7

Use of Flow Cytometry in the *In Vitro* and *In Vivo* Analysis of Tolerance/Anergy Induction by Immunocamouflage

Duncheng Wang, Wendy M. Toyofuku, Dana L. Kyluik and Mark D. Scott
Canadian Blood Services and The Department of Pathology and Laboratory Medicine and Centre for Blood Research at The University of British Columbia Canada

1. Introduction

Organ and tissue transplantations (including blood transfusions) are a critical care component for many life-threatening diseases. In transfusion and transplantation medicine, the concept of "self" is of crucial importance. Immunological "self" in transfusion medicine is primarily mediated by the ABO/RhD blood group antigens, though several hundred blood groups exist that can cause problems especially in the chronically transfused patient. For most other tissues, "self" is imparted by the major histocompatibility complex (MHC) proteins which provide a means for identifying, targeting and eliminating foreign (allorecognition) or diseased cells while preserving normal tissue. If the differences between donor and recipient occur only at minor MHC molecules (or non-ABO blood group antigens) or the transplanted antigens are only weakly antigenic or immunogenic, successful engraftment may result. In contrast, if significant differences in the exceedingly polymorphic MHC loci are present, or other highly antigenic (*e.g.,* ABO) or immunogenic antigens are present, allorecognition of the donor tissue occurs leading to rejection. Importantly, while rejection may be manifested as either Host versus Graft or Graft versus Host Disease (HVGD and GVHD, respectively), both are mediated by T lymphocyte (T cell) activation, differentiation and proliferation consequent to allorecognition. [Cote *et al.*, 2001] In this chapter, we will demonstrate how we have utilized flow cytometry to measure the induction of tolerance and/or anergy by polymer-mediated immunocamouflage using *in vitro* and *in vivo* models of allorecognition.

2. Allorecognition and allorejection

In HVGD, the host *(i.e.,* recipient) immune system recognizes (allorecognition) and rejects the allograft.[Li *et al.*, 2011, Dallman, 2001, Suthanthiran & Strom, 1995] Three major patterns of rejection can be identified based on the rapidity of graft injury: hyperacute (minutes to hours), acute (days to weeks) and chronic (weeks to years). [Goldstein, 2011, Battaglia, 2010, Weigt *et al.*, 2010] In hyperacute rejection not all parts of the graft are actively attacked. The primary site of injury is typically the vascular endothelium which can exhibit the ABO blood group antigens as well as MHC class I antigens. Damage is initiated by the binding of

complement fixing antibodies, complement activation, platelet aggregation, graft thrombosis, lytic damage, release of pro-inflammatory complement components (C3a and C5a) and leukocyte recruitment. These events result in the microvascular occlusion and the rapid loss of graft viability. Because hyperacute rejection occurs rapidly, few if any effective therapies for its prevention currently exist. Thus, many patients cannot be transplanted due to ABO-incompatibility or the presence of preformed anti-HLA antibodies. In contrast to hyperacute rejection, acute and chronic rejection are primarily cell mediated and initiated by T cell activation in context of mismatched MHC. Because of its central role, T cells have been the traditional focus of immunosuppressive drugs such as cyclosporin A. [Abadja *et al.*, 2009, Bonnotte *et al.*, 1996, Noris *et al.*, 2007]

GVHD is, essentially, a special category of HVGD.[Devetten & Vose, 2004] In GVHD, immunologically competent T cells, or their precursors, are transfused or transplanted into immunocompromised recipients. GVHD occurs most commonly in the setting of allogeneic bone marrow transplantation, but may also follow transfusion of whole blood into immunocompromised individuals and even some immunocompetent individuals. [Kleinman *et al.*, 2003] While both CD4+ (helper) and CD8+ (cytotoxic) T cells play a role in mediating tissue rejection in HVGD and GVHD, previous studies have found that depletion of MHC class II-recognizing CD4+ T cells to be most effective in preventing rejection.[Noris *et al.*, 2007] The MHC disparity between the donor and recipient induces the direct activation, differentiation and proliferation of either the recipient's (HVGD) or donor (GVHD) naïve T cells into effector subsets. Moreover, with regards to GVHD, surprisingly few T cells are necessary to induce a fatal outcome. Animal models suggest that as few as 10^7 donor lymphocytes/kg of recipient weight are sufficient to induce GVHD; though the degree of host immunosuppression and the overall disparity of the HLA antigens will exert a significant effect on the probability of GVHD induction.

