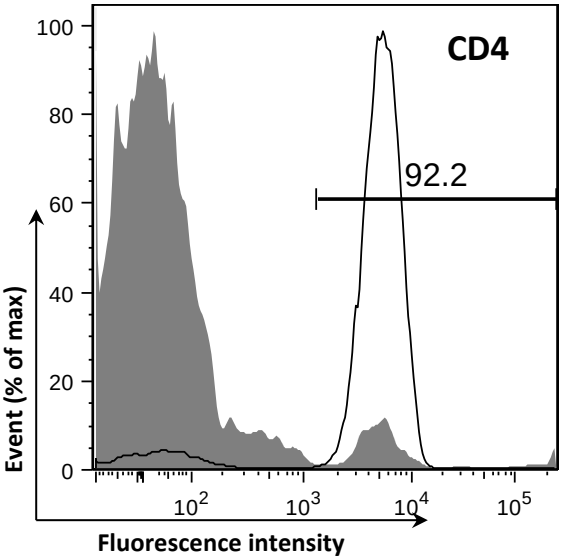


Figure 4.1.7 Isolation of $CD4^+$ cells from human PBMC. $CD4^+$ cells were isolated using the CD4 isolation kit by negative selection. The purity of $CD4^+$ cell was tested by flow cytometry.

PSTreg and NTreg exhibited the conventional human $CD4^+CD25^+CD127^{low/-}$ $Foxp3^+$ phenotype (Figure 4.1.8 A). There was no significant difference in the fraction of Foxp3-positive PSTreg and NTreg in both the $CD3^+CD4^+$ cells and the total cell populations (Figure 4.1.8 B, C, Table 4.1.1).

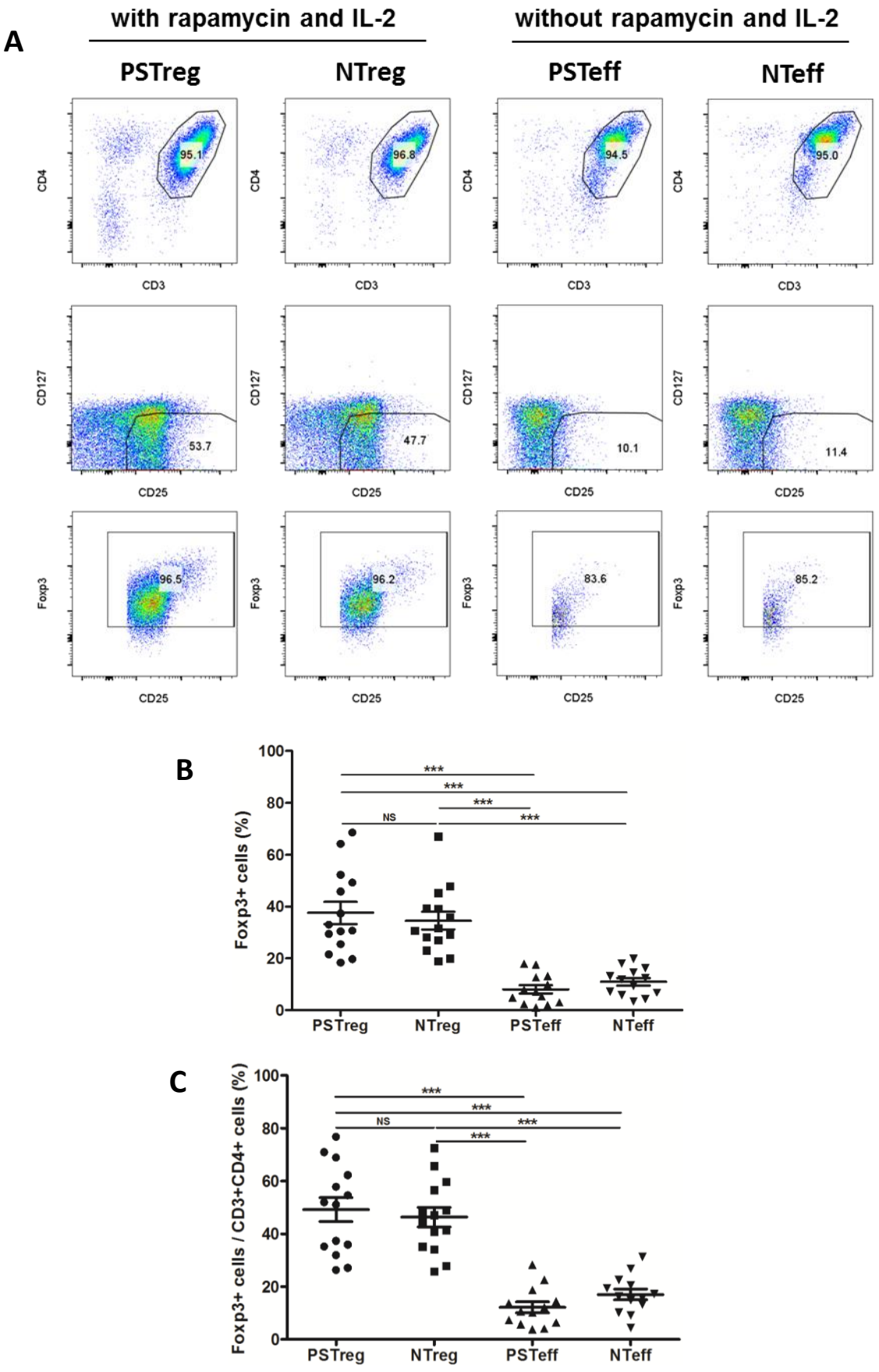


Figure 4.1.8 PSTreg can be generated using PS ivtRNA loaded tolDC. $CD4^+$ T cells were isolated and cocultured with PS antigen-expressing DC or mock-loaded DC for 10 days in the

presence of IL-2 w/o rapamycin. (A) Flow cytometry gating strategy for quantification of $CD4^+CD25^+CD127^{low/-}Foxp3^+$ Treg cells. (B, C) Ratio of PSTreg and NTreg in the alive cell population and in the $CD3^+CD4^+$ population after 10 days coculture. For comparison PSTeff and NTeff were analyzed. Data represent 14 independent experiments from 8 donors. ANOVA with Bonferroni's Multiple Comparison Test was used to determine the statistical significance. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. NS, non-significant, $p > 0.05$. Error bars: s.e.m.

Table 4.1.1 Frequency of $CD4^+CD25^+CD127^{low/-}Foxp3^+$ cells in PSTreg and NTreg and PSTeff and NTeff populations

	PSTreg	NTreg	PSTeff	NTeff
Treg in $CD3^+CD4^+$ cell fraction (%)	49.1±4.5	46.3±3.7	12.1±2.1	17.0±2.0
Treg in live cell fraction (%)	37.5±4.3	34.5±3.4	8.1±1.6	11.0±1.5

PSTreg expressed significantly more *Foxp3* mRNA than NTreg (Figure 4.1.9). However, NTreg inhibited *SATB1* mRNA expression, which at low expression is considered as a new marker of Treg, compared with PSTreg and non-specific Teff (NTeff) (Figure 4.1.9). GARP is known as an activation marker of Treg: PSTreg expressed significantly more *GARP* mRNA than NTreg (Figure 4.1.9).

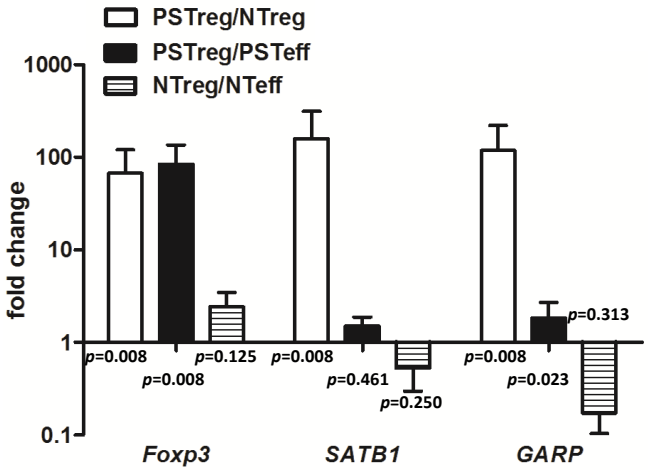


Figure 4.1.9 PSTreg express high levels of *Foxp3*, *SATB1* and *GARP*. Relative quantification of *Foxp3*, *SATB1* and *GARP* mRNA in PSTreg and NTreg was done by RT-PCR, NTreg and PSTeff served as control for PSTreg, NTeff as control for NTreg. *ACTB, G6PDH or CYPB* mRNA was used as endogenous reference gene for each sample. Data represent 8 independent experiments from 6 donors. Wilcoxon signed-rank test was used to determine p values. Error bars: s.e.m.

Furthermore, PSTreg exhibited a memory phenotype compared with NTreg, showing CD45RA$^-$Foxp3$^+$, and expressed significant lower levels of CCR7 (Figure 4.1.10 A, B). Moreover, CCR4 was significantly upregulated on PSTreg compared with NTreg (Figure 4.1.10 B). Similar to PSTreg, PSTeff also showed a more prominent memory phenotype (CD45RA$^-$CCR7^{low}) than NTeff (Figure 4.1.10 C, D).

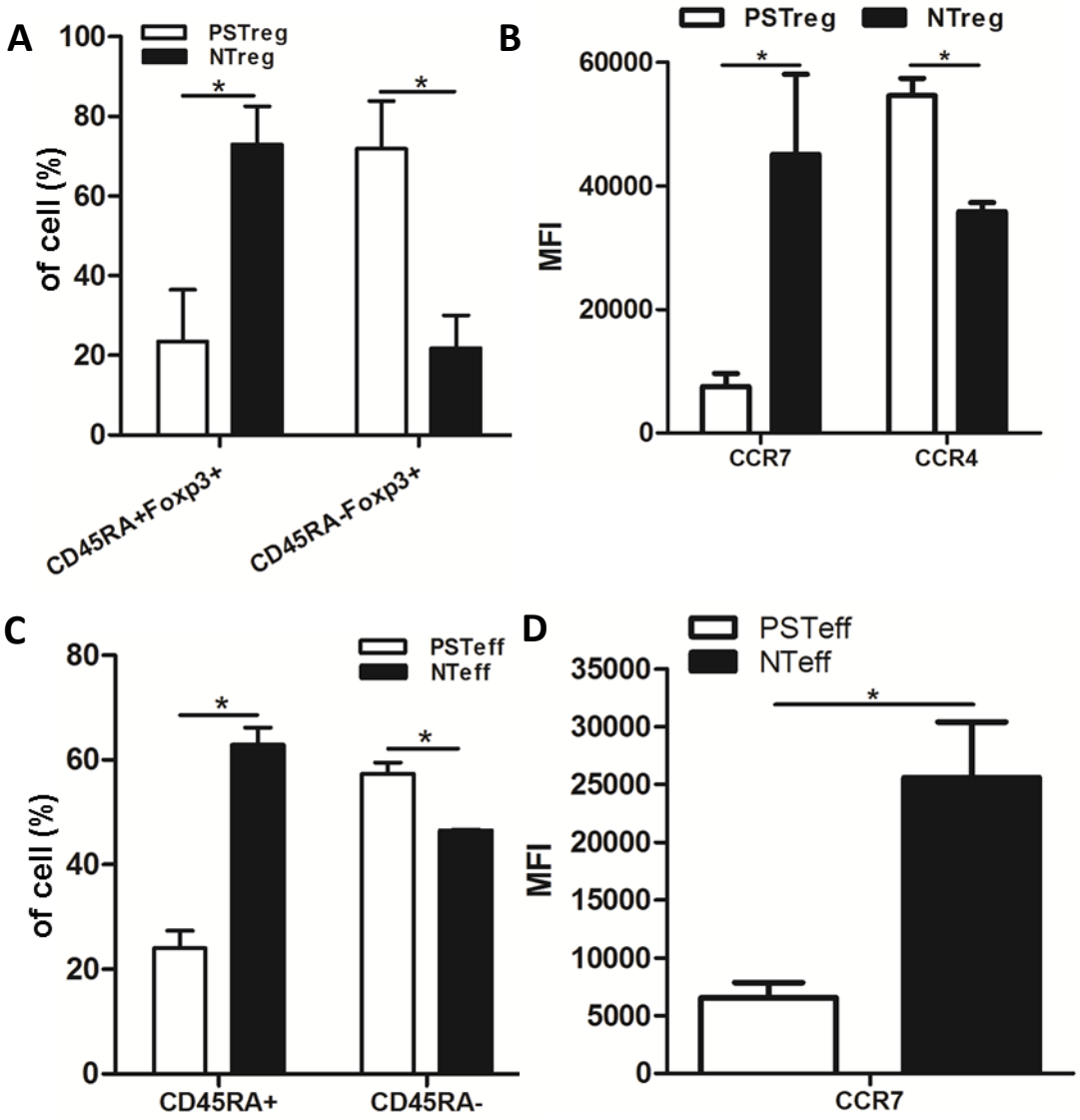


Figure 4.1.10 PSTreg express memory phenotype and high CCR4. The percentage of CD45RA$^-$Foxp3$^+$ population and CD45RA$^+$Foxp3$^+$ population in PSTreg and NTreg are shown in (A), the expression of CCR7 and CCR4 are shown in (B). The percentage of CD45RA$^-$ population and CD45RA$^+$ population in PSTeff and NTeff are shown in (C), the expression of CCR7 is shown in (D). Turkey test was used to determine p values. Error bars: s.e.m. n=3

To characterize cytokine production of PSTreg, culture supernatants of cocultures were harvested after coculture for 7 h and 96 h. The concentrations of IL-10, TGF-β1, IFN-γ, IL-12p70 and IL-17A were measured (Figure 4.1.11 A). PSTreg expressed significantly higher concentrations of IL-10 and TGF-β1 compared with both NTeff and PSTeff. Although not statistically significant they also showed higher concentrations than found for NTreg. PSTeff expressed the highest levels of IFN-γ, which was significantly higher than that found for PSTreg. IL-17A, an indicator of plasticity and instability of Treg, was undetectable in PSTreg and NTreg (data not shown). IL-12p70 was also not expressed in PSTreg and NTreg (data not shown).

Expression levels of *TGFB1* and *IL-10* mRNA in PSTreg were significantly higher than in NTreg and PSTeff, which showed 7.5- and 23.6- fold and 16.4- and 5071-fold higher than the expression levels of NTreg and PSTeff, respectively. NTreg also upregulated significantly more *TGFB1* and *IL-10* mRNA than NTeff (Figure 4.1.11 B). These results were consistent with those obtained at the protein level.

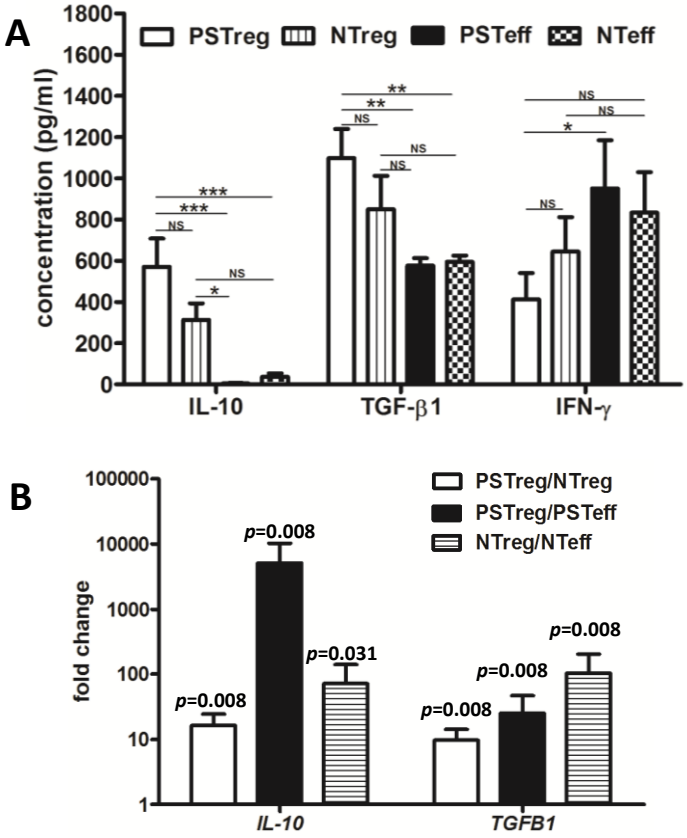


Figure 4.1.11 PSTreg express high levels of IL-10 and TGF-β1. Supernatants of cocultures were harvested for analyzing IL-10, TGF-β1, and IFN-γ. Relative quantification of *IL-10* and

TGFB1 mRNA in PSTreg and NTreg was done by RT-PCR, NTreg and PSTeff served as control for PSTreg, NTeff as control for NTreg. *ACTB, G6PDH or CYPB* mRNA was used as endogenous reference gene for each sample. Data represent 8-20 experiments from 6-7 donors. ANOVA with Bonferroni's Multiple Comparison Test was used to determine the statistical significance of cytokine production on protein level (A). Wilcoxon signed-rank test was used to determine statistical significance of mRNA fold change (B). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. NS, non-significant, $p > 0.05$. Error bars: s.e.m.

PSTreg expressed significantly more *EBI3* and *IL-12A* mRNA compared to NTreg, and significantly more *IL-12A* mRNA compared to PSTeff (Figure 4.1.12). This indicates that PSTreg produce more IL-35 than NTreg. PSTreg did not express significantly higher amounts of *IL-27A* mRNA in comparison to NTreg and PSTeff. Both PSTeff and NTeff expressed 47.1- and 843-fold more *IL-12B* mRNA than PSTreg and NTreg, however, the data did not meet statistical significance because only 4 samples out of 8 contained detectable *IL-12B* RNA (Figure 4.1.12).

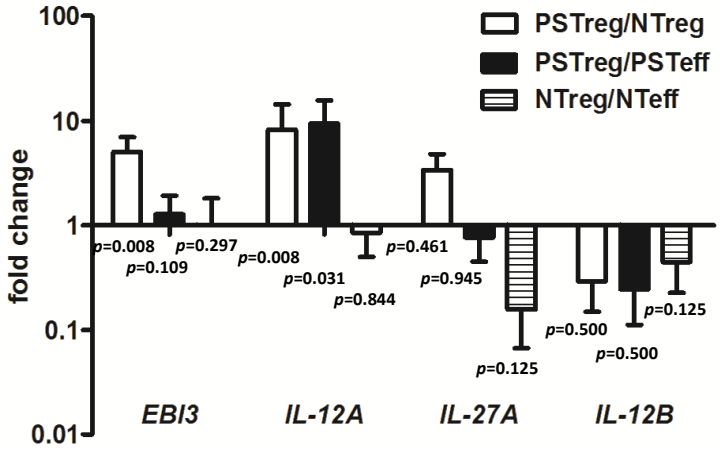
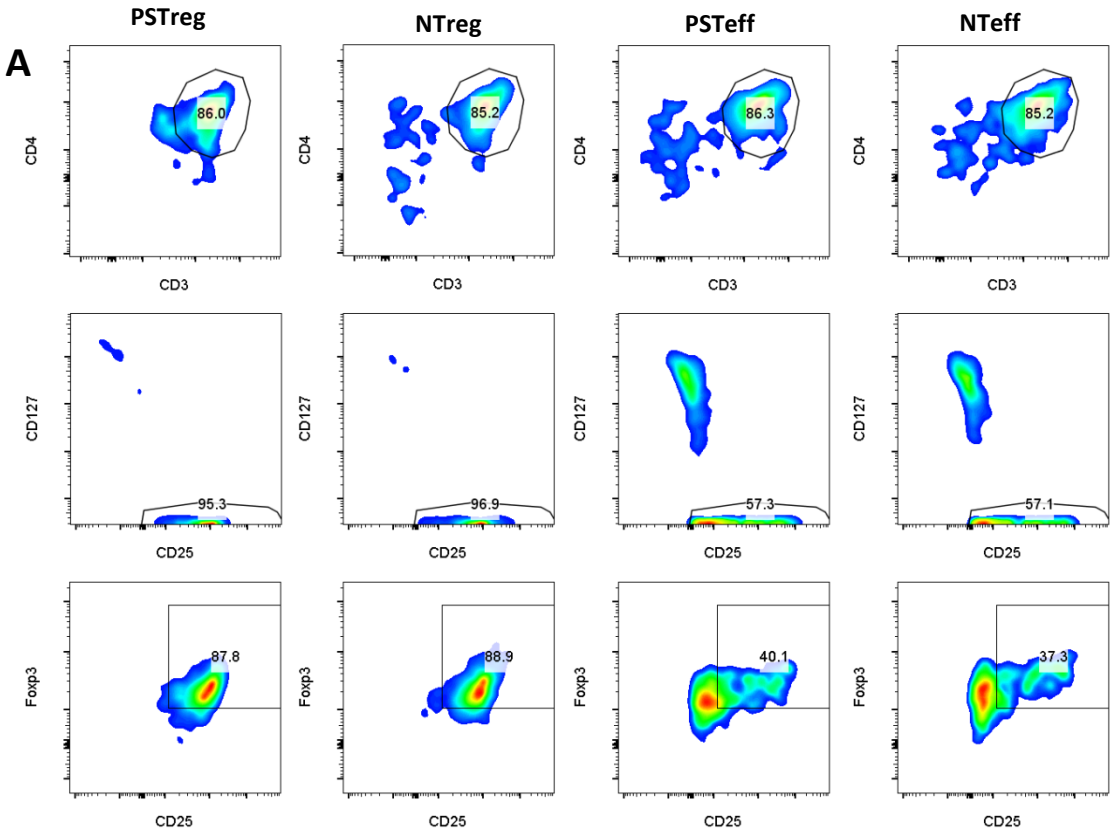


Figure 4.1.12 PSTreg express high levels of IL-35 gene family related mRNA. Relative expression of *EBI3, IL-12A, IL-27A, IL-12B* mRNA in PSTreg is shown. NTreg and PSTeff served as control for PSTreg, NTeff as control for NTreg. *ACTB*, *G6PDH* or *CYPB* mRNA were used as endogenous reference gene for each sample. Data represents 8 independent experiments from 6 donors. Wilcoxon signed-rank test was used to determine statistical significance. Error bars: s.e.m.

To test the stability of PSTreg, the purified PSTreg and NTreg cells were restimulated with PS antigen loaded or mock loaded tolDC or C5-DC. Upon restimulation, PSTreg maintained Foxp3 expression (Figure 4.1.13 A) and kept the more prominent memory phenotype than NTreg (Figure 4.1.13 B): $CD45RA^-$

CCR7^{low}Foxp3^{+}, and expressed significantly higher levels of CCR4 than NTreg (Figure 4.1.13 C). NTreg exhibited also CD45RA^{-}Foxp3^{+} phenotype upon restimulation, but at a significant lower level than PSTreg. PSTeff also maintained the more prominent memory phenotype than NTeff: CD45RA^{-} CCR7^{low} (Figure 4.1.13 D, E).



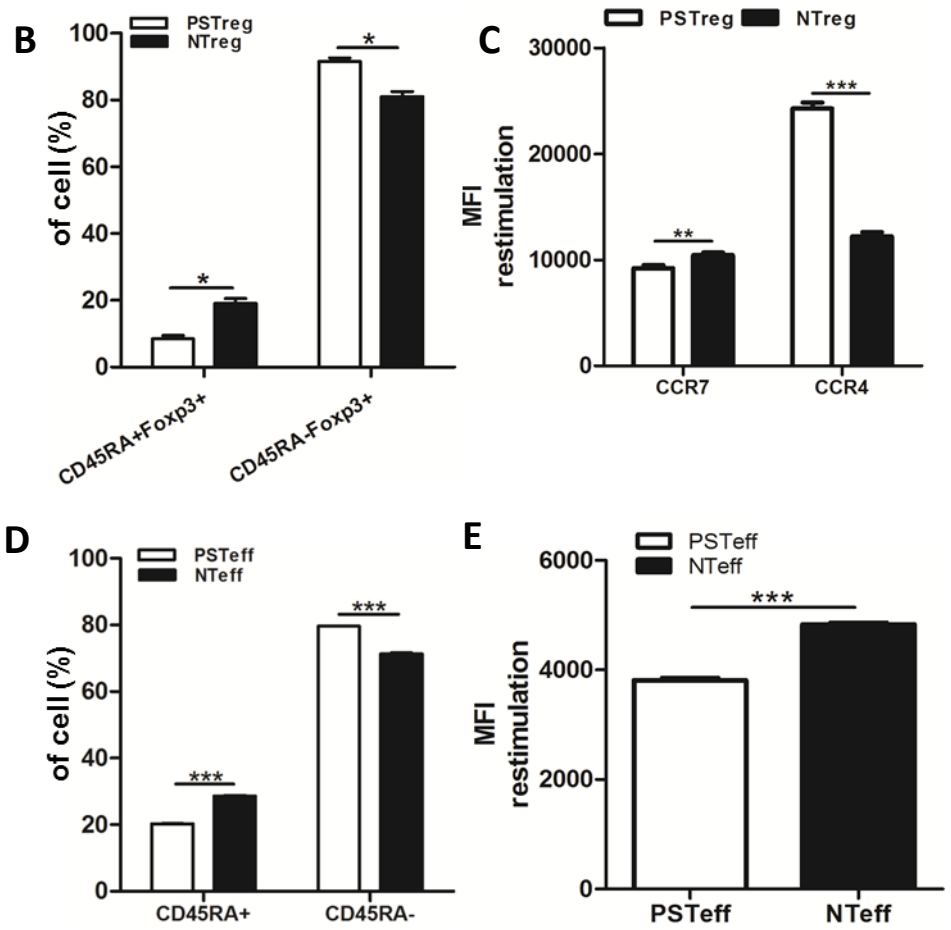


Figure 4.1.13 PSTreg kept Foxp3 expression, memory phenotype and high CCR4 after restimulation. PSTreg, NTreg and PSTeff, NTeff were harvested after cocultured with DC for 10 days. Then PSTreg and NTreg were purified, and all cells were restimulated again with DC for 10 days. (A) Gating strategy for PSTreg and NTreg after restimulation for 10 day. PSTeff and NTeff are shown as controls. (B) Percentage of $CD45RA^{+}Foxp3^{+}$ and $CD45RA^{-}Foxp3^{+}$ populations in PSTreg and NTreg after restimulation. (C) CCR7 and CCR4 expression in PSTreg and NTreg after restimulation. (D) Percentage of $CD45RA^{+}$ and $CD45RA^{-}$ in PSTeff and NTeff after restimulation. (E) CCR7 MFI of PSTeff and NTeff after restimulation. Turkey test was used to determine statistical significance. ***$p < 0.001$, *$p < 0.05$. Error bars: s.e.m.

By depletion of nTreg, residual $CD4^{+}$ T cells were used as precursor cells to generate PSTreg. PSTreg can also be generated from these cells (Figure 4.1.14).

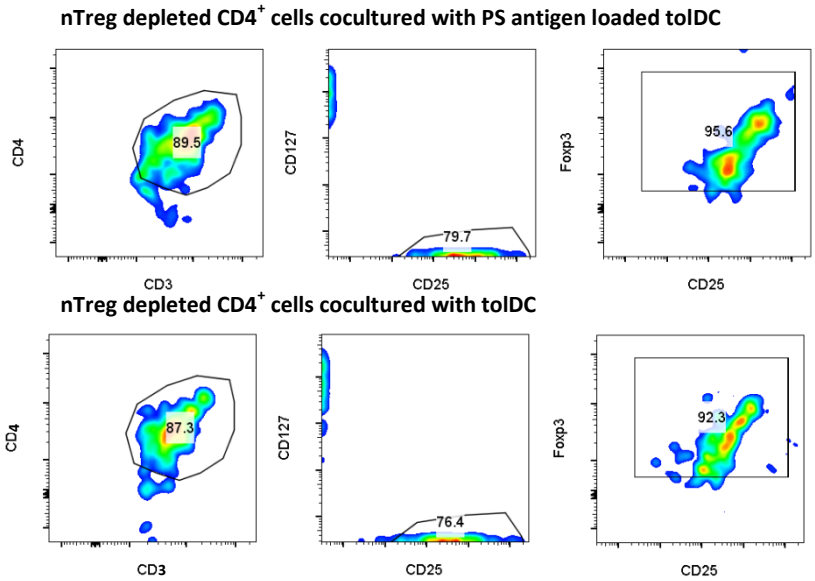


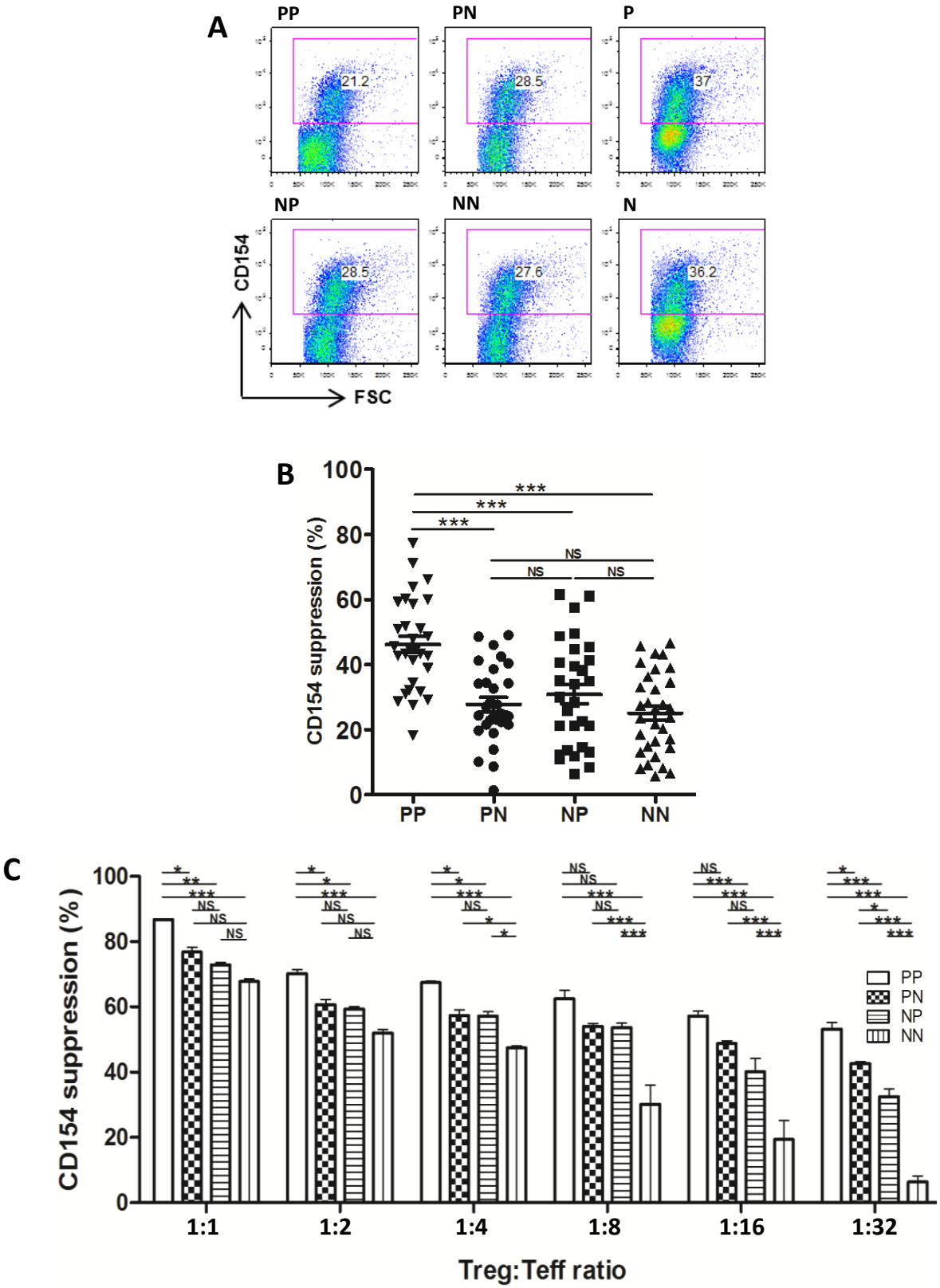
Figure 4.1.14 Generation of PSTreg using nTreg depleted CD4$^+$ cells as precursors. After depletion of nTreg, residual CD4$^+$ cells were cocultured with PS antigen loaded or mock loaded tolDC or C5-DC. After coculture for 10 days, the cells were harvested and phenotyped. Gating strategy of PSTreg and NTreg generated from nTreg depleted CD4$^+$ cell is shown.

These results confirmed that CD4$^+$CD25$^+$CD127$^{-/low}$Foxp3$^+$CD45RA$^-$ CCR7^{low}CCR4^{high}GARP^{high} PSTreg with high IL-10, TGF-β1 and IL-35 expression could be generated with our method.

4.1.4 PSTreg demonstrate specific immunosuppressive activity

CD154 is an early activation marker of Teff, which is expressed in the first few hours after Teff activation. After coculture of porcine-specific and non-specific Treg and Teff cells for 7 h, the cells were harvested and tested for CD154 expression in the PSTeff and NTeff cells by flow cytometry (Figure 4.1.15 A, B). Expression of CD154 was significantly suppressed in the PSTeff/PSTreg coculture group (PP) group compared with the other groups: CD154 expression in PSTeff was suppressed by nearly 50% in the PP group at ratio 1:1 (Figure 4.1.15 B). PSTreg specific suppressive function towards PSTeff was observed in different ratios, and still remained at a ratio of 1:32 (Figure 4.1.15 C). However, CD154 suppression of PN groups and NP groups showed no significant

difference at the different ratios, except ratio 1:32. In comparison to nTreg, PSTreg also exhibited a significant higher suppressive function towards PSTeff in the CD154 expression assay (Figure 4.1.15 D).



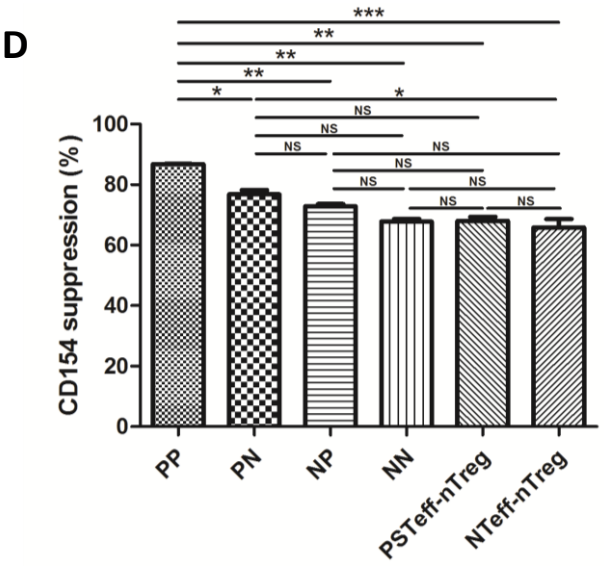
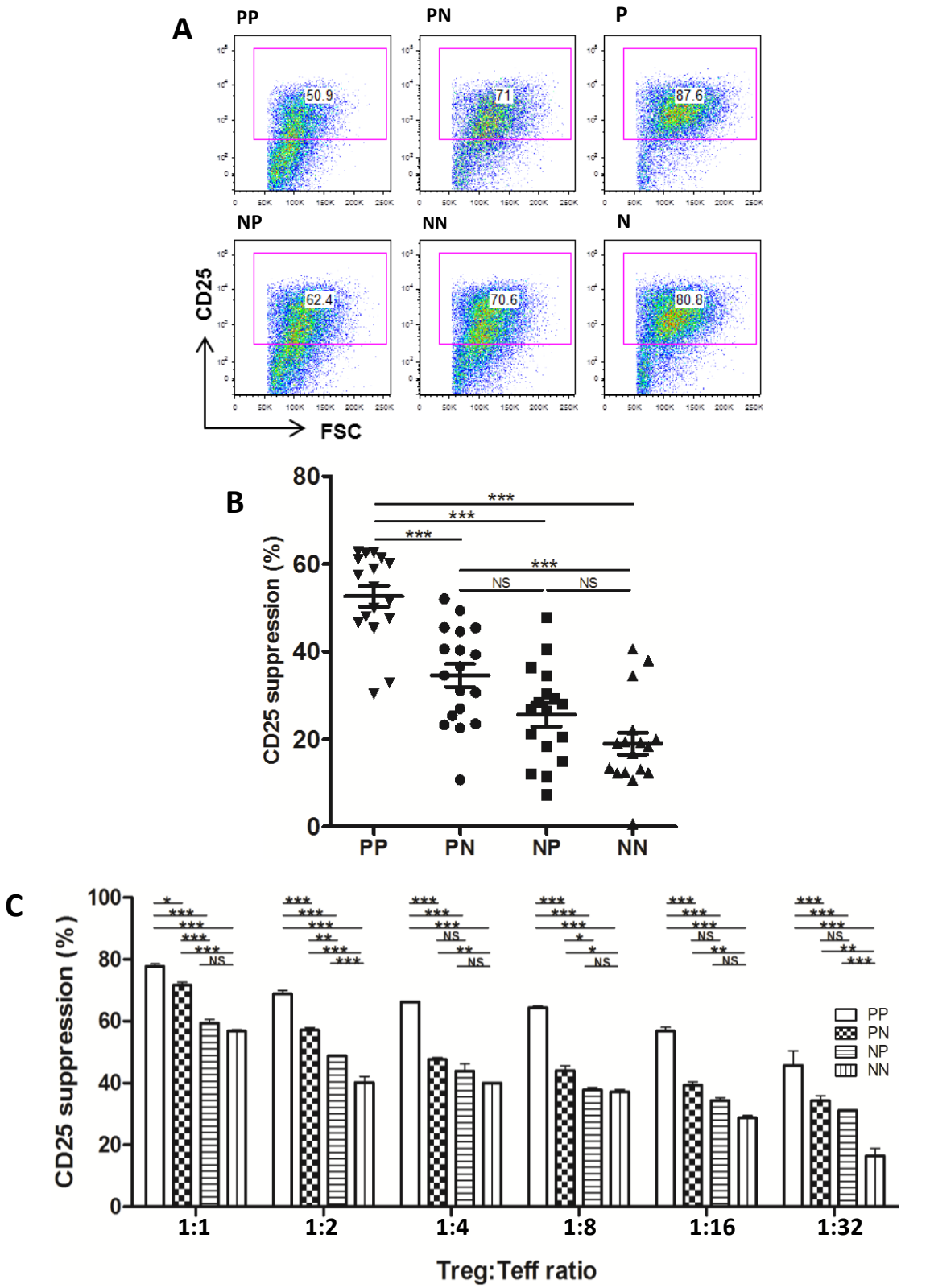


Figure 4.1.15 PSTreg show porcine-specific immunosuppressive activity after 7 h coculture. To determine the specificity of PSTreg, PS/NTreg and PS/NTeff were seeded in 96 U-well plates with CD3/CD28 microbeads in various ratios, NTeff and PSTeff were set up as control. After 7 h, cells were harvested and CD154 expression was measured by flow cytometry. A sample of Treg to Teff ratio at 1:1 is shown in (A), CD154 suppression ratio at Treg to Teff ratio at 1:1 is shown in (B), data represents 15 independent experiments from 8 donors. CD154 suppression ratio of Treg to Teff at different ratio is shown in (C), n=3. nTreg were used as control for PSTreg specificity: (D) suppression of CD154, n=3. ANOVA with Bonferroni's Multiple Comparison Test was used to determine the statistical significance. ***$p < 0.001$. **$p < 0.01$. *$p < 0.05$. NS, non-significant, $p > 0.05$. Error bars: s.e.m.

After 4 days of coculture, CD25, an intermediate activation marker of Teff, was measured to evaluate longer term regulatory function of PSTreg. As expected, the expression of CD25 was also suppressed significantly in the PP group compared to the other groups at different ratios, and PSTreg specific suppression was also retained at a ratio of 1:32 (Figure 4.1.16 A, B, C). However, PSTreg showed no significant suppressive function towards NTeff in the ratios 1:1, 1:4, 1:8, 1:16 (Figure 4.1.16 C). PSTreg also demonstrated a significant higher suppressive function towards PSTeff in the CD25 expression assay compared to nTreg (Figure 4.1.16 D).


A
PP
PN
P
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NN
N
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71
87.6
62.4
70.6
80.8
CD25
FSC
B
CD25 suppression (%)
PP
PN
NP
NN
NS
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CD25 suppression (%)
Treg:Teff ratio
1:1
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1:4
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PP
PN
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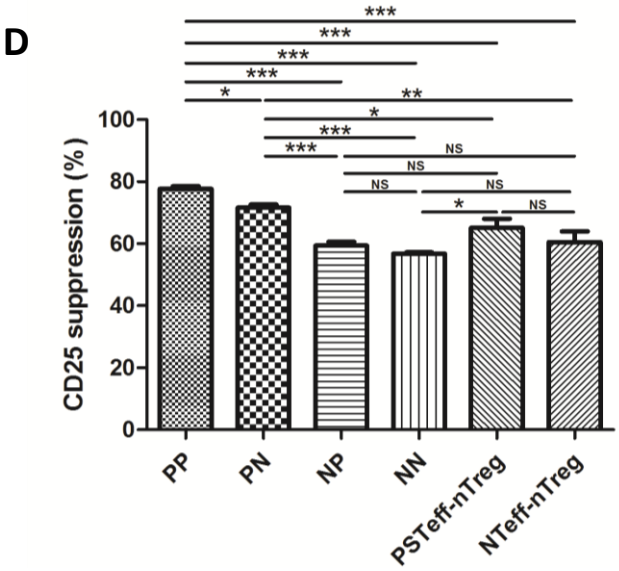
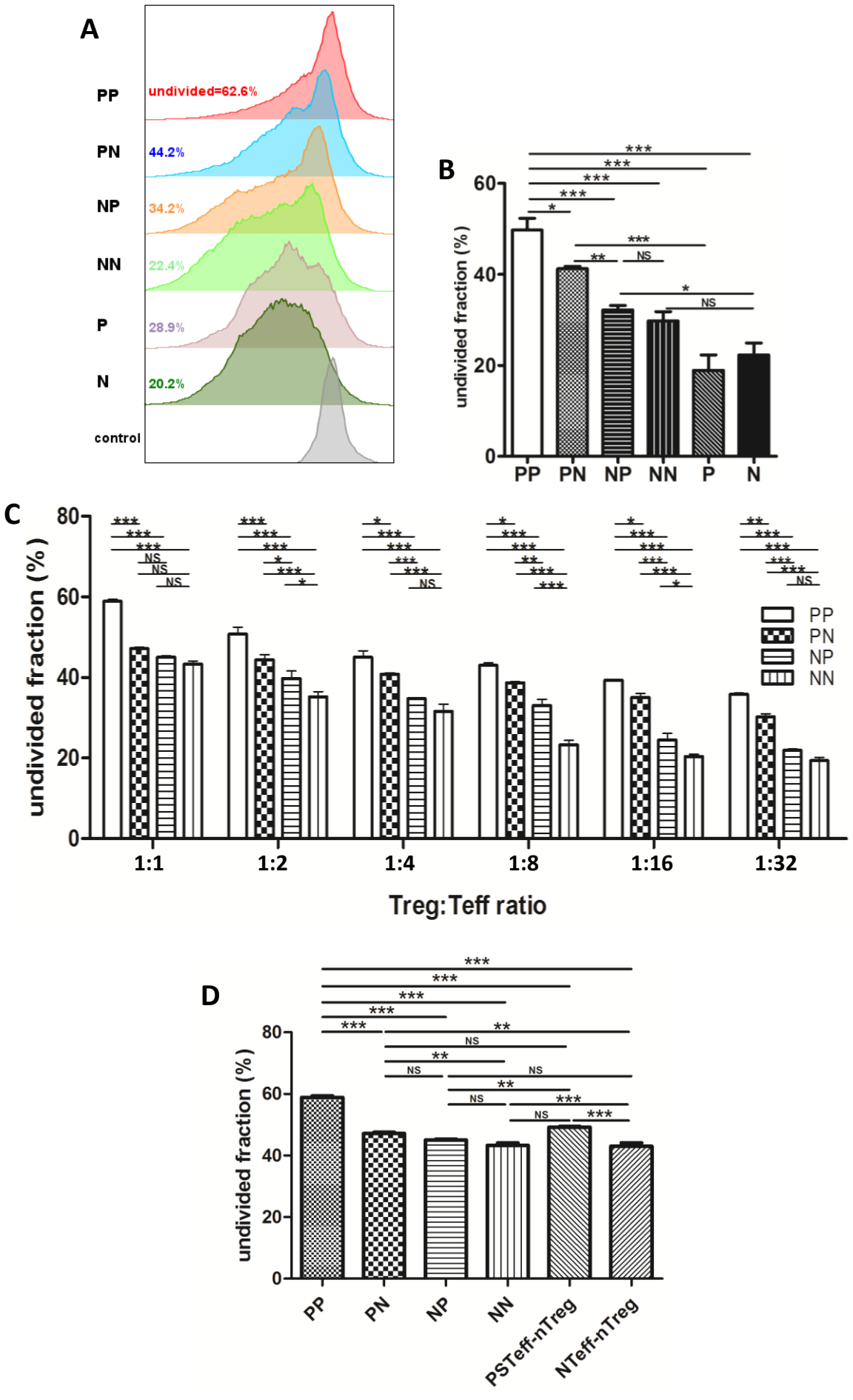


Figure 4.1.16 PSTreg show porcine-specific immunosuppressive activity after 96 h coculture. To determine the specificity of PSTreg generated with our method, PS/NTreg and PS/NTeff were seeded in 96 U-well plates with CD3/CD28 microbeads in different ratios, NTeff and PSTeff were set up as control. After 96 h, the expression (A) and suppression ratio (B) of CD25 on Teff at Treg to Teff ratio at 1:1 were measured using flow cytometry. Data represents 10 independent experiments of 6 donors. CD25 suppression ratio of Treg to Teff at different ratio is shown in (C), n=3. nTreg were used as control for PSTreg specificity: (D) suppression of CD25, n=3. ANOVA with Bonferroni's Multiple Comparison Test was used to determine the statistical significance. ***$p < 0.001$. **$p < 0.01$. *$p < 0.05$. NS, non-significant, $p > 0.05$. Error bars: s.e.m.