The risk of donor tissue rejection (and to a lesser extent GVHD) has severely impacted the advancement of transplantation medicine. To counter these risks, pharmacologic interventions have been employed. Indeed, over the last 40 years, the significant improvements in transplantation success have been achieved primarily through immunosuppressive drugs that attenuate chronic tissue rejection. Because of the central importance of the T cell in graft rejection, thes pharmacological approaches have almost exclusively targeted T cell activation or proliferation (Figure 1A). [Allison, 2000] Perhaps the most prominent of these drugs has been cyclosporin, which blocks activation of resting T cells by inhibiting the signaling pathway necessary for the transcription of interleukin (IL)-2 and the high affinity IL-2 receptor. As a consequence, production of both IL-2 and its receptor are significantly diminished, thus removing the autocrine and paracrine stimulation necessary for T cell proliferation. Unfortunately, these drugs are nondiscriminatory and target not only the alloreactive, but all T cell proliferation leading to the induction of a general, non-selective, immunosuppressive state that is linked to both a chronic susceptibility to infective agents and an increased cancer risk. Moreover, even with pharmacologic intervention, long-term graft (as well as patient) survival is often problematic. In part, graft failure is a side effect of the immunosuppressive therapy as current pharmacologic agents exert both organ specific and systemic toxicity.

Hence, both HVGD and GVHD are T cell-mediated events that require antigenic recognition of the foreign tissue and the elicitation of a T cell-mediated immune response. These effector

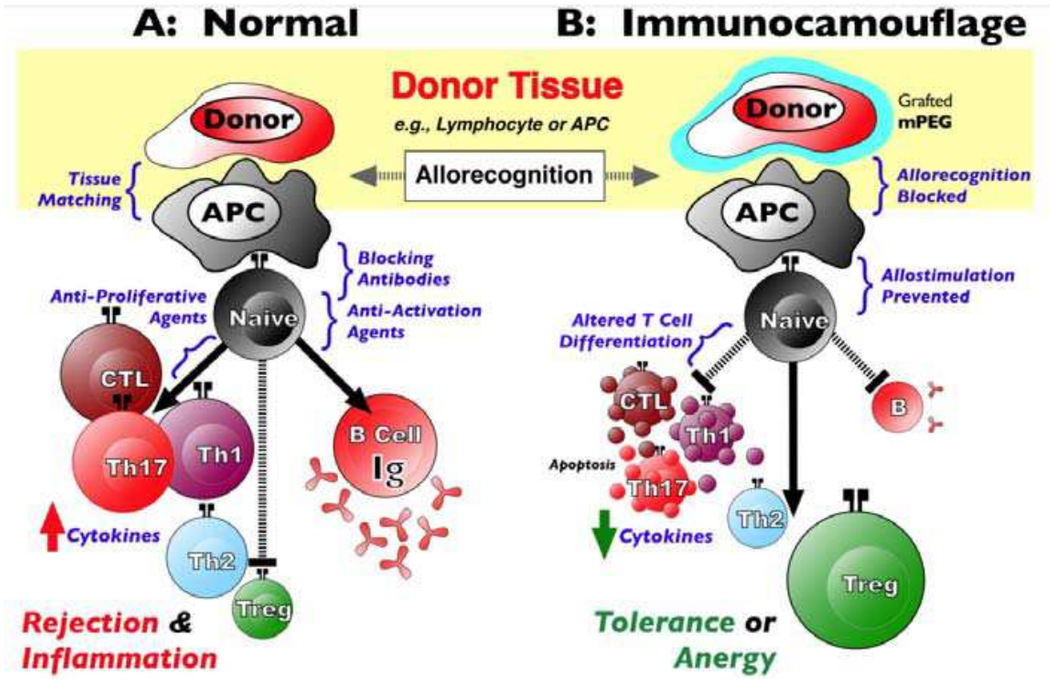


Fig. 1. Immune modulation via pharmacologic and immunocamouflage therapy. Panel A: Current pharmacologic therapy almost exclusively targets T cell activation and proliferation consequent to allorecognition. Response to non-self is in large part mediated by cell-cell interactions between Antigen Presenting Cells (APC; *e.g.*, dendritic cells) and naïve T cells. This cell-cell interaction is characterized by essential adhesion, allorecognition and co-stimulation events. Consequent to allorecognition, a proliferation of pro-inflammatory T cells (*e.g.*, CTL, Th17, Th1 populations) and decrease in regulatory T cells (Treg) is observed. Current therapeutic agents are primarily cytotoxic agents preventing T cell activation (*e.g.*, cyclosporine and rapamycin) or T cell proliferation (*e.g.*, methotrexate, corticosteroids and azathioprine). Additionally some blocking antibodies have been investigated. Panel B: In contrast, immunocamouflage of donor cells results in the disruption of the essential cell-cell interactions decreasing T cell proliferation and altering differentiation patterns (decreased Th17 and increased Tregs). In aggregate, these polymer induced changes induces a tolerogenic/anergic state both *in vitro* and *in vivo*. Size of T cell population denotes increase or decrease in number. Size of B cell indicates antibody response. Modified from: Wang *et al.* (2011). [Wang *et al.*, 2011]