Likewise, in the T cell proliferation assay (Figure 4.1.17 A, B, C), the undivided population of Teff in the PP group was significantly higher than in the other groups at different ratios, which indicated that the proliferation of PSTeff was inhibited by PSTreg. As demonstrated above, PSTeff proliferation was significantly inhibited by PSTreg than by nTreg (Figure 4.1.17 D).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PN
44.2%
NP
34.2%
NN
22.4%
P
28.9%
N
20.2%
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B
undivided fraction (%)
PP
PN
NP
NN
P
N
C
undivided fraction (%)
PP
PN
NP
NN
1:1
1:2
1:4
1:8
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1:32
Treg:Teff ratio
D
undivided fraction (%)
PP
PN
NP
NN
PSTeff-nTreg
NTeff-nTreg

Figure 4.1.17 PSTreg suppress PSTeff proliferation. After coculture of PS/NTreg and PS/NTeff together with CD3/CD28 microbeads for 96 h, Teff proliferation was evaluated using the CellTrace™ CFSE Cell Proliferation Kit, an example is shown in (A) and the undivided ratio is shown in (B) at Treg to Teff ratio at 1:1. Data represents 8 independent experiments of 5 donors. Undivided ratio of Treg to Teff at different ratios is shown in (C), n=3. nTreg were used as control for PSTreg specificity: (D) shows Teff undivided fraction in the Teff proliferation assay, n=3. Bonferroni's Multiple Comparison Test was used to determine the statistical significance. ***$p < 0.001$. **$p < 0.01$. *$p < 0.05$. NS, non-significant, $p > 0.05$. Error bars: s.e.m.

Restimulated PSTreg kept their specific suppressive function towards PSTeff with respect to the activation markers CD154 and CD25 (Figure 4.1.18 A, B).

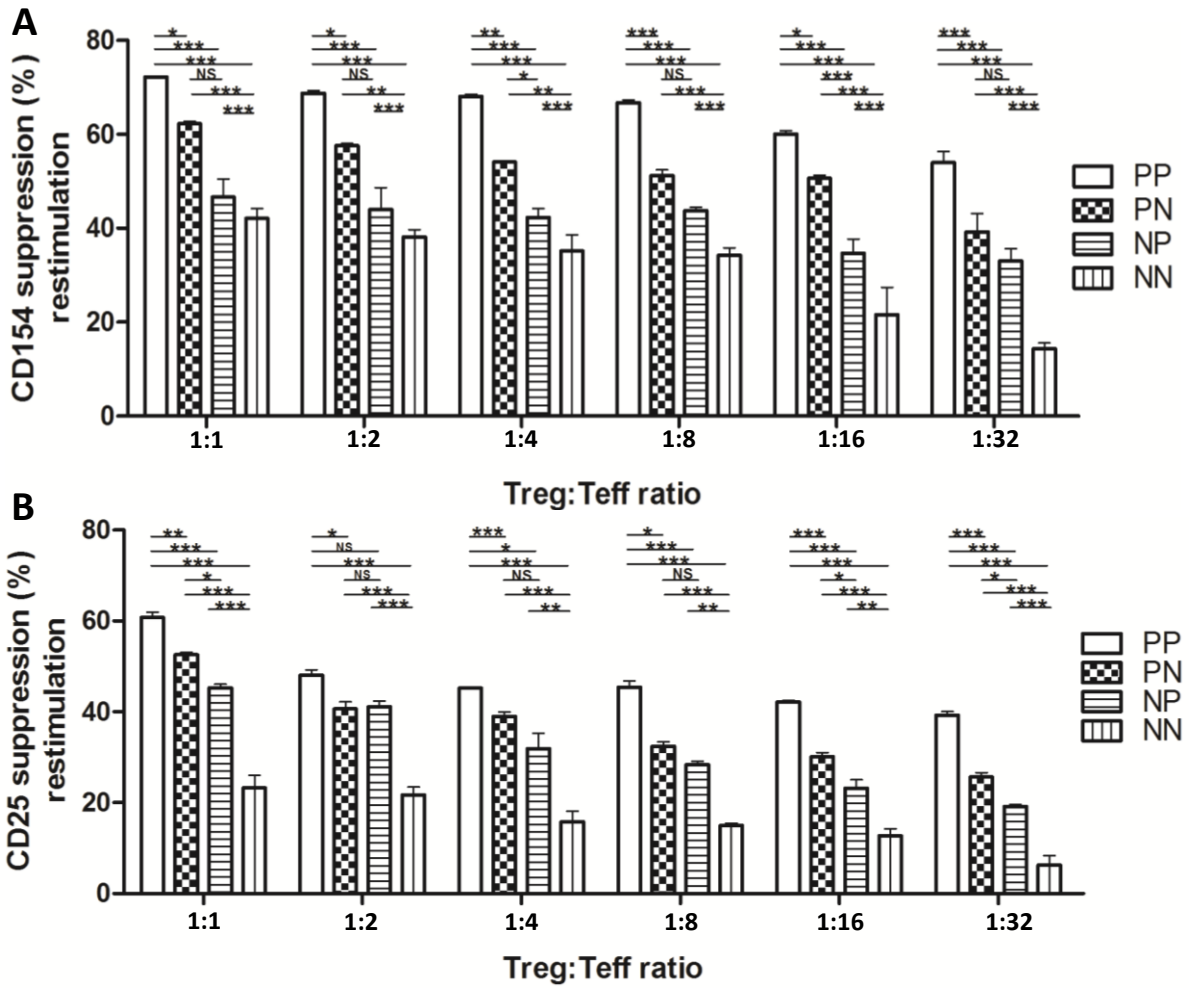


Figure 4.1.18. PSTreg retained specificity after restimulation. PSTreg, NTreg and PSTeff, NTeff were harvested after cocultured with DC for 10 days. Then PSTreg and NTreg were purified, and all cells were restimulated again with DC for 10 days. The specificity was tested by suppression of Teff activation marker at different ratios after restimulation, (A) CD154 suppression, (B) CD25 suppression. n=3. ANOVA with Bonferroni's Multiple Comparison Test

was used to determine the statistical significance. ***$p < 0.001$. **$p < 0.01$. *$p < 0.05$. NS, non-significant, $p > 0.05$. Error bars: s.e.m.

PSTreg generated from nTreg depleted residual $CD4^+$ cells showed no significant difference in suppressive function (Figure 4.1.19).

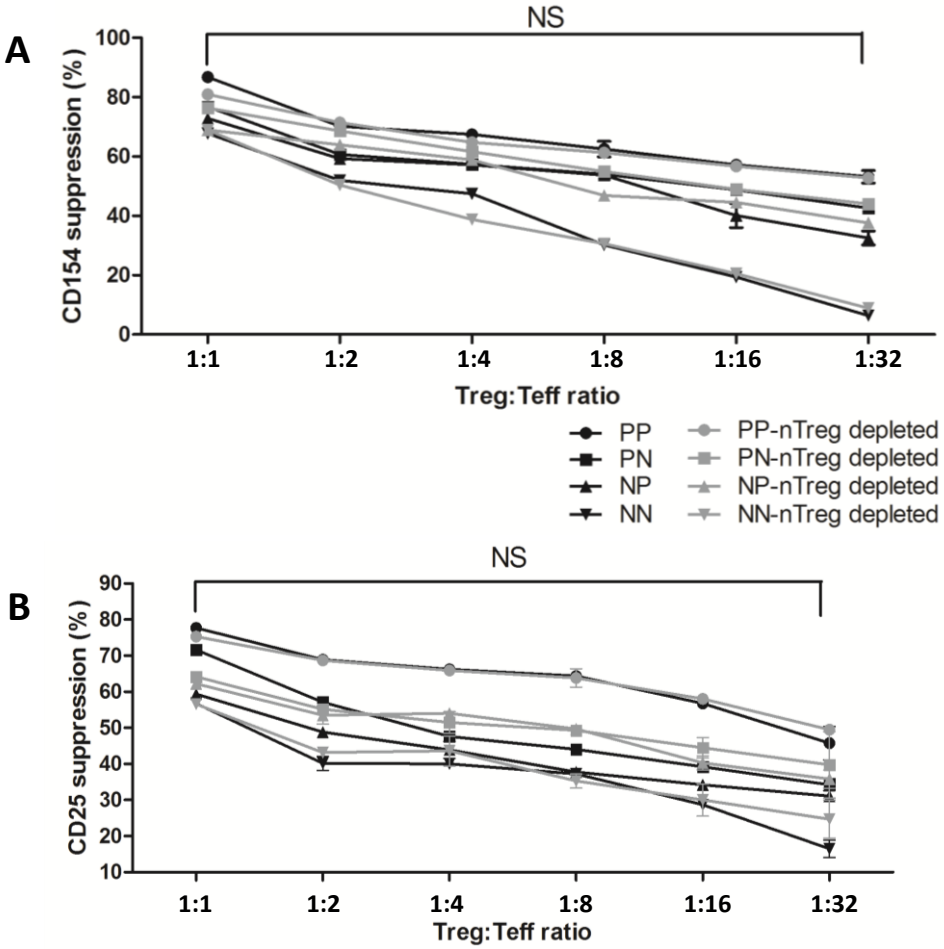


Figure 4.1.19: Generation of PSTreg using nTreg depleted $CD4^+$ cells as precursors. After depletion of nTreg, remaining $CD4^+$ cells were cocultured with PS antigen loaded or mock-loaded tolDC or C5-DC. After coculture for 10 days, suppression assays were set up and the comparison of nTreg depleted and non-depleted $CD4^+$ cell induced PSTreg function is shown in (A, B). Turkey test was used to determine the statistical significance.

These experiments revealed the high specificity of PSTreg towards PSTeff.

4.1.5 PSTreg express high amounts of IL-10, TGF-β1, and IL-35 after interaction with PSTeff

To further evaluate PSTreg functionality, cell supernatants were harvested after the functional assay to measure cytokine expression.

As shown in Figure 4.1.20, high IL-10 levels were observed already after 7 h coculture of PSTreg with PSTeff. After coculture for 96h, the amount of IL-10 in the supernatant increased slightly. In the PN, NP and NN coculture groups much lower levels of IL-10 were found.

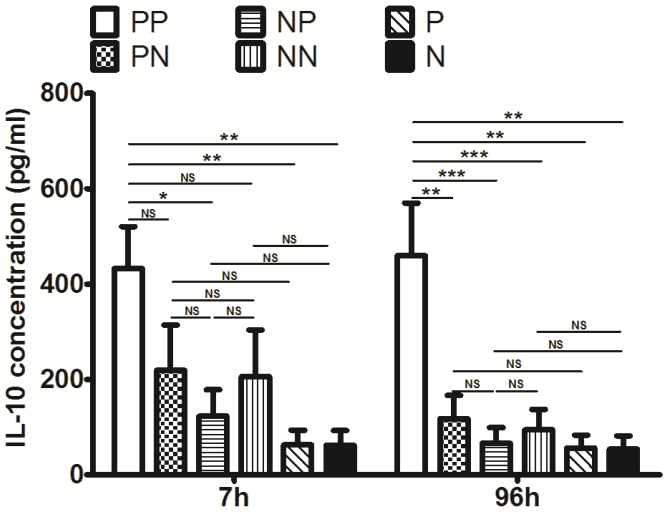


Figure 4.1.20 PSTreg express high amounts of IL-10. Supernatants were harvested at the 7 h and 96 h time point. IL-10 was quantified with BD™ CBA Flex Set and ELISA. Data represents 4-14 independent experiments from 4-8 donors. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, NS, non-significant, $p > 0.05$. ANOVA with Bonferroni's Multiple Comparison Test was used to determine the statistical significance. Error bars: s.e.m.

In the first 7 h, TGF-β1 secretion in the PP group was significantly higher than in the NN coculture group, and in P and N cells. At the 96 h time point, almost all groups produced increased amounts of TGF-β1 except the NP group. Although the PP group maintained the highest production of TGF-β1, this was not statistically significant (Figure 4.1.21).

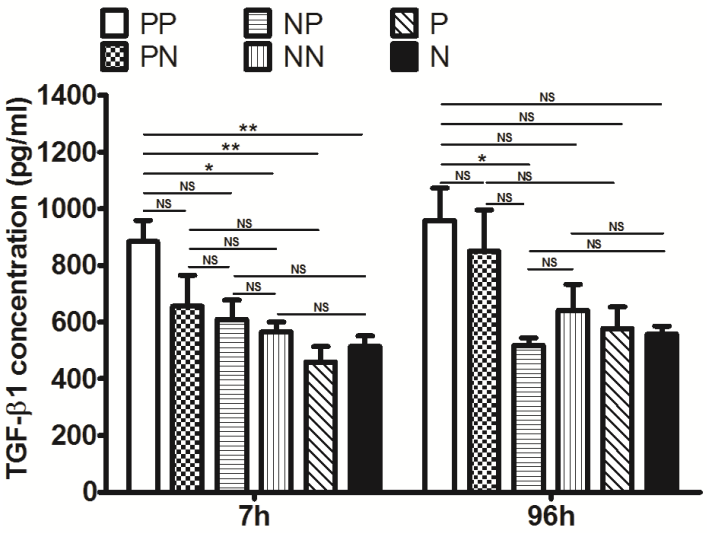


Figure 4.1.21 PSTreg express high amounts of TGF-β1. Supernatants were harvested at the 7 h and 96 h time point. TGF-β1 was quantified with BD™ CBA Flex Set and ELISA. Data represents 4-14 independent experiments from 4-8 donors. $^{**}p < 0.01$, $^{*}p < 0.05$, NS, non-significant, $p > 0.05$. ANOVA with Bonferroni's Multiple Comparison Test was used to determine the statistical significance. Error bars: s.e.m.

As expected, P and N cells secreted high amounts of IFN-γ after stimulation with CD3/CD28 beads. The production of IFN-γ was significantly inhibited in the PSTreg NTreg coculture group. In the first 7 h, PSTreg remarkably inhibited IFN-γ production of PSTeff, although not of statistical significance, while after 96 h incubation, all Treg with Teff coculture groups accumulated IFN-γ (Figure 4.1.22).

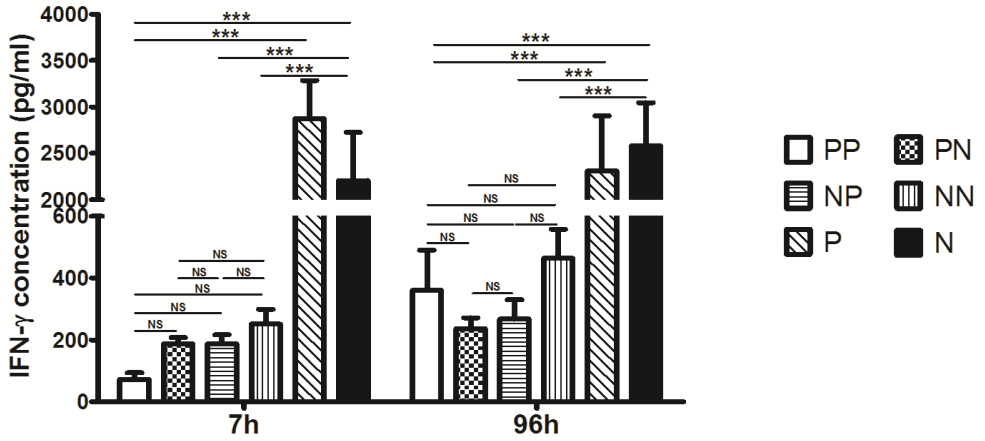


Figure 4.1.22 IFN-γ secretion during the functional assay. Supernatants were harvested at the 7 h and 96 h time point. IFN-γ was quantified with BD™ CBA Flex Set. Data represents 4-

14 independent experiments from 4-8 donors. ***$p < 0.001$, NS, non-significant, $p > 0.05$. ANOVA with Bonferroni's Multiple Comparison Test was used to determine the statistical significance. Error bars: s.e.m.

IL-17A and IL-12p70 secretion were also measured in all groups. IL-12p70 production was only observed in a few samples of P and N cells (data not shown). All coculture groups failed to secrete IL-17A and IL-12p70 after stimulation with CD3/CD28 beads (data not shown).

As expected, consistent with protein data, *IL-10* mRNA expression of the PP group was significantly higher compared to other groups (Figure 4.1.23). Relative expression of *TGFB1* mRNA in the PP group was significantly higher upregulated compared to the NN group, however, the relative high expression compared to PN and NP groups did not meet statistical significance.

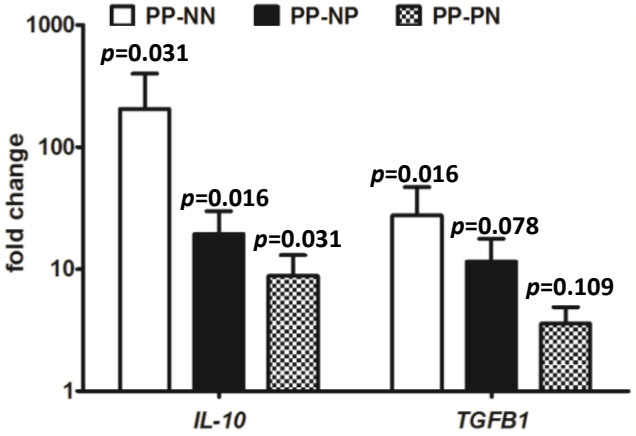


Figure 4.1.23 PSTreg express high levels of *IL-10* and *TGF-β1* mRNA after 96 h coculture. The cells of the functional assay were harvested, RNA was isolated and quantified by RT-PCR. Relative expression of *IL-10, TGFB1* mRNA in PP to other groups is shown, PN, NP, NN served as control. Data represents 7 independent experiments from 6 donors. *ACTB, G6PDH* or *CYPB* mRNA were used as endogenous reference gene for each sample. Wilcoxon signed-rank test was used to determine statistical significance. Error bars: s.e.m.

As shown in Figure 4.1.24, the expression of both *EBI3* and *IL-12A* mRNAs, encoding the two components of IL-35, was significantly upregulated in the PP group compared to the other groups. *IL-12A* mRNA was upregulated remarkably compared to *EBI3* mRNA. In contrast, *IL-27A* mRNA expression was not significantly upregulated in the PP group compared to the other groups, and in only 4 of the 7 Treg/Teff coculture samples *IL-27A* was expressed. We

also measured *IL-12B* mRNA expression, which was not detected in the Treg and Teff groups (data not shown).

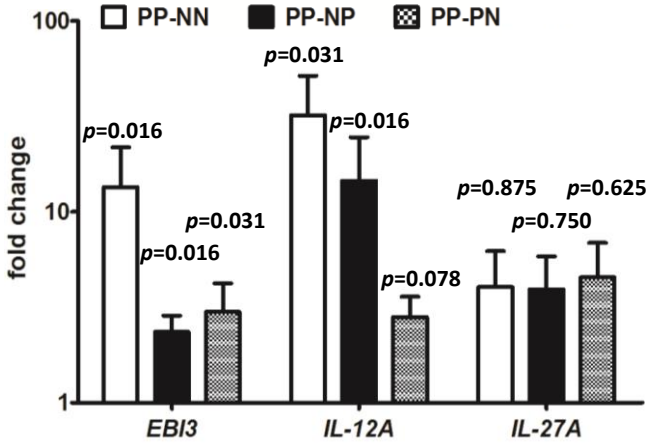


Figure 4.1.24 PSTreg express high levels of the IL-35 family related gene mRNA after 96 h coculture. The cells of the functional assay were harvested, RNA was isolated, and quantified by RT-PCR. Relative expression of *EBI3, IL-12A, IL-27A* related mRNA in PP to other groups is shown, PN, NP, NN served as control. Data represents 7 independent experiments from 6 donors. *ACTB, G6PDH* or *CYPB* mRNA was used as endogenous reference gene for each sample. Wilcoxon signed-rank test was used to determine statistical significance. Error bars: s.e.m.

These results indicate that all three anti-inflammatory cytokines were upregulated at the mRNA level.

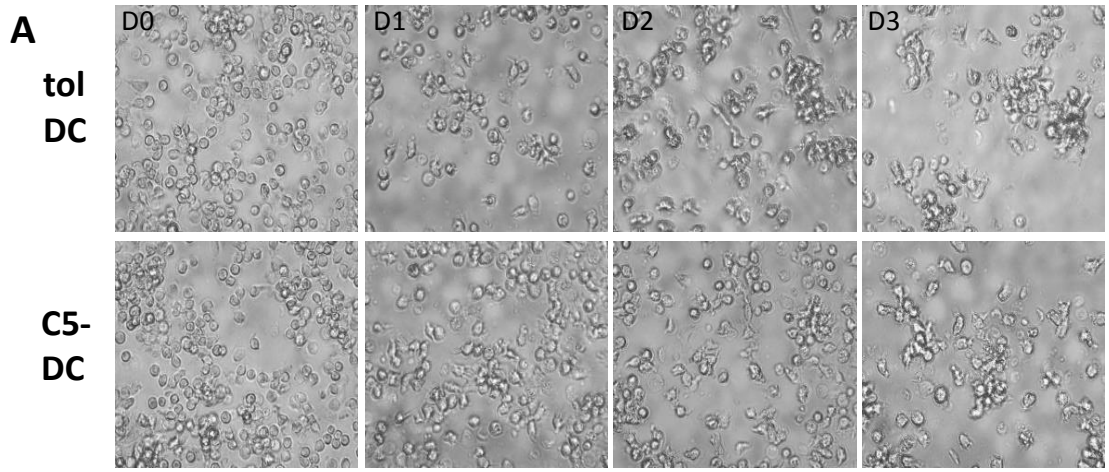
4.2 Generation of tolDC and PSTreg in the baboon system

4.2.1 TolDC express IL-10, TGF-β1, IL-35, IL-27, B7-H1, and B7-DC

In order to characterize baboon tolDC generated with the novel 3-day method, phenotype and cytokine secretion at protein and RNA levels compared to C5-DC were demonstrated in this study.

As shown in Figure 4.2.1 A, at day 0, cells maintained round shape in culture. The cells developed into dendritic shape on day 1 after culture with GM-CSF and IL-4 for 24 h. At day 2, tolDC developed less branched projections than non-tolerogenic C5-DC after adding IL-10 and TGF-β1 to the culture. Following maturation at day 3, tolDC developed less branched projections than C5-DC.

As expected, tolDC expressed significantly lower levels of CD83 compared with C5-DC. In addition, CD80 was less well expressed by tolDC compared to C5-DC, with respect to MFI. In contrast, significantly higher levels of B7-H1 and B7-DC expression were detected on tolDC (Figure 4.2.1 B-C). CCR7 was expressed on both tolDC and C5-DC (Figure 4.2.1 D).



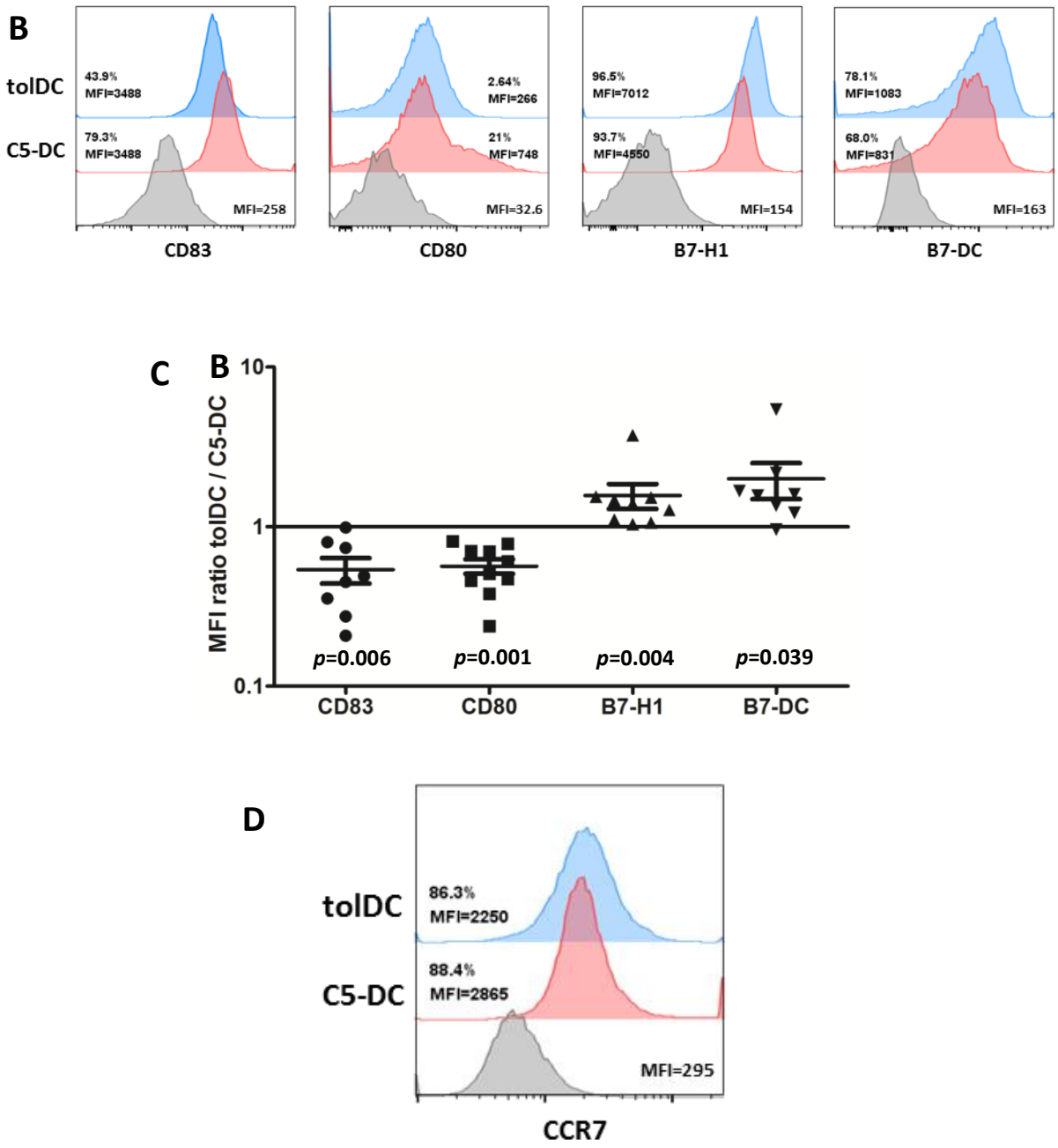


Figure 4.2.1 Baboon DC generated from monocytes with anti-inflammatory cytokines exhibit a tolerogenic phenotype. (A) Microscope photographs during DC generation. Blue lines represent tolDC, red lines represent non-tolDC, grey lines represent unstained control. (B) Phenotype analysis was done using flow cytometry. (C) shows dot chart of MFI ratio. Data represent 9 independent experiments from 6 baboons. Wilcoxon signed-rank test was used to determine the p values. Error bars: s.e.m. (D) CCR7 expression on tolDC and C5-DC. Data represents 2 independent experiments from 2 baboons.

The baboon-derived tolDC produced significantly more IL-10 at the protein level when compared to C5-DC. On the other hand, C5-DC culture supernatant

contained high amounts of IL-12p40 whereas tolDC produced no detectable IL-12p40 (Figure 4.2.2).

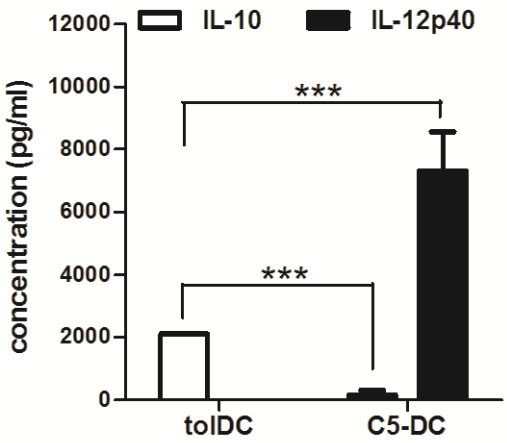


Figure 4.2.2 Baboon tolDC generated by our fast 3-day method produce anti-inflammatory cytokines. IL-10 and IL-12p40 quantification was done by ELISA. These data represent 6 independent experiments from 5 baboon donors. Turkey Test was used to determine the statistical significance. ***$p < 0.001$. Error bars: s.e.m.

Consistently, *IL-10* mRNA was significantly upregulated in all samples of baboon-derived tolDC prepared from different animals relative to C5-DC (Figure 4.2.3). However, only half of the different samples showed elevated *TGFB1* mRNA in tolDC relative to C5-DC (Figure 4.2.3). With respect to IL-35-related genes (Figure 4.2.3), an upregulation of *IL-12A* mRNA in tolDC was observed, although the data meet no statistical significance (p=0.063). The similar trend was also observed for *EBI3* mRNA, which was upregulated in most samples of tolDC. *IL-27A* mRNA was significantly upregulated in all tolDC samples of different baboons. Consistent with protein levels, *IL-12B* mRNA was found to be downregulated in tolDC compared to C5-DC in all samples, although again the data meet no statistical significance (p=0.063).

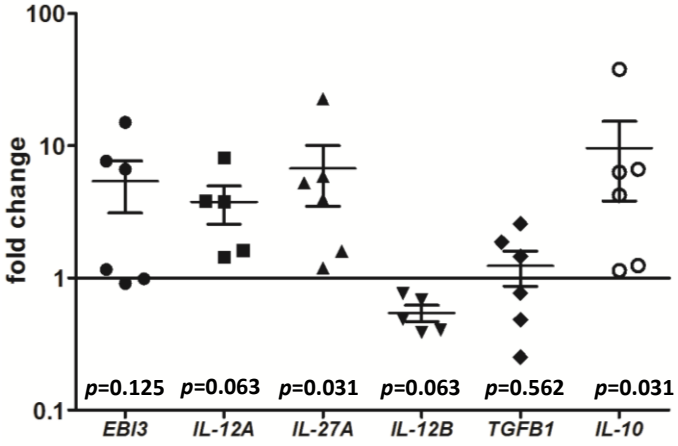


Figure 4.2.3 Baboon tolDC upregulated anti-inflammatory cytokine mRNA. RNA was isolated from tolDC and C5-DC, *EBI3, IL-12A, IL-27A, IL-12B*, *IL-10*, *TGFB1* mRNA were quantified by RT-PCR. *GAPDH* in each sample was used as endogenous reference gene. These data represent 6 independent experiments from 5 baboons. Wilcoxon signed-rank test was used to determine the p values. Error bars: s.e.m.

4.2.2 Baboon DC electroporation with porcine ivtRNA

Following electroporation of PS ivtRNA into tol/C5-DC, porcine MHC-I was used again as a surrogate marker to evaluate PS ivtRNA expression in DC after 24 h incubation. As shown in Figure 4.2.4, porcine MHC-I expression was detectable at 24 h after electroporation in both types of DC.

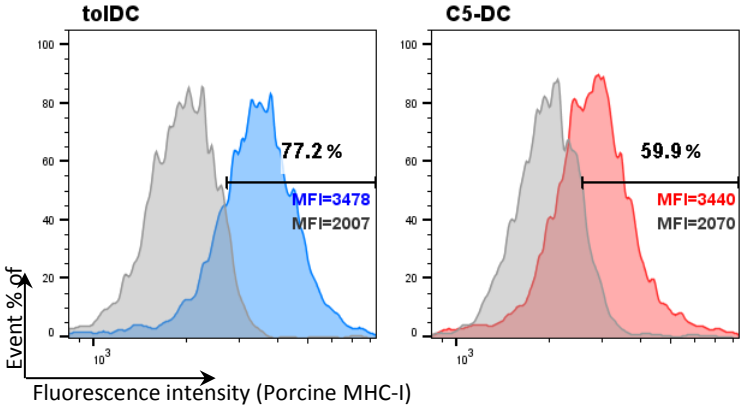
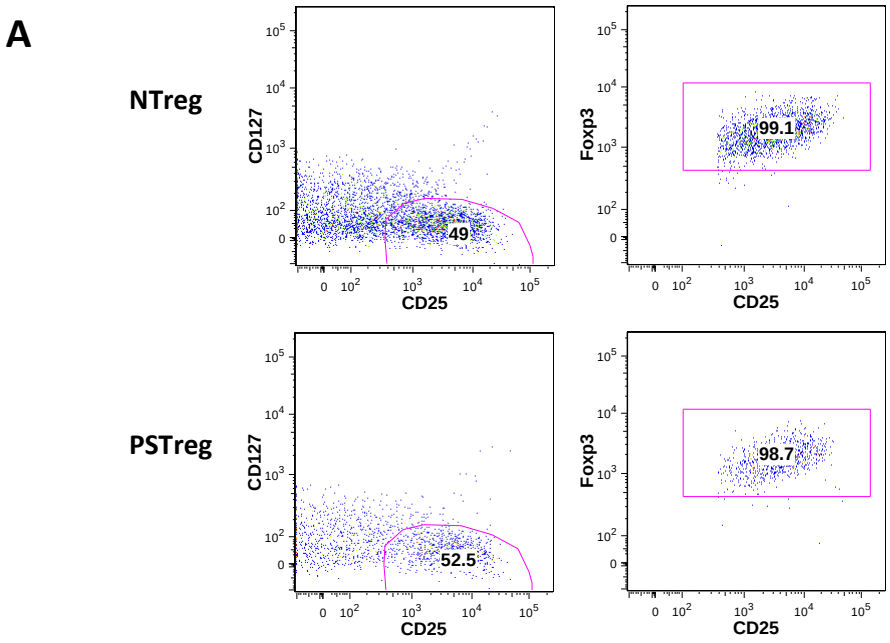


Figure 4.2.4 TolDC and C5-DC successfully express porcine antigen following electroporation of PS ivtRNA. Porcine MHC-I expression was analyzed by flow cytometry, mock-electroporated tolDC and C5-DC were used as control (grey peak).

4.2.3 Baboon PSTreg and PSTeff can be generated with baboon tolDC and C5-DC, respectively

Baboon PSTreg were induced from naïve $CD4^+$ T cells by coculture with porcine antigen-loaded tolDC in medium supplemented with a high concentration of IL-2 and rapamycin. In parallel, baboon PSTeff were generated from cocultures of naïve $CD4^+$ positive T cells with porcine antigen-loaded C5-DC, in medium supplemented with a lower concentration of IL-2.

Baboon PSTreg and NTreg generated using tolDC showed a similar phenotype as conventional human Treg: $CD3^+CD4^+CD25^+CD127^{low/-}Foxp3^+$ (Figure 4.2.5 A). After coculture for 10 days with PS ivtRNA-loaded baboon-derived tolDC supplemented with IL-2 and rapamycin, the yield of PSTreg was significantly greater than that found for NTreg induced in cocultures with baboon-derived tolDC not loaded with porcine antigen (Figure 4.2.5 B). Furthermore, PSTreg exhibited a $CD45RA^{low}$ phenotype compared with NTreg which displayed a $CD45RA^{high}$ phenotype (Figure 4.2.5 C, D).



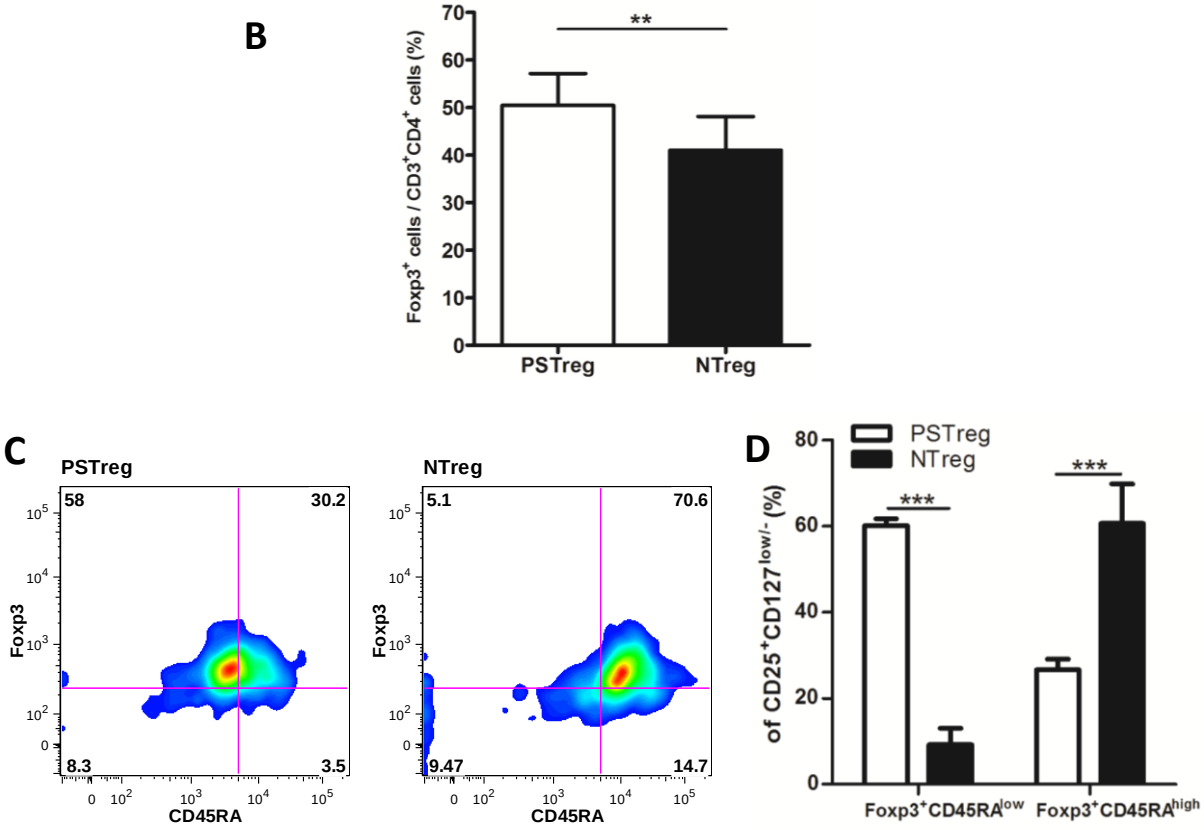
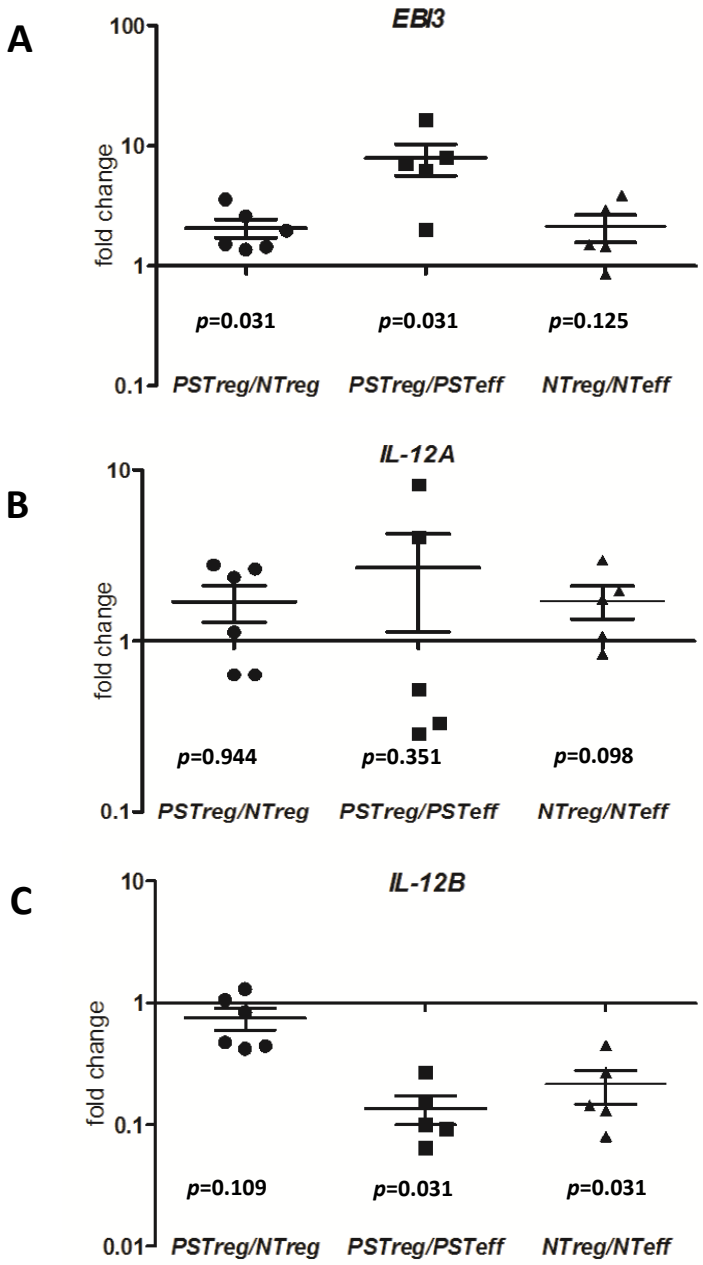


Figure 4.2.5 Baboon PSTreg can be generated using porcine-antigen-loaded baboon tolDC. After coculture of $CD4^+$ T cells with PS ivtRNA loaded tolDC or mock-loaded tolDC in medium supplemented with IL-2 and rapamycin for 10 days, cells were harvested and phenotyped using flow cytometry. Gating strategy of $CD25^+CD127^{low/-}Foxp3^+$ population from $CD3^+CD4^+$ gated cells is shown in (A). The percentage of $CD3^+CD4^+CD25^+CD127^{low/-}Foxp3^+$ Treg in $CD3^+CD4^+$ T cells is shown in (B). 7 independent experiments from 5 baboons. Expression of Foxp3 and CD45RA, gated out from the $CD3^+CD4^+CD25^+CD127^{low/-}$ population of PSTreg and NTreg is shown in (C). A bar chart demonstrating the $Foxp3^+CD45RA^{high}$ and $Foxp3^+CD45RA^{low}$ population is shown in (D). Data represent 2 individual experiments from 2 baboons (n=3). ANOVA with Bonferroni's Multiple Comparison Test and turkey test were used to determine the statistical significance. $^{**}p < 0.01$. $^{***}p < 0.001$. Error bars: s.e.m.