cells arise via differentiation and proliferation of naïve T cells into proinflammatory and cytotoxic subsets (*e.g.*, Th17, Th1, CTL). While a number of different approaches to reduce the risk of tissue rejection are currently being examined, these strategies are, typically, unilaterally targeted to specific components of the allorecipient's T cell activation/ proliferation pathway (Figure 1A). These include blocking monoclonal antibodies directed against the TCR, CD4, co-stimulatory ligands and receptors, adhesion molecules, and cytokine receptors. Some of these approaches have undergone clinical testing (*e.g.*, Anti-CD3 monoclonal antibodies) and demonstrated some promising effects. However, concurrent with the anti-rejection efficacy, these agents have been plagued by both significant toxicity and an inability to adequately eliminate or inhibit reactive T cells. Hence, these approaches

are not commonly used at most transplant institutions. Clinical trials have also tested the use of pharmacological inhibitors of T cell proliferation and differentiation. Antiproliferative agents such as azathioprine and methotrexate interfere with cellular functions by limiting the metabolites necessary for DNA synthesis (Figure 1). However, these compounds also demonstrate significant toxicity to organs characterized by high proliferation rates (*e.g.,* gastrointestinal tract) and/or drug metabolism (liver), thus limiting their practical application.

Consequently, novel approaches that effectively attenuate the risk of HVGD/GVHD and directly target the difficult challenges of the inherent antigenicity and immunogenicity of human (and possibly xenogeneic) donor tissues would be of significant value to transplantation medicine. Biologically, the most attractive strategy to improve donor tissue engraftment and to simultaneously reduce drug toxicity, would be to induce immune tolerance and/or anergy in the recipient's immune system thereby negating the need for toxic immunosuppressive pharmacologic agents. Tolerance may be viewed as a relatively specific non-responsiveness to a unique antigen while anergy is a more broad-spectrum attenuation of the immune response. As with cell-mediated rejection, tolerance and anergy are also mediated by a T cells subset (T regulatory cells; Tregs). [Muller *et al.*, 2011]

3. Immunocamouflage: Concept and mechanism of action

To prevent allorecognition and alloimmunization, our laboratory has pioneered the 'immunocamouflage' of donor cells and tissues (Figure 1B). [Bradley *et al.*, 2002, Bradley & Scott, 2004, Chen & Scott, 2001, Chen & Scott, 2003, Le & Scott, 2010, McCoy & Scott, 2005, Murad *et al.*, 1999a, Murad *et al.*, 1999b, Rossi *et al.*, 2010a, Rossi *et al.*, 2010b, Scott *et al.*, 2003, Scott *et al.*, 1997, Sutton & Scott, 2010] The immunocamouflage of cells and tissues is created by the covalent grafting of safe, non-toxic and low-immunogenic biocompatible polymers such as methoxypoly(ethylene glycol, PEGylation) and hyperbranched polyglycerols (HPG) to the surface of cells. The efficacy of immunocamouflage is dependent upon both the density and depth (*i.e.,* thickness) of the polymer layer. As shown in Figure 2, a rigid linear molecules lacks any significant radius of gyration (R_g; space filling) resulting in a poor or absent camouflaging (*a,b*) of the membrane antigens. In contrast, polymers with either high intra-chain flexibility (*e.g.,* methoxypoly(ethylene glycol); mPEG; *c,d*) or inherent density (*e.g.,* hyperbranched polyglycerols; HPG; *e*) exhibit either a significant radius of gyration (mPEG) or space filling capacity (HPG). Thus, while mPEG is a linear molecule, its high degree of intra-chain flexibility (due to the repeating, highly mobile, ethoxy units) gives rise to its expansive Rg. Moreover, due to its hydroscopic nature, the heavily hydrated polymer is able to sterically occlude a large three dimensional volume (Figure 2). Consequent to the space filling capacity of mPEG, the immunocamouflage of surface membrane proteins and carbohydrates (potential antigenic sites) occurs. In addition, the mPEG coating obscures the inherent electrical charge associated with surface proteins since the charged molecules become buried beneath the viscous, hydrated, neutral PEG layer thus further diminishing the antigenic/immunologic character of the cell surface. [Bradley *et al.*, 2002, Bradley & Scott, 2004, Le & Scott, 2010] As denoted in Figure 2, the longer the mPEG polymer chain the larger the membrane surface area covered by this steric shield (c, d and stippled area). In contrast to the flexible linear mPEG, HPG molecules are highly branched structures that exhibit limited flexibility but, due to its extensive branching, create a dense steric shield (Figure 2e). [Rossi *et al.*, 2010a, Rossi *et al.*, 2010b]

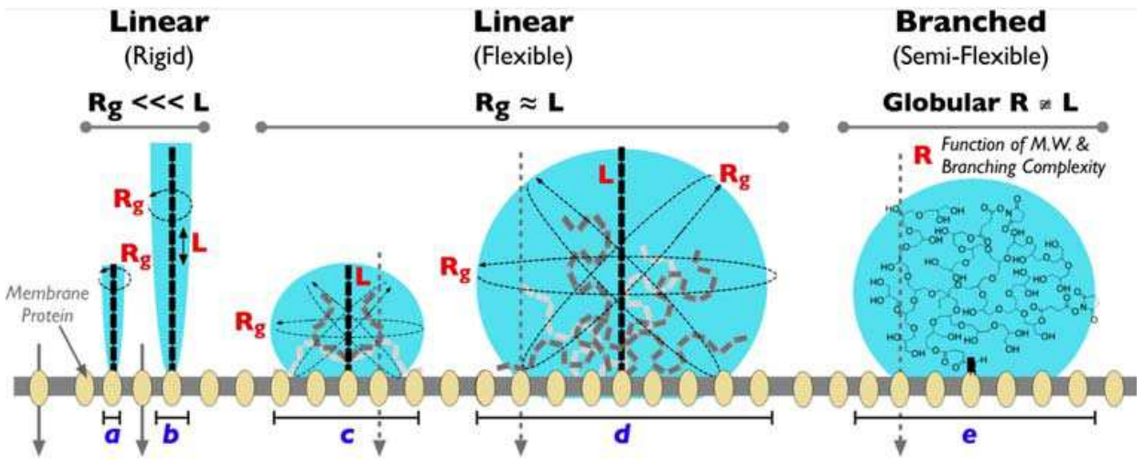


Fig. 2. Immunocamouflage of cells is produced via the grafting of polymers to membrane proteins. The linear flexible molecule and branched, semi-flexible, grafted polymers creates a steric barrier preventing the approach and binding of proteins (*e.g.*, antibodies) and cells (*e.g.,* APC or T cells) but along for metabolite (dashed arrows; *e.g.,* glucose) uptake. In contrast, rigid linear polymers produce a minimal steric barrier. Some polymers, such as mPEG, also very effectively camouflage membrane surface charge. Result present in this chapter all refer to methoxy(polyethylene glycol) [mPEG], a linear flexible polymer (*c, d*). L=polymer length as denoted by molecular weight while R_g is the radius of gyration

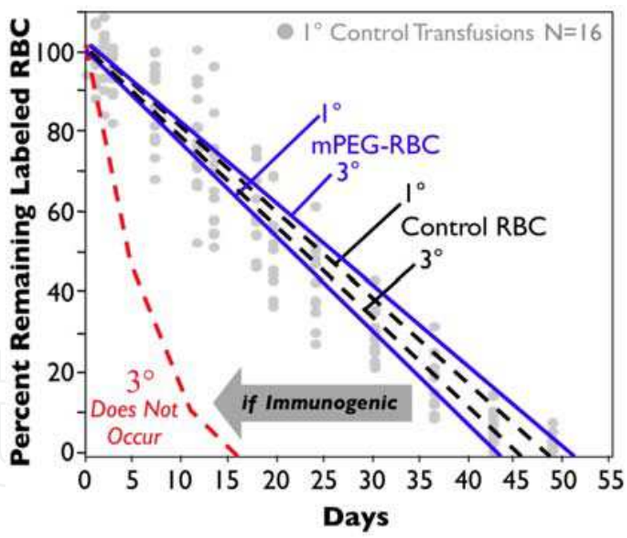


Fig. 3. Immuno-camouflage of murine RBC with mPEG (SVAmPEG; 20 kDa) results in normal *in vivo* survival. Donor RBC survival was measured via flow cytometry and detection of a fluorescent marker (PKH-26) inserted into the RBC membrane via a lipid tail. The theoretical effect of the production of anti-mPEG antibodies is shown by the red dashed line representing a tertiary transfusion of modified RBC

Previous studies in our laboratory on red blood cells, lymphocytes, pancreatic islets and viral models have demonstrated that the immunocamouflage of tissues is effective, reproducible and does not impair tissue function. [Bradley *et al.*, 2002, Bradley & Scott, 2004,

Chen & Scott, 2001, Chen & Scott, 2003, Le & Scott, 2010, McCoy & Scott, 2005, Murad *et al.*, 1999a, Murad *et al.*, 1999b, Rossi *et al.*, 2010a, Rossi *et al.*, 2010b, Scott *et al.*, 2003, Scott *et al.*, 1997, Sutton & Scott, 2010] For example, the PEGylated red blood cell function (*i.e.,* O_2 delivery and cellular deformability) were unaffected by the grafted polymer and exhibited normal *in vivo* circulation and lifespan (~50 day) in a murine transfusion model even after repeated transfusions (Figure 3). With relevance to tissue transplantation, our studies have demonstrated that transfusion of immunocamouflaged allogeneic murine splenocytes prevents allorecognition by either the donor cells (*e.g.,* Transfusion-Associated Graft vs. Host Disease model) or the recipients (*e.g.*, graft rejection model) immune system. [Chen & Scott 2003, Chen & Scott 2006] The loss of allorecognition is not accompanied by any systemic or local toxicity. More surprisingly, recent adoptive transfer studies within our laboratory using a murine model have demonstrated that immunocamouflaged leukocytes may be able to induce long-lasting systemic immunotolerance. [Wang *et al.*, 2011] An important and exceptionally powerful tool in assessing the efficacy of immunocamouflage in both *in vitro* and *in vivo* modeling systems has been flow cytometry.