Concerning the IL-35 gene family members (Figure 4.2.6), *EBI3* mRNA of all samples of different baboons was significantly upregulated in PSTreg compared with NTreg and PSTeff. We detected 1.01- and 7.93-fold higher expression of *EBI3* mRNA than in NTreg and PSTeff, respectively. However, the expression of *IL-12A* mRNA was not significantly upregulated in PSTreg in general, which was 0.67- and 1.70-fold higher than in NTreg and PSTeff, 2 out of 6 samples and 3 out of 5 samples showed downregulation in comparison to NTreg and PSTeff, respectively. *IL-27A* mRNA in PSTreg, on the other hand, was maintained

almost at the same level as in NTreg, and was downregulated in all samples compared to PSTeff, although again the data meet no statistical significance. *IL-12B* mRNA in PSTreg was maintained almost at the same level and showed a slight decrease compared with NTreg. As expected, PSTeff expressed significantly more *IL-12B* mRNA than PSTreg. Similar to PSTreg, NTreg showed upregulation of *EBI3* and *IL-12A* mRNA, downregulation of *IL-27A* mRNA expression, although without statistical significance, but a significant downregulation of *IL-12B* mRNA compared to NTeff.



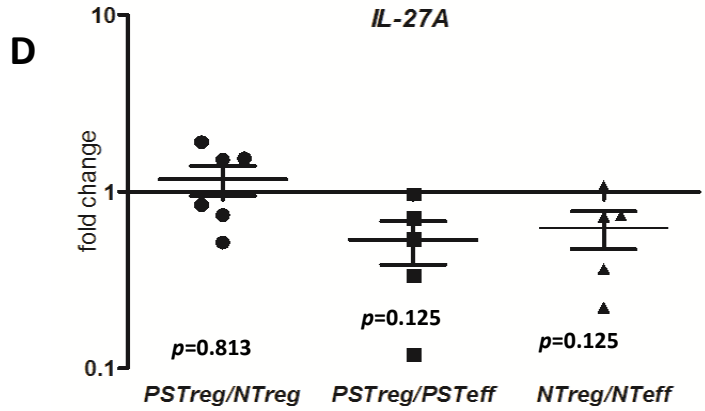


Figure 4.2.6 Baboon PSTreg express high amounts of *EBI3* mRNA towards PSTeff. *EBI3* (A), *IL-12A* (B), *IL-12B* (C) and *IL-27A* (D) mRNA were quantified by RT-PCR. NTreg and PSTeff served as control for PSTreg, NTeff as control for NTreg. *GAPDH* mRNA was used as endogenous reference gene for each sample. Data represent 5-6 independent experiments from 5-6 baboons. Wilcoxon signed-rank test was used to determine the p values.

IL-10 was quantified in the supernatants of cultured porcine-specific and non-specific Treg/Teff (Figure 4.2.7). Results showed that PSTreg supernatants contained significantly higher levels of IL-10 than PS/N Teff supernatants. Although the results did not meet statistical significance, NTreg produced less IL-10 than PSTreg at the protein level.

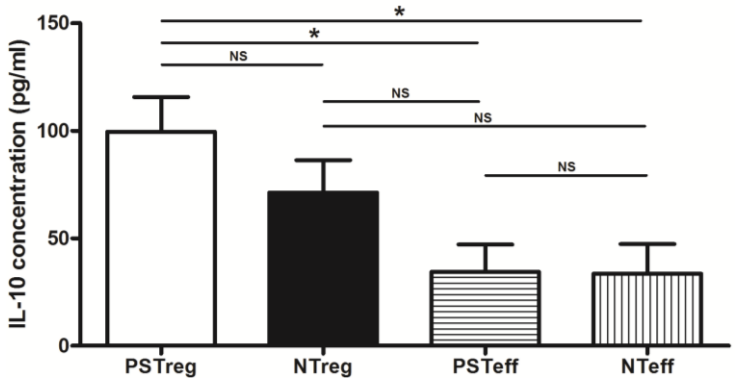


Figure 4.2.7 Baboon PSTreg secrete high amounts of IL-10. Supernatants of PS/N Treg/Teff were harvested to evaluate IL-10 protein level by ELISA. 7 independent experiments from 5 baboons. ANOVA with Bonferroni's Multiple Comparison Test was used to determine the statistical significance. *$p < 0.05$, NS, non-significant, $p > 0.05$. Error bars: s.e.m.

Consistent with the protein expression data, PSTreg showed significantly elevated expression of *IL-10* mRNA compared with NTreg and PSTeff, respectively. NTreg also upregulated *IL-10* mRNA compared to NTeff in most samples (Figure 4.2.8 A). *TGFB1* mRNA was significantly elevated in PSTreg relative to NTreg and PSTeff (4.56- and 16.48-fold higher than PSTreg, respectively). NTreg also expressed more *TGFB1* mRNA compared to NTeff (p=0.063) (Figure 4.2.8 B).

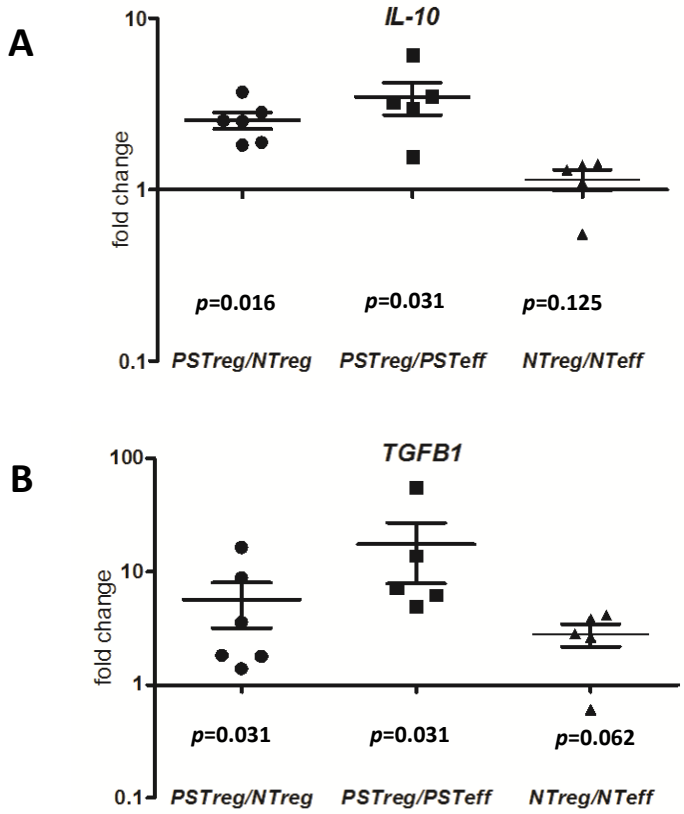


Figure 4.2.8 Baboon PSTreg express high amounts of *IL-10* and *TGFB1* mRNA. *IL-10* (A) and *TGFB1* (B) mRNA were quantified in PSTreg and NTreg by RT-PCR. NTreg and PSTeff served as controls for PSTreg, NTeff as control for NTreg. *GAPDH* mRNA was used as endogenous reference gene for each sample. Data represent 5-6 independent experiments from 5-6 baboons. Wilcoxon signed-rank test was used to determine the p values.

As a marker of activated Treg and as a receptor associated with latent TGF-β expression on the Treg plasma membrane, *GARP* mRNA was significantly higher expressed in PSTreg than in NTreg and PSTeff, respectively. NTreg also showed an upregulation compared to NTeff, which expressed 0.51-fold more *GARP* mRNA than NTeff although without statistical significance (Figure 4.2.9).

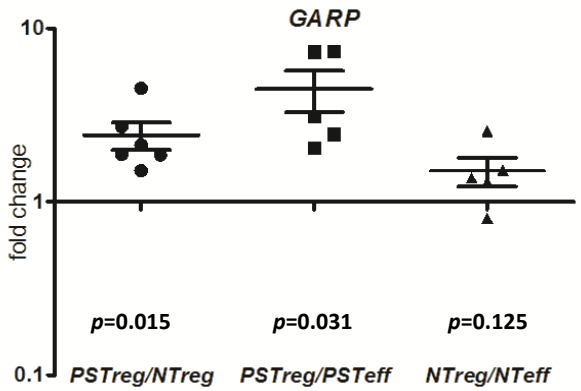


Figure 4.2.9 PSTreg express high amounts of *GARP* mRNA. *GARP* mRNA was quantified by RT-PCR in PSTreg and NTreg. NTreg and PSTeff served as controls for PSTreg, NTeff as control for NTreg. *GAPDH* mRNA was used as endogenous reference gene for each sample. Data represent 5-6 independent experiments from 5-6 baboons. Wilcoxon signed-rank test was used to determine the p values.

4.2.4 Baboon PSTreg showed specific immunosuppressive activity

After coculture of PSTeff and PSTreg (PP) for 7 h together with CD3/CD28 beads, cells were harvested and tested for the early activation marker CD154 on PSTeff (Figure 4.2.10 A, B). PSTeff and NTreg (PN), NTeff and PSTreg (NP), NTeff and NTreg (NN) were set up as Treg/Teff coculture controls. NTeff and/or PSTeff (C) together with CD3/CD28 beads were set up as Teff controls. Results showed that PSTreg significantly suppressed PSTeff CD154 expression: 72.60±3.341% of the mean of CD154 expression was suppressed in PSTeff after interaction with PSTreg for 7 h. However, the CD154 expression in NTeff was not suppressed significantly by PSTreg in comparison to the suppression by NTreg, suppression ratios of which were 48.67±4.731% and 43.46±3.994%, respectively. CD154 suppression ratio of PSTeff was 52.34±5.694% after interaction with NTreg.

Furthermore, high IL-10 levels were observed after 7 h following coculture of PSTreg and PSTeff (Figure 4.2.10 C). The secretion of IL-10 was 4.31-, 4.50- and 4.53-fold higher than in the PN, NP and NN groups. We also detected IL-10 in 2 out of 5 samples of the Teff control groups, however, in mean it was only 6.64±4.019 pg/ml (Figure 4.2.10 C).

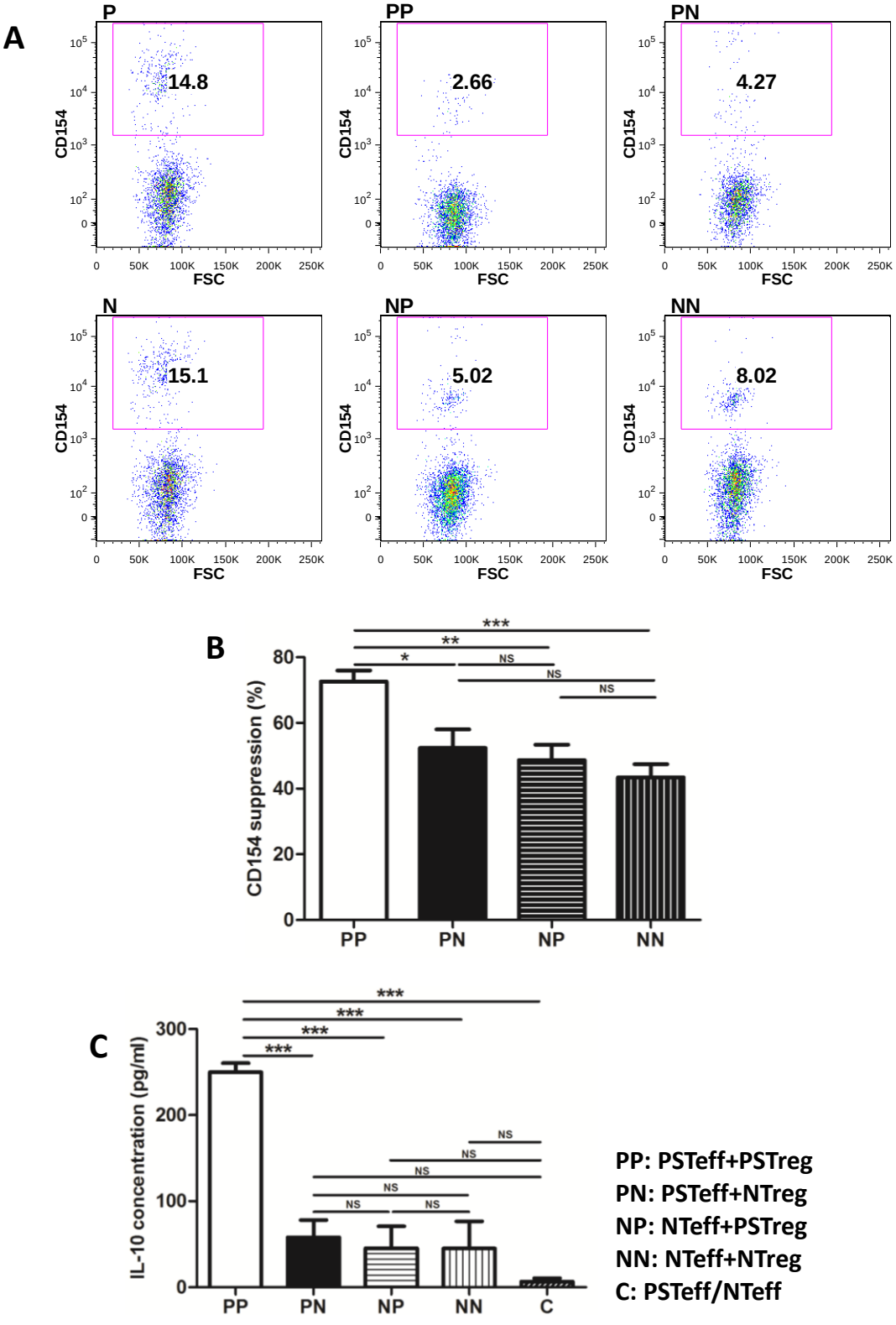
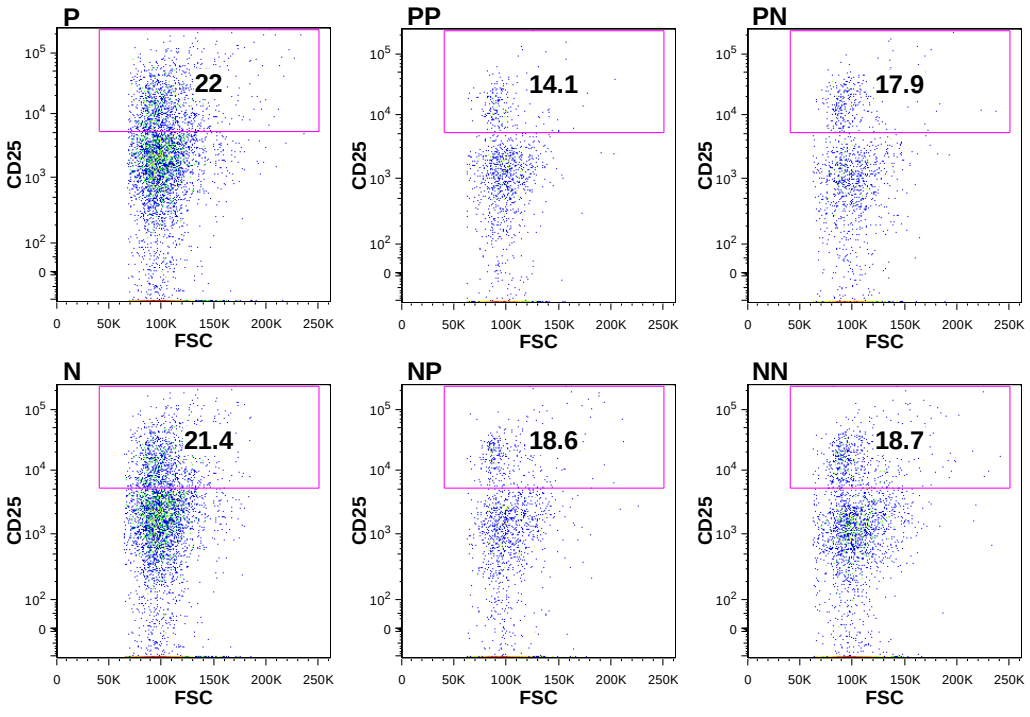


Figure 4.2.10 Baboon PSTreg showed specific immunosuppressive activity towards CD154 expression on PSTeff and expressed high amounts of IL-10 after 7 h coculture. PS/N Treg/Teff were seeded in 96 well plates (ratio 1:1) and stimulated with CD3/CD28 beads. After incubation for 7 h, cells were harvested to measure CD154 expression by flow cytometry, an example is shown in (A), and the suppression ratio is shown in (B). Data represent 5 independent experiments from 5 baboons. IL-10 was quantified in the

supernatant by ELISA (C). Data represent 5 independent experiments from 5 baboons. ANOVA with Bonferroni's Multiple Comparison Test was used to determine the statistical significance. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, NS, non-significant, $p > 0.05$. Error bars: s.e.m.

CD25, as an intermediate activation marker of Teff, was tested for expression in PS/NTeff after interaction with PS/NTreg for 4 days (Figure 4.2.11 A, B). 39.73±4.282% of CD25 expression in mean of PSTeff was suppressed by PSTreg, which was significantly higher than in the other groups. NTreg only suppressed 25.63±3.508 % of CD25 expression in PSTeff. PSTreg showed no specific suppressive function of NTeff: PSTreg only suppressed 22.86±3.095% of CD25 expression in NTeff, and there was no significant difference compared to a 20.41±2.769% suppression ratio of CD25 in NTeff interacting with NTreg. IL-10 cytokine accumulated in cocultures of all groups (Figure 4.2.11 C). The culture medium of the PP group contained the highest amount of IL-10 compared to the other groups. Consistently, the IL-10 level in the NP group was not significantly higher than in the NN group. In the PS/NTeff control group (C), the level of IL-10 also increased from 6.64±4.019 pg/ml at 7 h to 86.15±52.625 pg/ml at 96 h.

A



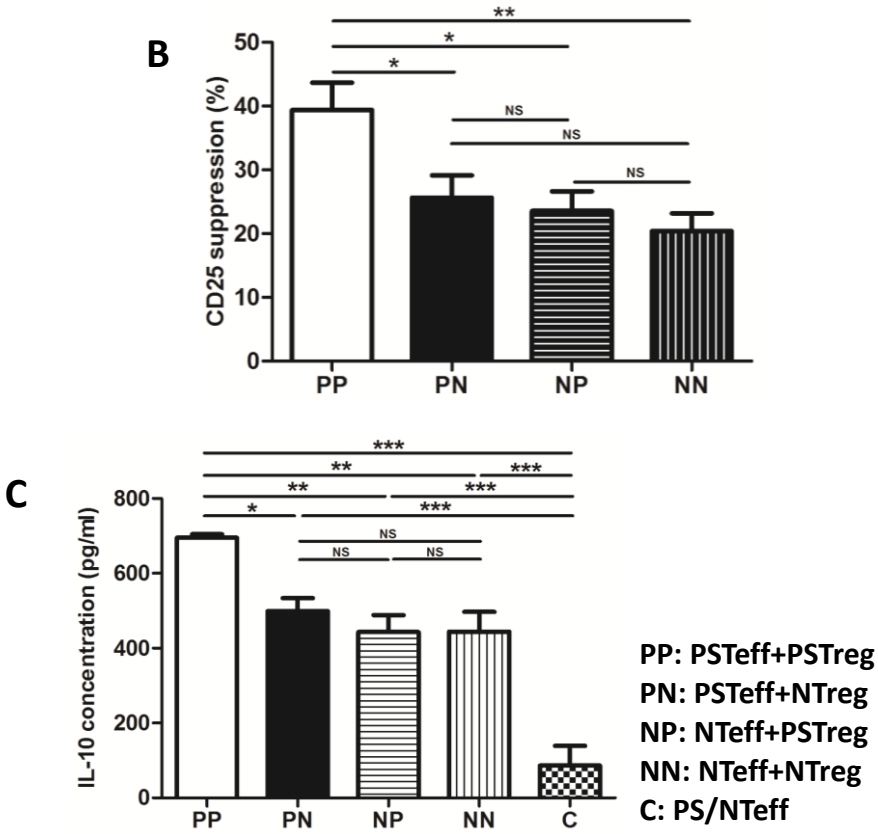


Figure 4.2.11 Baboon PSTreg showed specific immunosuppressive activity towards CD25 expression on PSTeff and expressed high amounts of IL-10 after 4 days coculture. After incubation for 4 days, cells were harvested to analyze CD25 expression. An example is shown in (A), suppression ratio is shown in (B). Data represent 4 independent experiments from 4 baboons. IL-10 was quantified in the supernatant after 4 day coculture by ELISA (C). Data represent 4 independent experiments from 4 baboons. ANOVA with Bonferroni's Multiple Comparison Test was used to determine the statistical significance. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, NS, non-significant, $p > 0.05$. Error bars: s.e.m.

4.3 Immune monitoring of baboons pre and post porcine heart transplantation

4.3.1 Immune monitoring of baboons receiving conventional immunosuppressive treatment

Five baboons were included in group 1. Donor pigs had the genotype *GGTA1* homozygous knockout/heterozygous *hCD46*, and the donor pig of baboon 49 had the genotype *GGTA1* homozygous knockout/heterozygous *hCD46*/heterozygous *HLA-E*. All baboons in this group received a pre-treatment in the last week before heterotopic intrathoracic transplantation consisting of bortezomib, cyclophosphamide, and anti-CD20 mAb. Post transplantation, they got anti-CD20 mAb, ATG, tacrolimus, MMF, methylprednisolone, cyclophosphamide, bortezomib, and heparin. Furthermore, they all received total lymphoid irradiation (TLI) on day 5 post transplantation (except baboon 46: day 9) (Abicht, Mayr et al. 2015). The monitoring of immune cells, including total and activated T cells ($CD25^+CD69^+$), monocytes, NK cells and B cells, was done by using flow cytometry. The immune monitoring time points are shown in table 4.3.1.

Table 4.3.1 Time points of immune monitoring for baboons in group 1.

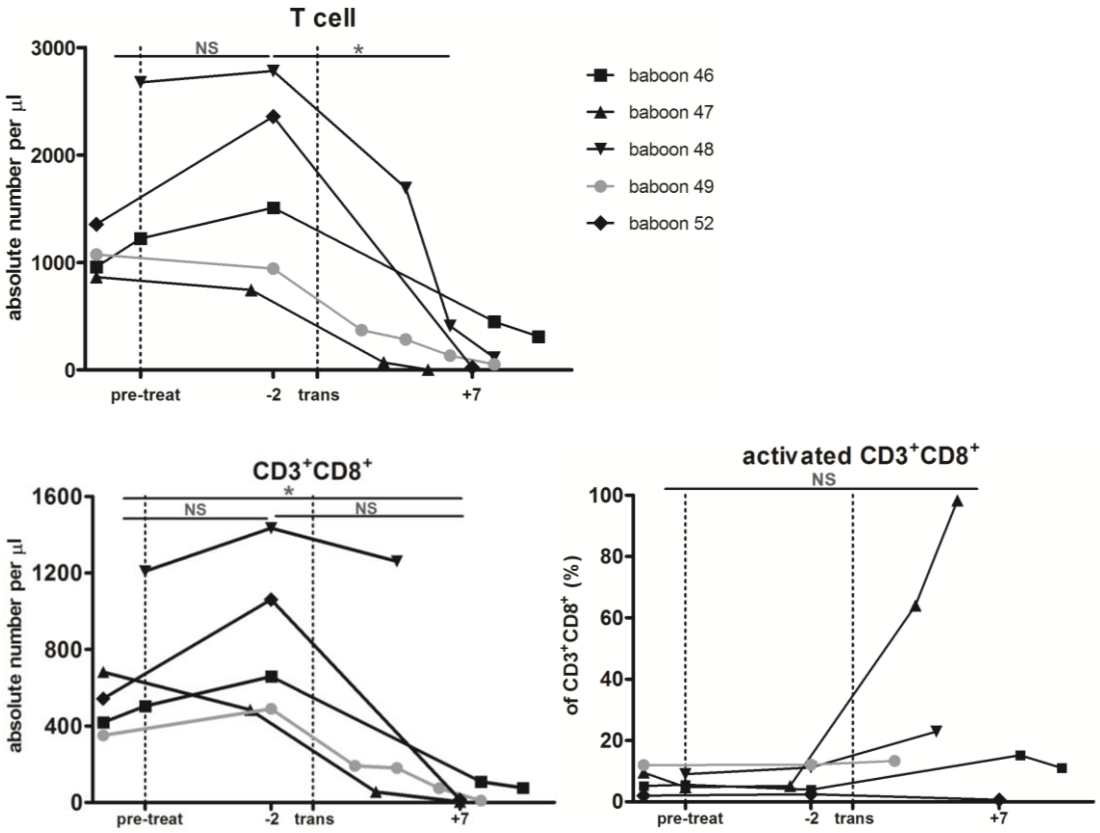
	Survival [days]	Overall survey assay [day]	Determination of Treg cells [day]	Determination of T cell activation[day]
Baboon 46	17	-23, -7, -2, 8, 10, 17	-23, -7, -2, 8 10	-23, -7, -2, 8 10
Baboon 47	7	-37, -7, -3, 3, 5, 7	-37, -7, -3	-37, -7, -3, 3, 7
Baboon 48	15	-7, -2, 4, 6, 8, 11, 14	-7, -2, 4	-7, -2, 4
Baboon 49	35	-8, -2, 2, 4, 6, 8, 15	-8, -2, 2, 4, 6, 8	-8, -2, 2
Baboon 52	7	-35, -2, 7	-35, -2, 7	-35, -2, 7

Negative numbers mean pre transplantation, positive numbers mean post transplantation. Transplantation was done at day 0.

In this group of baboons, the number of total T cell, and of the $CD3^+CD8^+$ and $CD3^+CD4^+$ populations was unchanged after pre-treatment (except for baboon

52 where these populations were increased significantly after pre-treatment) (Figure 4.3.1). Activated $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells as well as $CD3^+CD4^+CD25^+CD127^{low/-}Foxp3^+$ Treg remained unchanged during pre-treatment (Figure 4.3.1).

Following transplantation, the number of total T cells, $CD3^+CD8^+$, and $CD3^+CD4^+$ T cells decreased significantly (Figure 4.3.1). However, the activated fraction of $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells increased after transplantation although without statistical significance. In baboon 47, the activated $CD3^+CD8^+$ and $CD3^+CD4^+$ T cell populations were upregulated to 98.3 % and 99.3 % at the last time point after transplantation, respectively. In Baboon 49 and baboon 52, the Treg percentages among $CD3^+CD4^+$ T cells were also upregulated after transplantation. In baboon 46, the Treg percentage first increased until day 8 after transplantation and then decreased during the following two days (Figure 4.3.1). In baboon 48, Treg were downregulated following transplantation (Figure 4.3.1).



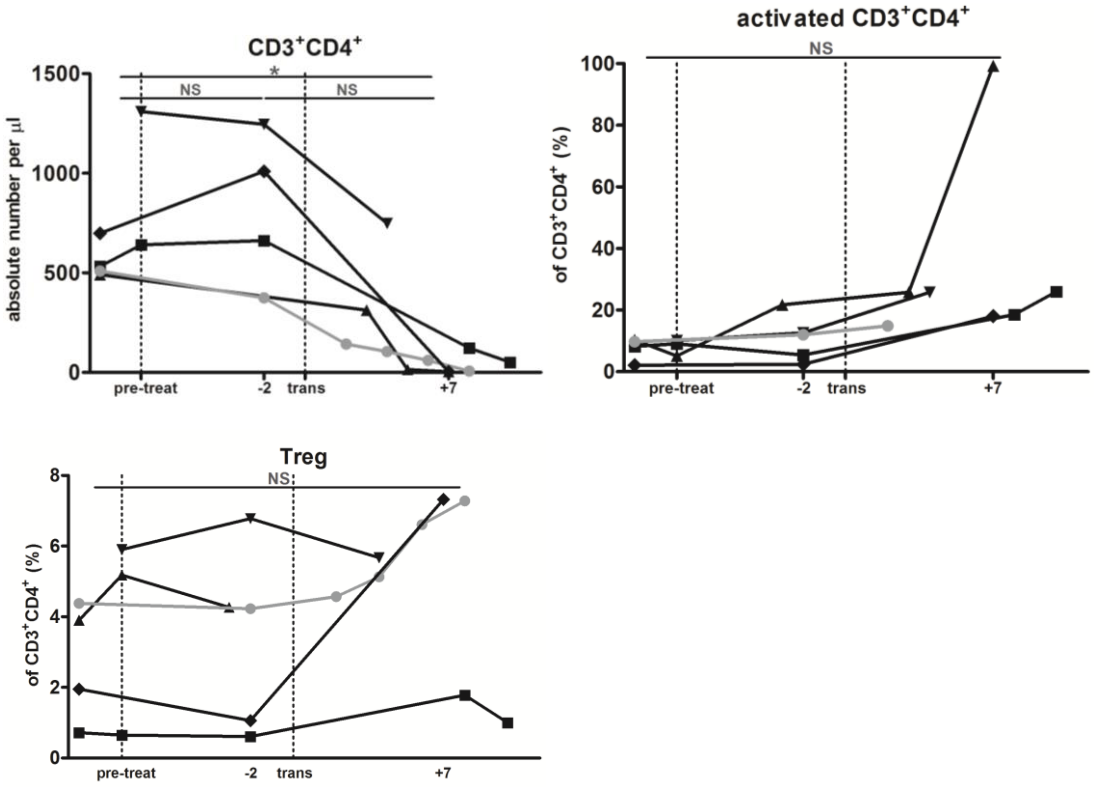


Figure 4.3.1 Immune monitoring of T cells in baboon group 1 pre and post xenotransplantation of porcine hearts using flow cytometry. T cells, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells are shown as absolute numbers, activated $CD3^+CD4^+$ and $CD3^+CD8^+$ cells as percent of $CD3^+CD4^+$ and of $CD3^+CD8^+$ T cells, respectively. Treg cells are shown as percent of $CD3^+CD4^+$ cells. Baboon 49 received a *GGTA1* homozygous knockout/heterozygous *hCD46*/heterozygous *HLA-E* pig heart transplant (grey line). Absolute T cells numbers were calculated using data from the overall survey assay and blood cell counts; activated $CD3^+CD4^+$, $CD3^+CD8^+$ cells ($CD25^+CD69^+$) were analyzed using the T cell activation panel; absolute numbers of $CD3^+CD8^+$, $CD3^+CD4^+$ cells of baboon 46, baboon 47, baboon 48 and baboon 52 were calculated using data from the T cell activation panel and blood cell counts; absolute numbers of $CD3^+CD8^+$, $CD3^+CD4^+$ cells of baboon 49 were calculated using data from the Treg determination assay and blood cell counts; Treg percentage was analyzed using the Treg determination assay. If not enough material was available, the curves end up before death of the baboons). Paired t-tests were used to determine the statistical significance.

Generally, the number of monocytes (Figure 4.3.2) of all baboons in group 1 retained unchanged after pre-treatment. Just after transplantation the monocytes of baboon 47 and 52 decreased dramatically. The monocytes of baboon 46, 48 49 were higher after transplantation, peaked at day 10, day 4 and day 2 post transplantation, respectively, and then decreased dramatically.

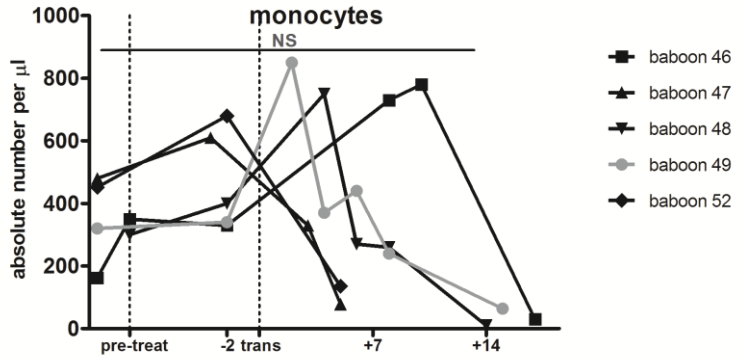


Figure 4.3.2 Immune monitoring of monocytes in baboon group 1 pre and post xenotransplantation of porcine hearts. Monocytes are shown as absolute numbers and were calculated using data from the overall survey assay and blood counts. Paired t-tests were used to determine the statistical significance.

The number of NK cells remained overall constant during pre-treatment and transplantation, except baboon 46, where the cells increased during pre-treatment. In baboon 49, receiving an HLA-E-expressing pig heart, the NK cells were present at low level through the pre-treatment and transplantation compared to the other baboons (Figure 4.3.3).

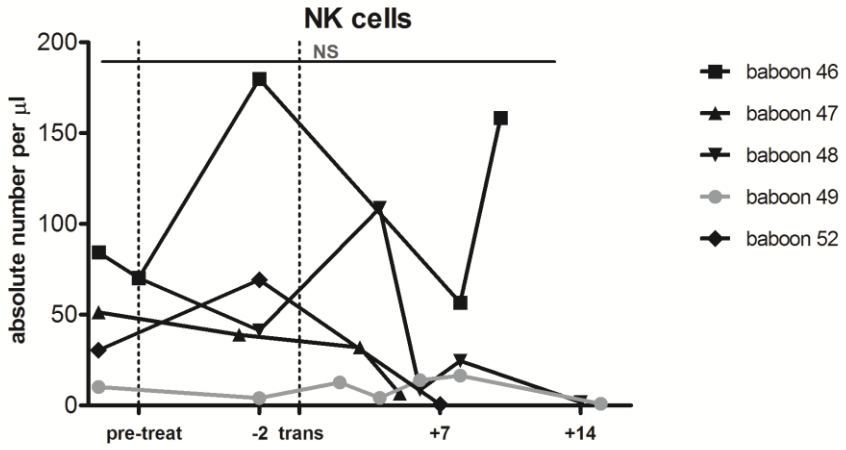


Figure 4.3.3 Immune monitoring of NK cells in baboon group 1 pre and post xenotransplantation of porcine hearts. NK cells are shown as absolute numbers and were calculated using data from the overall survey assay. Paired t-tests was used to determine the statistical significance.

The number of B cells decreased significantly after pre-treatment and remained at low level post transplantation in all baboons (Figure 4.3.4).

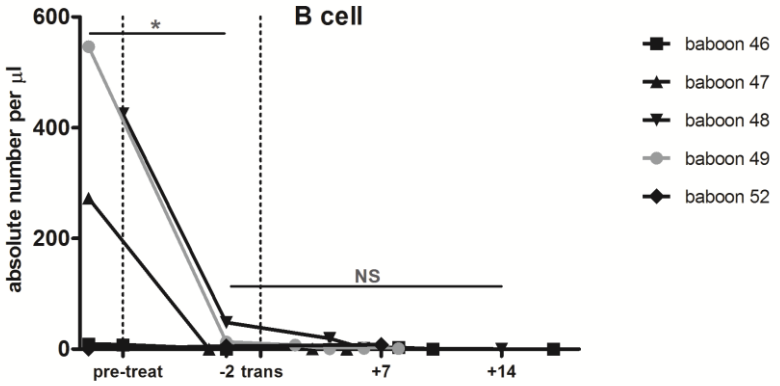


Figure 4.3.4 Immune monitoring of B cells in baboon group 1 pre and post xenotransplantation of porcine hearts. B cells are shown as absolute numbers, and were calculated using data from the overall survey assay. Paired t-tests were used to determine the statistical significance.

4.3.2 Immune monitoring of baboons receiving anti-CD40 immunosuppressive treatment

Two baboons were included in group two. Donor pigs had the genotype *GGTA1* homozygous knockout/heterozygous *hCD46*. Instead of bortezomib and cyclophosphamide, anti-CD40 mAb was given to block the interaction between APC and T cells. Heart transplantation was done intrathoracically at a heterotopic site. Immune monitoring time points for baboons in group 2 are shown in table 4.3.2. However, because the blood cell count data were not available for group 2, the data are shown only in percentage.

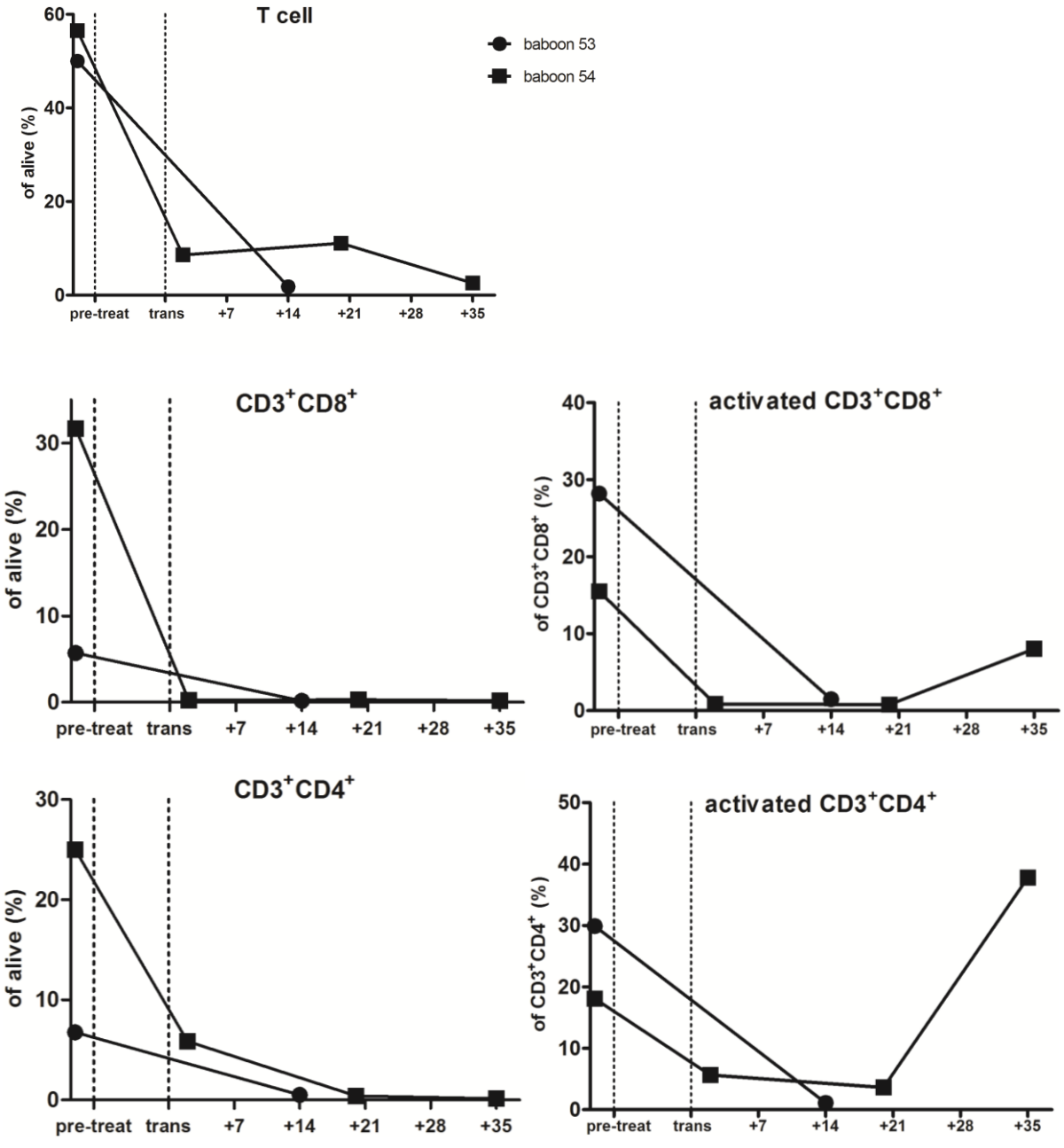
Table 4.3.2 Time points of immune monitoring for baboons in group 2

	Survival [days]	Overall survey assay [day]	Treg determination [day]	Determination of T cell activation [day]
Baboon 53	13	-31, 14	-31, 14	-31, 14
Baboon 54	35	-31, 2, 20, 35	-31, 2, 20, 35	-31, 2, 20, 35

Negative numbers mean pre transplantation, positive numbers mean post transplantation. Transplantation was done at day 0.

In these two baboons, the total T cell population, the $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells decreased after transplantation. The activated $CD3^+CD8^+$, $CD3^+CD4^+$ T cells,

and the Treg cells decreased after transplantation in baboon 53, However, at the last time point, activated $CD4^+$ and $CD8^+$ T cells increased in baboon 54 (Figure 4.3.5). Treg cells increased after transplantation in baboon 54, except at day 20 (Figure 4.3.5).



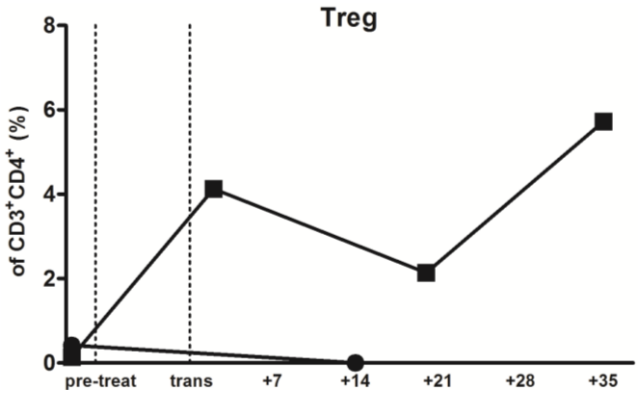


Figure 4.3.5 Immune monitoring of T cells in baboon group 2 pre and post xenotransplantation. Total T cells, $CD3^+CD4^+$, and $CD3^+CD8^+$ T cells are shown as percent of living mononuclear cells. Activated $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells are shown as percent of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, respectively. Treg as percent of $CD3^+CD4^+$ T cells. T cells were analyzed using the overall survey assay; $CD3^+CD4^+$, $CD3^+CD8^+$ T cells and activated $CD3^+CD4^+$, $CD3^+CD8^+$ T cells were analyzed using the T cell activation panel; Treg cells were analyzed using the Treg determination assay.

Monocytes increased after transplantation in baboon 53, while they slightly decreased in baboon 54 after transplantation (Figure 4.3.6).

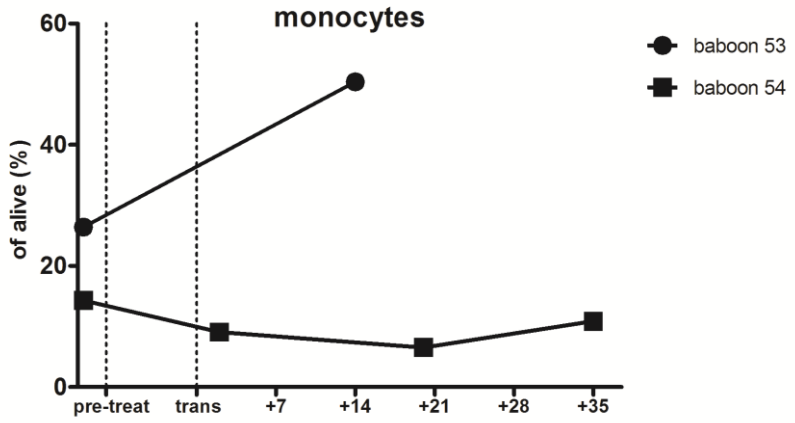


Figure 4.3.6 Immune monitoring of monocytes in baboon group 2 pre and post xenotransplantation of porcine hearts. The total population of monocytes is shown as percent of living mononuclear cells and was analyzed using the overall survey assay.