4. Assessing polymer-mediated immunomodulation by flow cytometry: T cell camouflage, differentiation and proliferation

Flow cytometry is essential in characterizing both *in vitro* and *in vivo* immunological response including cell proliferation (CSFE staining and murine H2 determination), cytokine expression (cytometric bead array), intra-cellular signaling cascades, lymphocyte differentiation (*e.g.,* Tregs and Th17 cells) and *in vivo* cell trafficking (*e.g.,* thymus, spleen, lymph node and blood). Indeed, as will be shown in this chapter, flow cytometry is an exceptionally powerful tool in investigating the induction of tolerance and/or anergy in experimental models.

Mechanistically, allorecognition requires multiple sustained interactions between the donor and recipient tissues for the activation, differentiation and proliferation of alloresponsive T cell to occur. These interactions, involving both external receptor-ligand interactions and intracellular signalling cascades fall within three general categories: 1) cell:cell adhesion events; 2) allorecognition; and 3) costimulation. [Chen & Scott, 2001, Murad *et al.*, 1999a] Blockade or attenuation of any (or all) of these events will reduce allorecognition and allograft rejection. The *in vitro* and *in vivo* effects of polymer-mediated immunocamouflage on these essential events are readily measured via flow cytometry.

4.1 Immunocamouflage of membrane markers

Multiple receptor-ligand interactions (encompassing adhesion, allorecognition and costimulation pathways) between the T cell and APC are essential for successful allostimulation. The membrane proteins involved in these cell:cell events have been well characterized over the last several years and often targeted by experimental therapies. Importantly, the efficacy of immunocamouflage can be readily assessed by the literal camouflage of these markers using flow cytometry.

Previously, we investigated the efficacy of immunocamouflage in a murine model of transfusion-associated graft versus host disease (TA-GVHD). [Chen & Scott, 2003, Chen & Scott, 2006] Using this model, flow cytometric analysis demonstrated that polymer grafting

significantly camouflaged membrane proteins involved in adhesion, allorecognition and co-stimulation events. As shown in Table 1, the immunocamouflage of allogeneic donor lymphocytes resulted in the efficient camouflage of donor leukocyte membrane markers. These membrane proteins are still present on the surface of the allogeneic leukocytes but are camouflaged by the grafted polymer from detection by anti-marker antibodies. Consequent to the polymer-mediated camouflage, signal transduction is block or significantly attenuated.

mM mPEG	Polymer *m.w.*	CD3ε	T cell receptor	CD11a	MHC Class II	CD4	CD25	CD28
0	-	100%	100%	100%	100%	100%	100%	100%
0.6	5 kDa	59*	20*	49*	100	75	ND	64*
	20 kDa	73*	14*	53*	100	84	67*	35*
1.2	5 kDa	53*	11*	39*	100	65*	62*	31*
	20 kDa	27*	2*	26*	45*	53*	67*	30*
2.4	5 kDa	38*	7*	30*	59*	7*	12*	31*
	20 kDa	3*	5*	7*	8*	3*	67*	40*

Table 1. The covalent grafting of mPEG to membrane proteins results in the global camouflage of multiple proteins crucial for effective allorecognition of foreign tissue. Values shown are "Percent Positive Cells" relative to the unmodified control (=100%). Results shown are the mean of a minimum of 3 independent experiments. * $p < 0.01$ compared to unmodified controls

4.2 Effect of immunocamouflage on cytokine-chemokine release

Consequent to the camouflage of the receptor-ligands necessary for alloresponsiveness, a dramatic reduction in cytokine/chemokine release is observed. Flow cytometry provides a valuable tool for the simultaneous measurement of cytokine (signalling molecules integral to the immune response) production, T cell proliferation and differentiation. Historically, cytokines were commonly measured using enzyme-linked immunosorbent assays (ELISA or EIA); a relatively low throughput system. Flow cytometry has greatly increased both the speed and sensitivity of cytokine measurements both *in vitro* and *in vivo*. One of the most powerful flow cytometric tools in this regard is the BD™ Cytometric Bead Array (CBA; BD™ Biosciences, San Diego, CA). There are significant advantages for using the CBA versus ELISA. The CBA Array system is a multiplex quantitive assay requiring fewer sample dilutions and utilizing a single set of standards to generate a standard curve for each analyte. Moreover, the throughput of the CBA is significantly greater than ELISA (*e.g.,* 1/2 day for the CBA Array versus 2-3 days for ELISA).