NK cells decreased after transplantation. Similar tendency was also found for NKT cells (Figure 4.3.7).

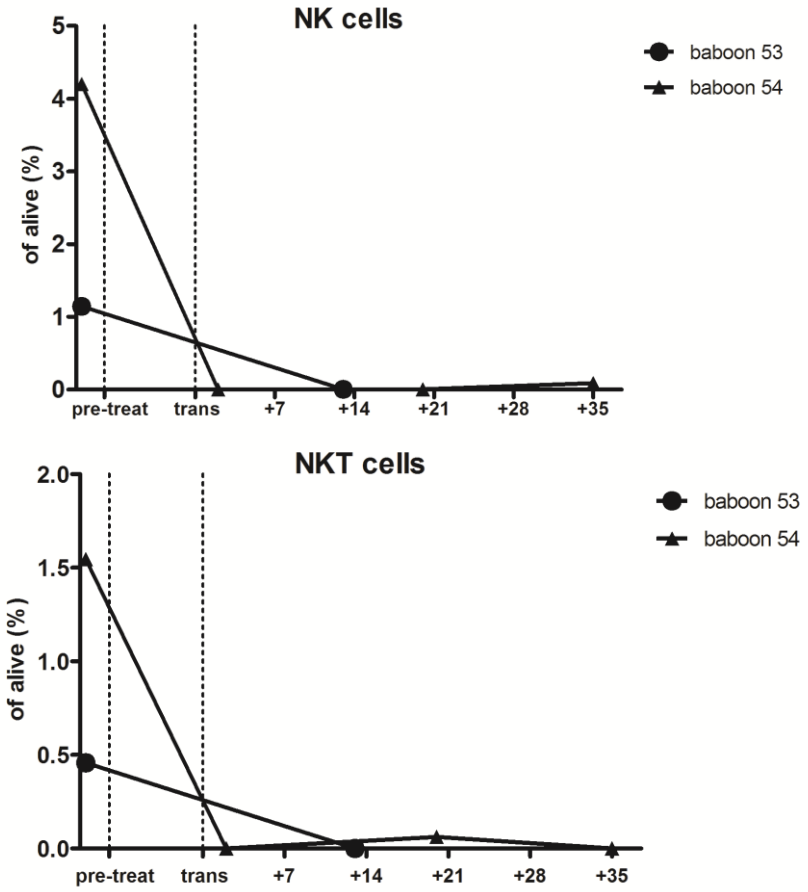


Figure 4.3.7 Immune monitoring of NK and NKT cells in baboon group 2 pre and post xenotransplantation of porcine hearts. NK cells and NKT cells are shown as percent of living mononuclear cells. They were analyzed using the overall survey assay.

As in group 1, B cells also decreased in group 2 after pre-treatment and transplantation (Figure 4.3.8).

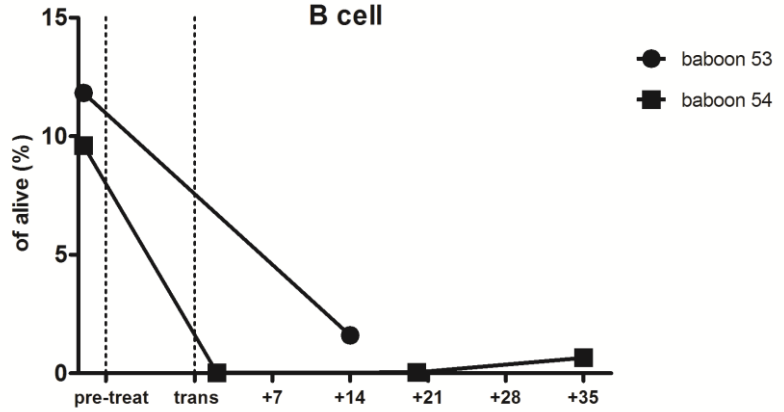


Figure 4.3.8 Immune monitoring of B cells in baboon group 2 pre and post xenotransplantation of porcine hearts. B cells are shown as percent of living mononuclear cells and were analyzed using the overall survey assay.

4.3.3 Immune monitoring of baboons receiving anti-CD40 or anti-CD40L immunosuppressive treatment without tacrolimus

In the third group, five baboons were included. Donor pigs had the genotype *GGTA1* homozygous knockout/heterozygous *hCD46*/ homozygous *hTM*. All baboons received a pre-treatment with ATG and anti-CD20 mAb. In subgroup A, anti-CD40 mAb was administered to baboon 55, baboon 57, and baboon 63. In subgroup B, anti-CD40L mAb was administered to baboon 60 and baboon 64. Tacrolimus was not used after transplantation. In this group an orthotopic transplantation was done. Immune monitoring time points for baboons in group 3 are shown in table 4.3.3.

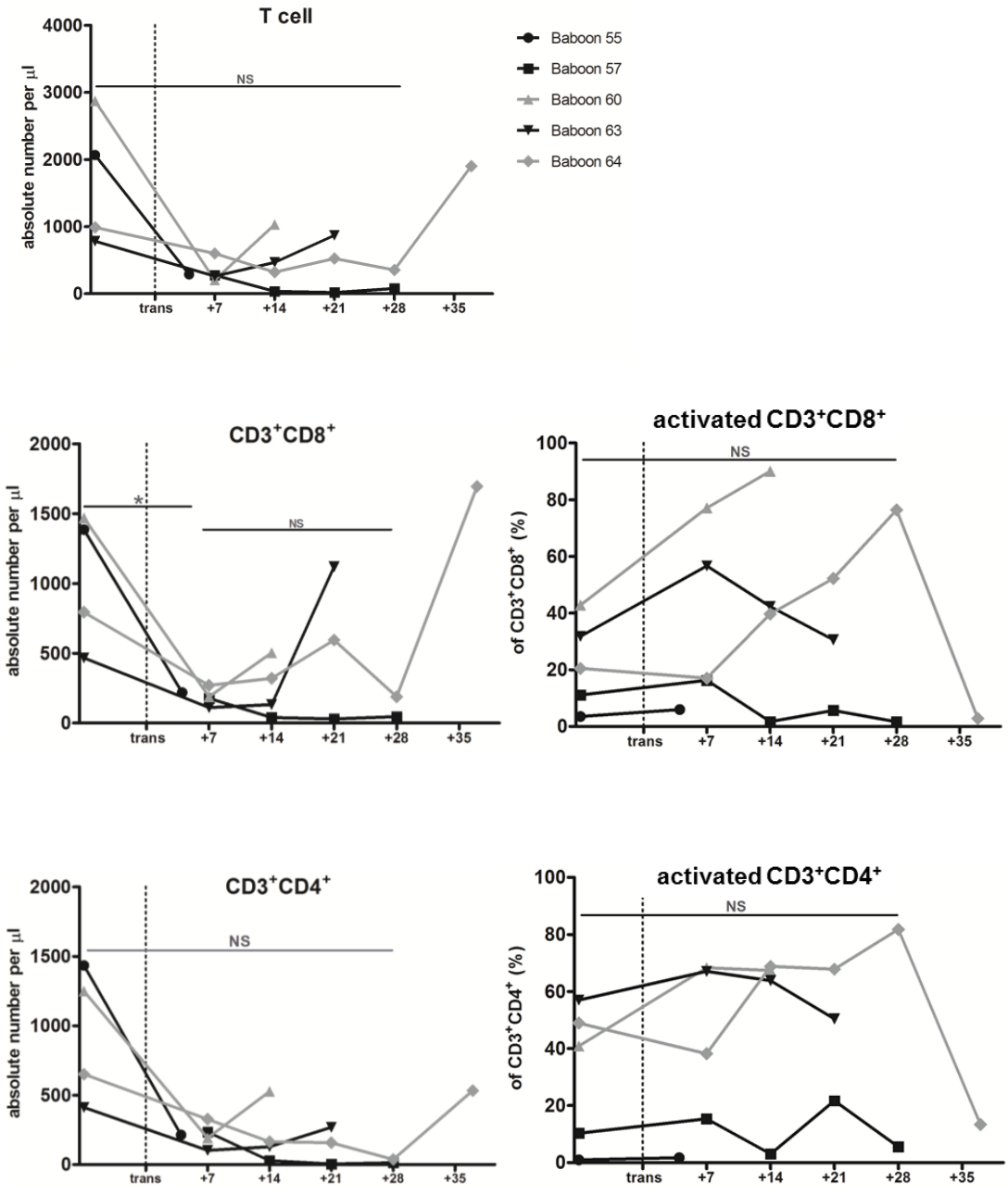
Table 4.3.3 Time points of immune monitoring for baboons in group 3

	Survival [days]	Overall survey assay [day]	Determination of Treg cells [day]	Determination of T cell activation [day]
Baboon 55	3	-2, 4	-2, 4	-2, 4
Baboon 57	30	-33, 7, 14, 21, 28	-33, 7, 14, 21, 28	-33, 7, 14, 21, 28
Baboon 60	18	-7, 7, 14	-7, 7, 14	-7, 7, 14
Baboon 63	27	-25, 7, 14, 21	-25, 7, 14, 21,	-25, 7, 14, 21,
Baboon 64	40	-138, 7, 14, 21, 28, 37,	-138, 7, 14, 21, 28, 37,	-138, 7, 14, 21, 28, 37,

Negative numbers mean pre transplantation, positive numbers mean post transplantation. Transplantation was done at day 0.

$CD3^+CD8^+$ T cell populations of all baboons significantly decreased until day 7 post transplantation (Figure 4.3.9). The total T cell population and the $CD3^+CD4^+$ also decreased after transplantation, but the data meet no statistical significance. Following this time point the T cells and $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells of baboon 60, baboon 63 and baboon 64 showed an increase. Although the number of these T cells populations, of baboon 57 remained at a low level after transplantation, at the last time point a slight increase was observed (Figure 4.3.9). The fraction of activated $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells increased in all the baboons (except baboon 64) in the first week post transplantation (Figure 4.3.9) although data meet no statistical significance. In baboon 64, the fraction of activated $CD8^+$ and $CD4^+$ T cells decreased during the

first week after transplantation, followed by an increase and peaked at day 28 post transplantation (Figure 4.3.9). Within the following 9 days a dramatic decrease was found in baboon 64 (Figure 4.3.9). Except for baboon 60, all baboons showed an upregulation of Treg cells during the first week after transplantation although the data meet no statistical significance. At the following time points a downregulation was detected in baboon 63 and 64. In Baboon 60 Treg cells were downregulated after transplantation, and then, a slight increase was followed during the next 7 days (Figure 4.3.9).



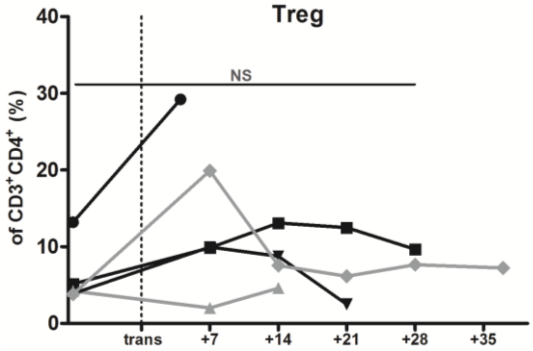


Figure 4.3.9 Immune monitoring of T cells in baboon group 3 pre and post xenotransplantation of porcine hearts. Total T cells, $CD3^+CD4^+$, and $CD3^+CD8^+$ T cells are shown as absolute numbers. Activated $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells are shown as percent of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, respectively. Treg as percent of $CD3^+CD4^+$ T cells. Baboon 55, baboon 57, and baboon 63 were treated with anti-CD40 mAb (black lines). Baboon 60 and baboon 64 were treated with anti-CD40L mAb (grey lines). T cells were calculated using data from the overall survey assay; $CD3^+CD4^+$, $CD3^+CD8^+$ T cells and activated $CD3^+CD4^+$, $CD3^+CD8^+$ T cells were analyzed using the T cell activation panel; Treg cells were analyzed using the Treg determination assay. Paired t-tests were used to determine the statistical significance. The absolute number of baboon 57 pre-transplantation could not be calculated because the blood cell count was unavailable.

Monocytes of all baboons increased during the first week after transplantation without statistical significance, and decrease afterwards (Figure 4.3.10).

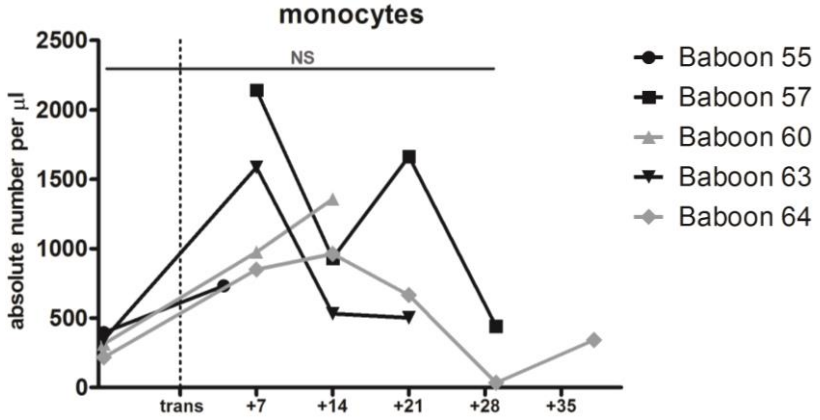


Figure 4.3.10 Immune monitoring of monocytes in baboon group 3 pre and post xenotransplantation of porcine hearts. The monocytes are shown as absolute numbers, calculated using data from the overall survey assay. Paired t-tests were used to determine the statistical significance.

As in group 2, NK cells significantly decreased after transplantation in all baboons (Figure 4.3.11). Baboon 57 and baboon 64 showed a slight recovery at

day 14 and day 28, followed by loss, respectively. Similar to NK cell, NKT cells also decreased significantly after transplantation (Figure 4.3.11).

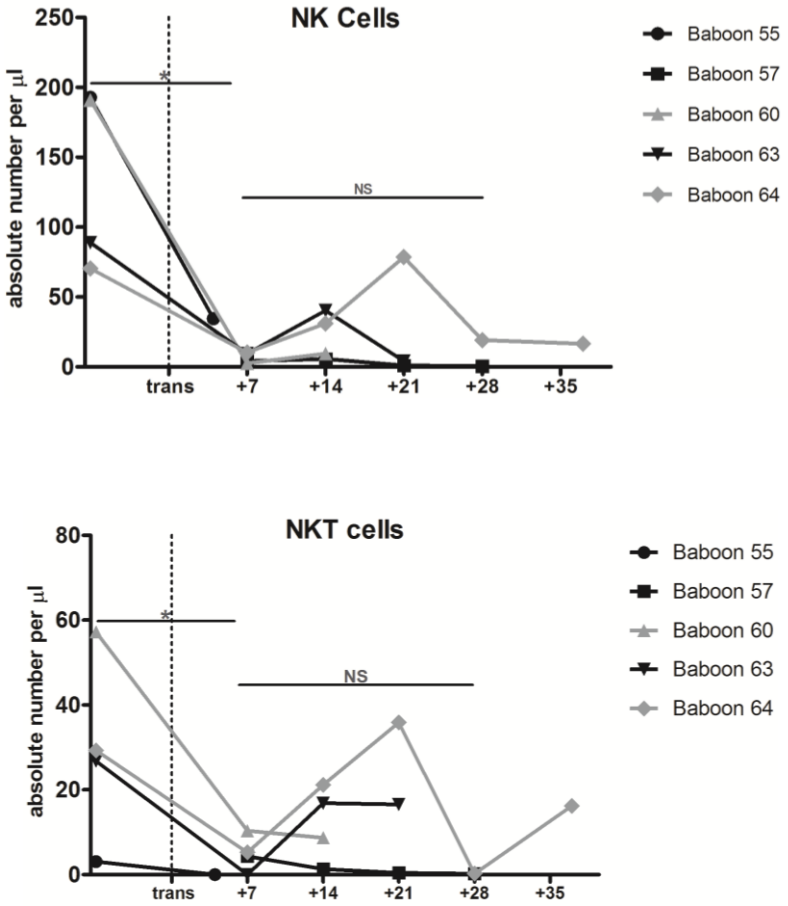


Figure 4.3.11 Immune monitoring of NK and NKT cells in baboon group 3 pre and post xenotransplantation of porcine hearts. The cells are shown as absolute numbers. NK cells and NKT cell numbers were calculated using data from the overall survey assay. Paired t-tests were used to determine the statistical significance.

Similar to group 1 and group 2, B cells were depleted in all baboons (Figure 4.3.12).

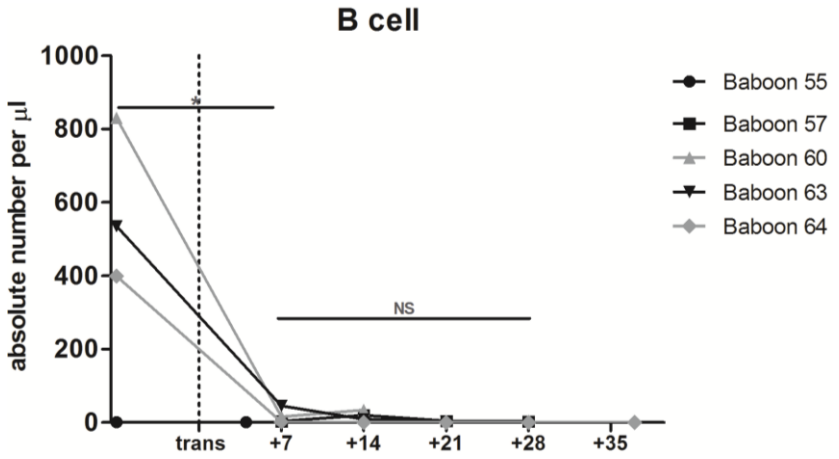


Figure 4.3.12 Immune monitoring of B cells in baboon group 3 pre and post xenotransplantation of porcine hearts. B cells are shown as absolute numbers, calculated using the data from the overall survey assay. Paired t-tests were used to determine the statistical significance.

5. Discussion

5.1 Generation of human PSTreg with PS ivtRNA loaded human tolDC

Several methods have been reported to generate tolDC and most require 6 or more days to obtain these cells. In this paper, we investigated a fast method that needs only 3 days to acquire functional tolDC. TolDC generated with this fast protocol retained a semi-mature state with preservation of CD14 expression and low level expression of CD83, CD80 and CD86. Elsewhere, high expression of B7-H1 and B7-DC was reported to be characteristic for tolDC (Chen, Zhang et al. 2007). B7-H1 and B7-DC, also termed PD-L1 and PD-L2 respectively, are ligands for PD-1 (Keir, Butte et al. 2008). PD-1 signal induces T cell inactivation via inhibition of TCR ligation by targeting PI3K/Akt and Ras/MEK/Erk pathway, and inhibits T cell proliferation by inhibiting cell cycle progression through Cdk2 regulation, and induce iTreg production via TGF-β independent Smad3 regulation (Yamazaki, Bonito et al. 2007). Our tolDC expressed significantly high levels of B7-DC and B7-H1. In some samples B7-H1 was downregulated, indicating some differences to the published reports of other tolerogenic DC. B7-H1 was found to be upregulated by IFN-γ (Schoop, Wahl et al. 2004, Abiko, Matsumura et al. 2015), which may explain its higher expression in some samples of C5-DC that were found to produce IFN-γ. *In vivo*, Treg are generated by DC that provide few or no inflammatory cytokines and costimulatory signals in the presence of low antigen levels (Maldonado and von Andrian 2010). Our tolDC shared these characteristics and were capable of inducing Treg *in vitro*. By electroporation, PS antigen was induced on DC generated with our fast 3 day protocol and no severe cell death was observed. It was also reported in our former research that the 3-day DC are more robust to electroporation than traditional 7-day DC (Burdek, Spranger et al. 2010).

Antigen-specific Treg were obtained in cultures using PS-antigen-loaded tolDC. In order to demonstrate the type of PSTreg, we use nTreg-depleted $CD4^+$ T cells as precursor to generate PSTreg. As shown in the result, PSTreg can be

generated with these cells, which indicates that in our method PSTreg are induced Treg but not expanded nTreg. As expected, Foxp3 was highly expressed in PSTreg but also in NTreg that were generated using non-antigen-loaded tolDC. The development of NTreg may have resulted from exposure of T cells to high levels of exogenous IL-2 and rapamycin (Yamazaki, Bonito et al. 2007). Foxp3 directly blocks SATB1 and indirectly induces microRNA which binds to the *SATB1* 3'untranslated region. Therefore, low *SATB1* expression was found to be characteristic for Treg and their suppressive function (Beyer, Thabet et al. 2011). In concordance, the NTreg in our studies showed low *SATB1* expression. While PSTreg had slightly higher expression of *SATB1* compared with PSTeff, they also showed lower expression of *SATB1* compared with NTeff. GARP expression is a marker of activated Treg (Abd Al Samid, Chaudhary et al. 2016), and can be used to isolate Treg with high suppressive function (Wang, Kozhaya et al. 2009, Abd Al Samid, Chaudhary et al. 2016), GARP is associated with Foxp3 expression (Probst-Kepper, Geffers et al. 2009). More importantly, GARP is involved in TGF-β expression by forming the GARP-LAP complex on the Treg surface (Wang, Zhu et al. 2012). In the cells studied here, higher expression of *GARP* was found in PSTreg compared to NTreg, indicating that foreign-antigen stimulation may activate Treg more efficiently than self-antigens. Consistently, a more memory phenotype was observed in our PSTreg, which showed $CD45RA^{-}CCR7^{low}$ phenotype (Rosenblum, Way et al. 2016). With IL-10 producing tolDC, induced Treg can be generated and exhibit a more activated phenotype that was also reported by others (Kryczanowsky, Raker et al. 2016). The high CCR4 expression again confirmed the high suppressive function of PSTreg, which demonstrated by others that the CCR4 expressing $CD45RA^{-}FOXP3^{high}CD4^{+}$ Treg are terminally differentiated and most suppressive (Sugiyama, Nishikawa et al. 2013). The stability of our PSTreg was demonstrated upon PS antigen loaded tolDC, by Foxp3 expression, an activated phenotype and specific suppressive function. Fast porcine-specific tolDC provide an effective tool to successfully generate PSTreg.

High expression of IL-10 and TGF-β1 and the lack of IL-12p70, confirmed the tolerogenic phenotype. In contrast, C5-DC expressed IL-12p70, but not IL-10, in accordance with their immunogenic phenotype. Although *EBI3* mRNA expression showed no significant difference between tolDC and C5-DC in most samples *EBI3* mRNA was downregulated in tolDC, which can be explained by a

previous report demonstrating that IFN-γ could induce *EBI3* expression in DC (Dixon, van der Kooij et al. 2015). *IL-27A* expression was upregulated more in C5-DC compared to tolDC, which was also consistent with the former report (Dixon, van der Kooij et al. 2015). In general, C5-DC, as immunogenic antigen-presenting cells, produce IL-27 and IL-12p70, but not IL-35. Our tolDC downregulate IL-27, and do not express IL-12p70, as another confirmation of the tolerogenic character of these cells.

As expected, PSTreg secreted high level of IL-10 and TGF-β1 at protein and RNA levels. Based on the mRNA expression of *EBI3* and *IL-12A*, it is evident that PSTreg were more strongly activated after exposure to foreign antigens compared with NTreg that were activated by self-antigens. Although compared with PSTeff *EBI3* upregulation in PSTreg meet no statistical significance, *IL-12A* was significantly upregulated, which confirms observations of others that human activated Treg upregulated predominantly more *IL-12A* than *EBI3* (Bardel, Larousserie et al. 2008). We speculate that our fully activated PSTreg produce IL-35. Because there is no direct method to measure IL-35 as a dimeric protein, we can only infer IL-35 production indirectly from their *EBI3* and *IL-12A* mRNA expression.

The functionality of PSTreg was clearly demonstrated: two activation markers of different activation stages were significantly suppressed on PSTeff after exposure to PSTreg compared with NTreg generated with mock-loaded tolDC and nTreg. Likewise, proliferation of PSTeff was inhibited significantly in the presence of PSTreg and PSTreg still retained the specific suppression towards PSTreg in lower ratio, indicating it is applicable in vivo. In contrast, PSTreg inhibited NTeff proliferation and the expression of activation markers of NTeff were significantly lower than in PSTreg compared to PSTeff, and in the ratio of 1:1, PSTreg showed no significant suppressive function to NTeff. This demonstrates that PSTreg generated with our method exhibit high suppressive activity only towards PSTeff and indicates that PSTreg are highly specific and do not mediate non-specific, unwanted immunosuppression. In comparison with nTreg isolated from the same donor at the same time, NTreg suppressive activity towards PSTeff and NTeff showed no significant difference to nTreg in the aspect of activation marker expression and Teff proliferation. NTreg showed higher suppressive activity than nTreg in the PSTeff CD25 expression assay. This indicates that NTreg generated with our mock-loaded tolDC exhibit

comparable function to nTreg in the aspect of Teff suppression. Moreover, IL-17A production was not detected, and PSTreg still showed the specific suppressive function after restimulation, which indicates high stability of our PSTreg.

IL-10 secretion was reproducibly observed during the first few hours of coculture of PSTreg and PSTeff, and became more pronounced after longer incubation periods. TGF-β1 also accumulated preferentially in PP cocultures, although differences with other coculture combinations were less prominent. In line with protein levels, PP cocultures contained the highest amounts of *IL-10* and *TGFB1* mRNA. Interestingly, from the statistical aspect, the increase of *IL-10* mRNA in PP was more pronounced than that of *TGFB1* mRNA, which indicates that IL-10 might be the major cytokine of the two involved in PSTreg suppressive function. An additional potential candidate involved in suppressive function of PSTreg is IL-35, as suggested by earlier studies (Collison, Workman et al. 2007). The mRNAs of IL-35 components *EBI3* and *IL-12A* were preferentially expressed in PSTreg upon interaction with PSTeff.

IFN-γ is a cytokine that mediates inflammation and causes potent immune regulatory effects. As expected, IFN-γ was highly expressed by PSTeff in the absence of PSTreg. Coculture of PSTeff with PSTreg strongly suppressed IFN-γ secretion. However, after extended coculture with PSTreg, IFN-γ production increased. Due to their plasticity Treg produce IFN-γ when they are recruited to the site of Th1-type inflammation, and there is evidence that IFN-γ also has immune inhibitory effects: .i.e. by inducing PD-L1 (Schoop, Wahl et al. 2004) and IDO (Jurgens, Hainz et al. 2009) expression in DC and upregulates the expression of *EBI3* (Dixon, van der Kooij et al. 2015). In addition, IFN-γ mediated protection in GvHD and closely associated with Treg development and function in GvHD sittings (Wang and Yang 2014). Therefore, it is conceivable that PSTreg profit from an IFN-γ environment produced by PSTeff through upregulation of *EBI3* expression, as shown in the results section.

Treg may inhibit cellular rejection in several ways: a) by secretion of suppressor cytokines, such as IL-10 and TGF-β, which inhibit effector T cells directly, b) by expression of high levels of CD25, leading to competition for IL-2 with effector T cells, c) by acting as cytotoxic cells that directly kill responder T cells, and d) by inducing expression of galectin-1 or other unknown molecules on the cell

surface leading to effector T cell cycle arrest (Shevach 2009). Thus, it is unlikely that PSTreg mediate their specific suppressive functions solely via secretion of anti-inflammatory cytokines. Further investigations will be required to elucidate the exact mechanisms that contribute to the distinct specificity of PSTreg described in these studies. Such analyses become feasible through the capacity of tolDC to induce these cells in a rapid and efficient manner, leading to generation of PSTreg with high stability.

PSTreg developed with this fast method represent Treg that exhibit a phenotype of activated cells and produce high levels of IL-10, TGF-β1, and IL-35 and also have porcine-antigen specificity. In contrast to the methods used by others our method uses ivtRNA to induce xeno-antigen specific tolDC to generate PSTreg, which is safe and ivtRNA is easy to generated in large amounts. A recent study reports large-scale expansion of Treg with CD3/CD28 beads together with rapamycin and IL-2 and then the cells were restimulated additionally with irradiated pig PBMC, but the specificity was lost following several restimulations (Jin, Lu et al. 2016). Our PSTreg generated with tolDC might provide a better protocol, but this should be demonstrated in the future. In the baboon system, enriched and expanded Treg can suppress xenogeneic immune responses, and it can be suggested that adoptive transfer of baboon Treg cells may be an approach to prevent xeno-graft rejection in a pig-to-baboon xenotransplantation model (Porter, Horvath-Arcidiacono et al. 2007, Singh, Horvath et al. 2009). Therefore, we successfully transferred our technique into the baboon system. Therefore, our method of tolDC generation with subsequent induction of porcine-specific Treg has the potential to be developed for use in porcine solid organ transplantation or porcine cell transplantation through adoptive cell transfer into host animals or human transplant patients in the future.

5.2 Generation of baboon PSTreg with PS ivtRNA loaded baboon tolDC

TolDC are defined as a subset of DC that induces immune tolerance in vivo. For example, oral tolerance is induced by the mucosal environment, which is rich in various anti-inflammation factors: TGF-β, retinoic acid, IL-10, vasoactive

intestinal peptide, thymic stromal lymphopoietin, and hepatocyte growth factor (Maldonado and von Andrian 2010, Bekiaris, Persson et al. 2014). DC present in the intestinal mucosa transfer intestinal tract samples to mesenteric lymph nodes in a CCR7-dependent manner (Bekiaris, Persson et al. 2014). DC in lung that induce tolerance keep a semi-mature status and their high expression of CCR7 enables them to migrate to the lymph nodes where they induce Treg. Resting pulmonary stromal cells produce TGF-β as in the mucosal environment (Lloyd and Hawrylowicz 2009, Bakocevic, Worbs et al. 2010). By mimicking in vivo environments where DC are involved in immune tolerance, we developed a protocol to generate baboon-derived tolDC using a maturation cocktail containing IL-10 and TGF-β1 as we have done this for human tolDC. Immature DC induce Treg in vivo, presumably due to the presentation of antigens to naïve T cells without costimulatory signals and cytokines (Maldonado and von Andrian 2010). Baboon tolDC generated with our fast 3 day protocol exhibit an immature status, demonstrated by low expression of CD83 in comparison to the control immune-activating C5-DC. Low expression of the costimulatory molecule CD80 and high expression of inhibitory B7-H1 and B7-DC molecules on tolDC**,** is seen to be characteristic of other tolDC. These characteristics are exemplified by the baboon-derived tolDC described here. Baboon TolDC as well as C5-DC displayed CCR7 expression, which would indicate a migratory capacity of both cell types to lymphatic tissues in vivo. IL-10 was highly expressed also by our baboon tolDC while IL-12p40 was not expressed at either the protein or RNA levels, again reflecting another characteristic of tolDC. Only half of the baboon tolDC samples upregulated *TGFB1* mRNA, which indicates a difference to human tolDC. However, non-upregulated TGF-β expression can be bypassed by high expression of B7-H1 and B7-DC molecules on tolDC in the aspect of Treg induction: the PD-1 pathway in T cell reduces the threshold of TGF-β mediated signal by inhibiting Cdk-2 mediated Smad3 phosphorylation, which results in enhanced Smad3 transactivation in a TGF-β independent manner (Boussiotis, Chatterjee et al. 2014). Similarly, administration of rapamycin in Treg induction also increases the responsiveness to the baseline level of TGF-β through constitutively phosphorylated Smad3 (Powell, Pollizzi et al. 2012). *IL-12A* mRNA of the IL-35 gene family was upregulated in tolDC compared to C5-DC. *EBI3* mRNA in tolDC of all samples was also upregulated compared to C5-DC although without statistical significance. This is similar to human tolDC

which upregulated *IL-12A* mRNA to a larger extent than *EBI3* (Dixon, van der Kooij et al. 2015). Another difference to human tolDC is the upregulation of IL-*27A* mRNA in all samples of baboon tolDC, compared to a downregulation known for human tolDC (Dixon, van der Kooij et al. 2015). Despite these subtle differences to reports of human tolDC, the baboon-derived tolDC generated with our method demonstrated a clear tolerogenic phenotype, and expressed high levels of IL-10, and potentially also high levels of IL-35 and IL-27.

Others have reported that baboon-derived porcine-specific and non-specific Treg exhibit similar phenotypes to those described for human Treg (Porter, Horvath-Arcidiacono et al. 2007). They were also found to produce high amounts of IL-10. In contrast to our results using human tolDC and $CD4^+$ T cell cocultures to induce various Treg subpopulations, baboon PSTreg were induced in significantly greater numbers than NTreg, and these cells expressed higher amounts of IL-10, as measured at both protein and RNA levels (although the data does not meet statistical significance at protein level, PSTreg secreted more IL-10 compared with NTreg in each individual experiment of different baboons). We assume that differences between the results of human and baboon experiments may be due to the baboon donors that share a more similar genetic background than the human PBMC donors. Also the environmental history of baboons and human donors differ substantially and may impact on cell differentiation in vitro. Although tolDC expressed less *TGFB1* mRNA compared to C5-DC in half of the samples, PSTreg and NTreg expressed higher amounts of *TGFB1* mRNA compared to PSTeff and NTeff, respectively. The high expression of *TGFB1* mRNA associated with upregulated *GARP* mRNA expression in PSTreg is of interest because GARP is the receptor of latent TGF-β on the Treg plasma membrane surface (Wang, Zhu et al. 2012). *GARP* mRNA was most highly expressed in PSTreg, demonstrating that PSTreg generated with PS ivtRNA-loaded tolDC were more highly activated by xeno-antigen compared to NTreg generated by coculture with non-loaded tolDC. Simultaneous to upregulated *GARP* expression, CD45RA expression was downregulated in PSTreg, again serving as evidence for greater PSTreg activity and higher suppressive function, as noted by others (Sakaguchi, Miyara et al. 2010). In terms of the IL-35 gene family, all samples showed greater upregulation of *EBI3* mRNA in PSTreg compared to NTreg and PSTeff. However, PSTreg *IL-12A* mRNA is not consistently upregulated in all samples compared to

NTreg and PSTeff. This differs from stimulated Treg in humans, in which *IL-12A* is more prominently upregulated than *EBI3* (Bardel, Larousserie et al. 2008). Like our observations in the human system, PSTreg show downregulation of *IL-12B* and *IL-27A* mRNA compared to PSTeff, although *IL-27A* mRNA downregulation in PSTreg meets no statistical significance. In total, despite subtle differences to parameters known for human cells, we could demonstrate that baboon PSTreg can be efficiently generated using PS loaded baboon-derived tolDC and these antigen-induced PSTreg express high levels of IL-10, TGF-β1. In this work, upregulation of IL-35 related genes, *EBI3* and *IL-12A*, were observed in tolDC and PSTreg, however, some of these data meet no statistical significance. Further research with more samples from more animals is needed to demonstrate the IL-35 expression in baboon.

The specificity of baboon PSTreg towards PSTeff could be clearly demonstrated: baboon PSTreg significantly suppressed early and intermediate activation markers of PSTeff. Moreover, PSTreg failed to demonstrate suppressive function towards NTeff. In correlation, IL-10 was highly expressed in the PSTeff/PSTreg coculture group compared to the other groups as measured at different time points. The NTeff/PSTreg group displayed no significantly higher level of IL-10 than the other Treg/Teff control groups. These results indicate that baboon PSTreg generated with our method exhibit a high specificity towards PSTeff and would provoke no unwanted immune responses.

The baboon is a non-human primate experimental animal that is used in important xenotransplantation research. This study provides a protocol allowing fast and efficient generation of baboon PSTreg using baboon-derived tolDC that express high levels of IL-10 and TGF-β1. Importantly, the PSTreg exhibit porcine-antigen specificity in their immune suppressive function, measured by several parameters. This approach enables our method of baboon-derived tolDC generation with subsequent induction of porcine-specific baboon Treg to be developed for use in porcine solid organ or cell xenotransplantation studies through adoptive cell transfer into host baboons.

5.3 Immune monitoring of pig heart-transplanted baboons

In group 1, baboons received a conventional immunosuppressive therapy. Bortezomib should reduce the effector functions of T cells mainly by increasing Treg populations (Pellom, Dudimah et al. 2015). Our results indicated that T cells were less influenced by administration of bortezomib and cyclophosphamide pre transplantation. The activation of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in group 1 remained unchanged after pre-treatment. Treg cells were also not significantly influenced by conventional immunosuppressive pre-treatment in group 1. The same was observed for monocytes. B cells of all baboon groups were successfully depleted by anti-CD20 mAb, as expected. Following transplantation, T cells did not recover. This is mainly caused by the administration of ATG, tacrolimus and MMF. ATG was given at the day of transplantation and the first four days post transplantation in this group of baboons. ATG depletes T cells and NK cells, drives DC towards a tolerogenic phenotype, and induces B cell apoptosis (Mohty 2007, Ruan, Czer et al. 2017). In some baboons an increase of Treg cells was observed. This was also reported by Feng et al. 2008 (Feng, Kajigaya et al. 2008) and Shimony et al 2012 (Shimony, Nagler et al. 2012). Furthermore, it was demonstrated by Broady et al 2009, that following ATG treatment T cells acquire an activated phenotype (Broady, Yu et al. 2009), this was also confirmed by the immune monitoring of our baboons. Therefore, Popow et al. recommend ATG preparations depleted of CD3-TCR complexes, CD2, and CD28 (Popow, Leitner et al. 2013). Tacrolimus inhibits IL-2 production and, thus should block T cell activation; MMF inhibits inosine monophosphate dehydrogenase (IMPDH), which is also needed for lymphocyte activation and function, reviewed by Ruan et al. 2017 (Ruan, Czer et al. 2017) and Gorantla et al. 2000 (Gorantla, Barker et al. 2000). Monocytes increased during the pre-treatment and dropped after transplantation, this might be caused by transplantation antigens that are taken up by monocytes causing them to mature into DC which downregulated CD14. DC presenting transplant antigens might also be an explanation for the emerging of activated T cells.

In baboons of group 2, anti-CD40 mAb was used to block the interaction between APC and T cells. Pre-treatment with anti-CD40 mAb, a higher dosage of ATG, and the administration of MMF and tacrolimus after transplantation

effectively depleted T cell populations, and suppressed activation of T cells. Similar as in group 1, increased T cell activation was observed in baboon 54 at the later time point after transplantation. ATG was administered only in the pre-treatment phase. Baboon 54, which survived longer, showed increased Treg numbers pre transplantation and after transplantation at a later time point. Baboon 54 showed a slight decrease of monocytes. On the contrary, baboon 53, which survived shorter in group 2, showed an increase of monocytes and decrease of Treg cells after pre-treatment and transplantation. Both NK and NKT cells were downregulated during the pre- and post-transplantation period. In contrast, an expansion of NKT cells was observed by Lan et al. 2001 in a mouse model (Lan, Zeng et al. 2001) and reviewed by Mohty 2007 (Mohty 2007).

In baboons of group 3, blockade of CD40 signal with anti-CD40 mAb or anti-CD40L mAb was performed, there was no observed difference between these two subgroups in respect of immune monitoring data and survival. ATG was administrated only during pre-treatment as in group 2. However, baboon 64, which was the longest survivor in group 3, showed a slight decrease of activated T cells after the first week after transplantation and then the cells increased further. However, the other baboons in this group showed the opposite situation. In most of the baboons Treg cells increased during pre-transplantation and the first week after transplantation. The NK and NKT cells in baboon 63 and 64 first decreased and then fluctuated but on a low level. In group 3, baboon 64 showed the longest survival, the major difference in immune monitoring was observed in the first week. Therefore based on the results seen in baboon 64, the decrease in activated T cells together with the increase in Treg in the first week after transplantation could be an indicator of longer transplant survival.

In group 1, baboon 49 received an HLA-E expressing pig heart which was different for other baboons in this group. While the NK cells in baboons of this group fluctuated dramatically, baboon 49 had very low numbers of NK cells before transplantation. It had the longest survival within this group. Thus, one might speculated that low NK cell numbers are beneficial for transplant survival. An association of NK cell numbers and HLA-E expression on the transplant cannot be drawn.

Taken together, although the groups are small and the results quite heterogeneous, our work demonstrates the relevance of immune monitoring for the schedule of immunosuppressive treatment pre and post transplantation. In future experiments proliferations assays using irradiated pig stimulator cells and baboon responder cells should be additionally performed to elucidate the time point which might be beneficial to perform adoptive therapy with porcine-specific Treg.

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Abbreviations

ANOVA	ONE-Way Analysis Of Variance
APC	antigen presenting cells
ATG	anti-thymocyte globulin
C	porcine-specific effector T cells/non-specific effector T cells control
C5-DC	non-tolerogenic dendritic cells
CCR7	C-C chemokine receptor type 7
CD	cluster of differentiation
cDNA	complementary DNA
CFSE	carboxyfluorescein succinimidyl ester
CNS	conserved non-coding sequences
CTLA	cytotoxic T-lymphocyte-associated protein
CYPB	cyclophilin B
DC	dendritic cells
DNA	deoxyribonucleic acid
EBI3	epstein-barr virus-induced gene 3
ELISA	enzyme-linked immunosorbent assays
Foxp3	forkhead box P3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GARP	glycoprotein A repetitions predominant
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glycophosphatidylinositol
GvHD	graft versus host disease
hDAF	human decay accelerating factor
HLA	human leukocyte antigen
ICOS	Inducible costimulator
IFN	Interferon
IL	interleukin
IMPDH	Inosine-5’-monophosphate dehydrogenase
iTreg	induced regulatory T cells
ivtRNA	in vitro transcripted RNA
JAK	janus kinases
LAP	latency-associated peptide
mAb	monoclonal antibody

MAC	membrane attack complex
MHC	major histocompatibility complex
MIRL	membrane inhibitor of reactive lysis
MMF	mycophenolate mofetil
MMR	macrophage mannose receptor
mRNA	messenger RNA
mTOR	mechanistic target of rapamycin
N	non-specific effector T cells
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor-κB
NK cells	natural killer cells
NKT	natural killer T cells
NN	non-specific effector T cells + non-specific regulatory T cells
NOD	non-obese diabetic
NP	non-specific effector T cells + porcine-specific regulatory T cells
NRP1	Neuropilin 1
NTeff	non-specific effector T cells
nTreg	natural regulatory T cells
NTreg	non-specific regulatory T cells
P	porcine-specific effector T cells
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-L1	programmed death-ligand 1
PD-L2	programmed death-ligand 2
PGE_2	prostaglandin E2
PN	porcine-specific effector T cells + non-specific regulatory T cells
PP	porcine-specific effector T cells + porcine-specific regulatory T cells
PS	porcine-specific
PSTeff	porcine-specific effector T cells
PSTreg	porcine-specific regulatory T cells
R848	resiquimod
RA	rheumatoid arthritis
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SATB1	special AT-rich sequence-binding protein-1
SCR	short consensus repeats

STAT	signal transducer and activator of transcription
Tcon	conventional T cells
TCR	T Cell Receptor
Teff	effector T cells
Tfr	T follicular regulatory cells
TGF	transforming growth factor
Th1	T helper 1 cells
Th17	T helper 17 cells
Th2	T helper 2 cells
TLR	toll like receptor
tolDC	tolerogenic dendritic cells
trans	transplantation
Treg	regulatory T cells
TSDR	regulatory T cells-specific demethylation region

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