The CBA system is a multiplexed bead based immunoassay used to simultaneously quantitate multiple soluble cytokines within a single sample by fluorescence-based emission. This assay allows multiple simultaneous cytokine determinations in serum, plasma, cell lysates or tissue culture supernatants. As shown in Figure 4, each sample preparation can contain multiple bead populations, each with distinct fluorescence intensity. Each of these bead

populations (capture beads) are coated with an antibody for a specific cytokine and essentially mimics an individually coated well in an ELISA plate. The capture beads are mixed with the sample of interest (standard or experimental sample) and a PE-conjugated detection antibody to form a sandwich complex and resolved using the FL3 or FL4 channel. The data is analyzed using specific software (FCAP Array™ and BD CBA™ analysis software; BD Biosicences, San Diego, CA and Soft Flow Inc, St. Louis Park, MN) to provide a quantitative value for the selected cytokines.

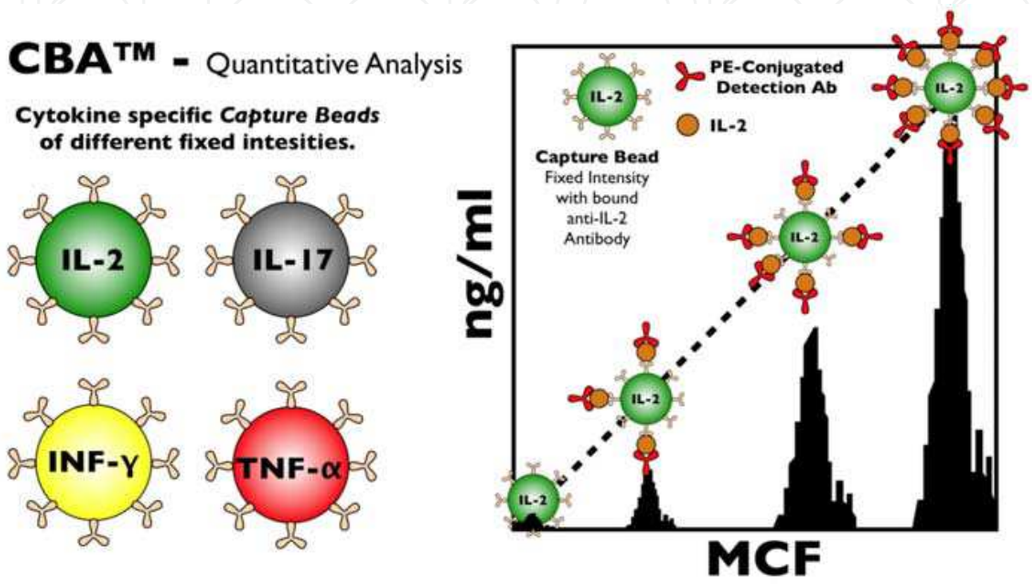


Fig. 4. Quantitative Cytometric Bead Array™. Capture beads are conjugated to analyte specific antibodies (*e.g.*, anti-Il-2, Il-17, INF-γ or TNF-α as shown). Each bead has a different fluorescent intensity allowing for analyate discrimination

For our experiments, a BD™ CBA Human Th1/Th2 Cytokine Kit (Catalog No. 550749) and Murine Th1/Th2 Cytokine Kit (Catalog No. 551287) were utilized to determine the effects of immunocamouflage on allorecognition *in vitro* and *in vivo*. For *in vitro* human or mouse studies, cell culture supernatants were collected and stored at -80°C prior to analysis. For flow cytometric analysis samples were incubated with desired detection kit (*e.g.,* Th1/Th2) capture beads and a PE-conjugated detection antibody and aquired using a BD FACSCaliburTM flow cytometer and the Cell Quest Pro Software. Cytokine protein levels were analyzed using the BD™ Cytometric Bead Array and FCAP Array™ analysis software (BD Biosicences, San Diego, CA and Soft Flow Inc, St. Louis Park, MN).

As demonstrated in Figure 5, resting human PBMC, as well as resting PEGylated PBMC, expressed little detectable IL-2, TNF-α or INF-γ. [Wang *et al.*, 2011] However, when two disparate populations (two-way mixed lymphocyte reaction; 2-way MLR) of PBMC are mixed together, allorecognition occurs resulting in very significant increases in these cytokines. In stark contrast to the positive control MLR, the PEGylation of either donor population results in an immunoquiescent state characterized by baseline (*i.e.,* resting PBMC) levels of these cytokines. Also shown in Figure 5 is a diagrammatic presentation of how the capture beads detect cytokine levels of the control and PEGylated MLR.

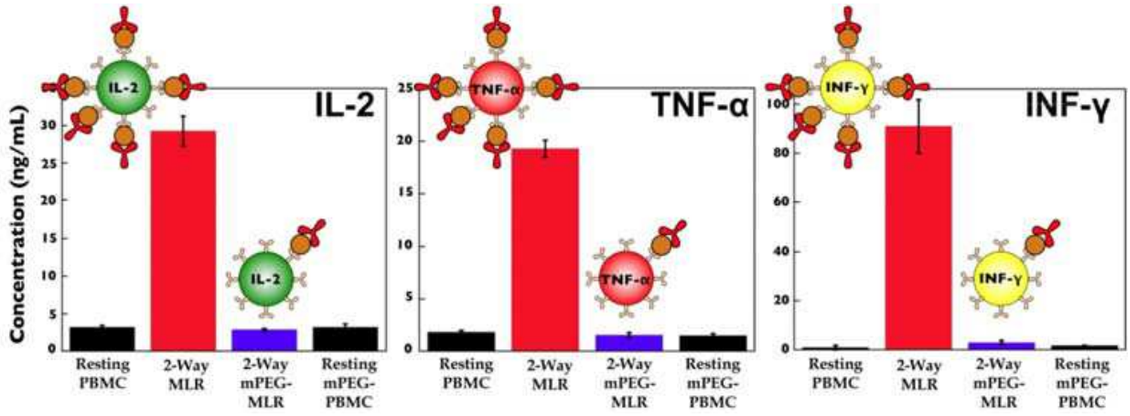


Fig. 5. Effect of immunocamouflage on cytokine secretion as measured by flow cytometry using the BD Cytometric Bead Array™ (CBA). Also shown is a diagrammatic representation of the capture beads and bound analayte for the control 2-way MLR and mPEG 2-way MLR. Modified from Wang *et al.* (2011). [Wang et al., 2011]

4.3 Effect of immunocamouflage on *In Vitro* and *In Vivo* T cell proliferation and differentiation

For decades, ^{3}H-thymidine incorporation was the standard for measuring cell proliferation in MLRs. While highly useful *in vitro*, this methodology is less useful *in vivo*. Moreover, radionucleotides bring additional regulatory burden to research laboratories. Hence, alternative approaches for measuring proliferation have been developed. Multiple flow cytometric tools are available for this purpose. Perhaps the most common of these flow methodologies is a dye dilution assay utilizing carboxyfluorescein succinimidyl ester (CFSE). Importantly, the CFSE assay is a valuable tool for both *in vitro* and *in vivo* proliferation assays.

CFSE was originally developed to label lymphocytes and track their *in vivo* migration over several months. [Weston & Parish, 1990] Subsequent studies demonstrated its utility in measuring lymphocyte proliferation both *in vitro* and *in vivo* consequent to the progressive dilution of CFSE fluorescence within daughter cells following each cell division. For cell staining, carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; a non-fluorescent precursor to CFSE), a highly cell permeable compound, is added to a cell population (*e.g.*, human PBMC or murine splenocytes) where it enters the cytoplasm of cells. Within the cell, intracellular esterases remove the acetate groups present on CFDA-SE and convert the molecule to the fluorescent ester, CFSE. The CFSE molecule is permanently retained within cells due to its covalent coupling to intracellular molecules via its succinimidyl group. The CSFE signal is extremely stable allowing for long-term studies and is not transferred to adjacent cells. However, upon allostimulation (or mitogen challenge) and proliferation, the CFSE dye is evenly diluted between the daughter cells and continues to be diluted with subsequent proliferation of these cells (Figure 6). With optimal labelling, 7-8 cell divisions can be identified before the CFSE fluorescence is indistinguishable from backgound autofluorescence. Thus, this assay thereby provides significant quantitative data as to the percentage of alloresponsive cells within a population as well as the degree of proliferation over a course of several days to weeks.

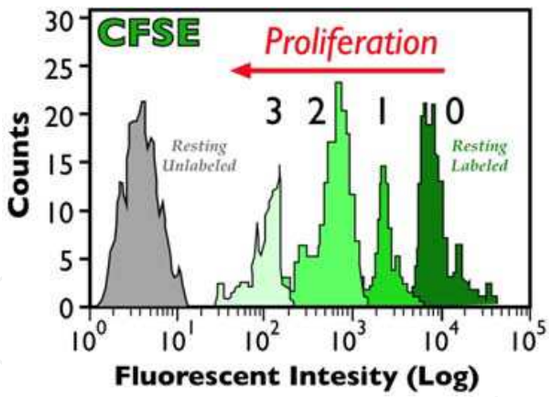


Fig. 6. Flow cytometric analysis of intracellular CFSE can be used to measure cell proliferation based on dye-dilution within the daughter cells (0-4). CFSE can be used both *in vitro* and *in vivo* where it can also be used to investigate cell trafficking. The grey peak represents unlabeled cells

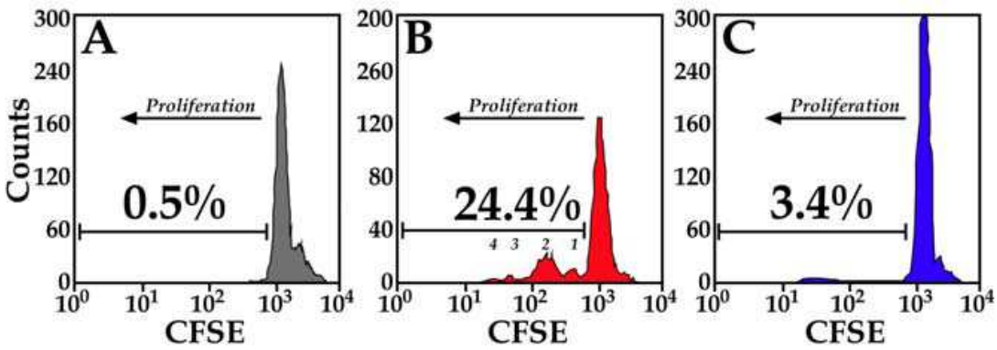


Fig. 7. Proliferation of CD4+ T cells in resting PBMC (A), Control MLR (B) and mPEG-MLR (C). Denoted in B are the proliferation (*1-4*) peaks of the alloresponsive subpopulation. Shown is the flow cytometric analysis of a representative experiment of human PBMC

The ultimate efficacy of immunocamouflage of allogeneic cells in MLRs is primarily assessed by the loss of proliferation. As shown in Figure 7, polymer grafting to either immunologically disparate population results in the loss of cell proliferation. [Wang *et al.*, 2011] Importantly, this is not due to any loss of viability of the polymer modified cells as direct mitogen stimulation yields normal nearly identical proliferation rates as unmodified cells (as measured by CFSE or ^{3}H-thymidine incorporation; not shown). The loss of proliferation is consequent to the global immunocamouflage of the receptor-ligand markers described in Table 1.

In vivo cell trafficking of allogeneic donor cells is also of importance in immunological studies. While this can be done using radiolabelled cells, it is a low resolution assay and is often quantitated as simply CPM per organ. In contrast, flow cytometry can provide significantly improved information. In murine studies it is possible to look at the proliferation of allogeneic donor cells based on flow cytometric analysis of the murine haplotype (H2). In an *in vivo* murine model of transfusion associated graft versus host disease (TA-GVHD) in which mice are transfused with allogeneic cells, significant proliferation of the donor cells is observed in lymphatic tissues such as the spleen after 28

days (Figure 8). [Chen & Scott, 2003, Chen & Scott, 2006] In contrast, if the allogeneic donor cells are PEGylated prior to their transfusion, minimal allorecognition of the foreign host tissues occurs and there is no significant proliferation of the donor splenocytes (Figure 8). While not shown, CFSE labelling can also be an important flow cytometric tool for simultaneously monitoring the trafficking and proliferation of donor cells *in vivo*. Both H2 markers and/or CSFE can be further coupled with the use of fluorescent antibodies against lymphocyte cell surface markers (*e.g.*, CD4, CD25, IL-17R, *etc.*) making it possible to follow *in vivo* lymphocyte differentiation. Due to the non-toxic nature of CFSE, labelled viable cells can be recovered via cell flow cytometric cell sorting for further *in vitro* analysis.

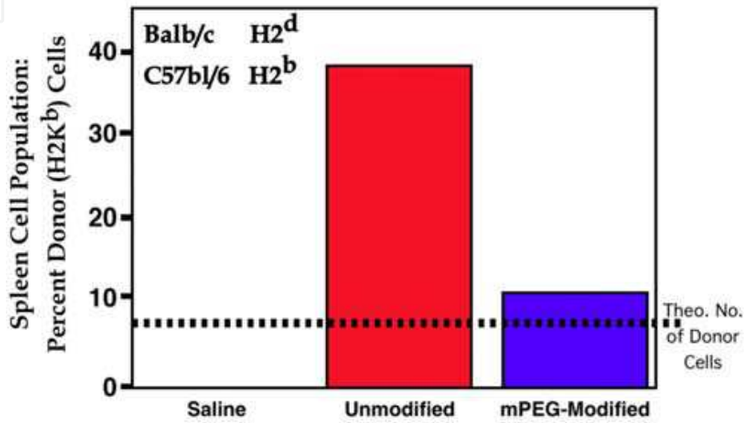


Fig. 8. Immunocompetent Balb/c mice ($H2K^d$) were transfused with 1.5 x 10^7 unmodified or mPEG-modified C57Bl/6 splenocytes ($H2K^b$) on days 0 and 14. At day 28, mice were sacrificed and the *in vivo* donor cell proliferation was assessed by flow cytometry using an anti-$H2K^b$ monoclonal antibody. Shown are the mean precent donor cells within the spleens of the recipient animals (n=4/group)

Differentiation of naïve T cells is an important regulator of the immune response or lack of response. [Heidt *et al.*, 2010, Nistala & Wedderburn, 2009, O'Gorman *et al.*, 2009, Oukka, 2007] As our understanding of the biological functions of T cells has expanded a vast array of T cell subsets, all of which have unique immunological functions and phenotypes, have been described. Indeed, T cell differentiation is a crucial indicator of whether an inflammatory or tolerogeneic response is induced consequent to exposure to control or polymer-modified allogeneic tissue. Fortunately, flow cytometry provides a high throughput tool for the analysis of T cell differentiation.

Consequent to the immunocamouflage of the receptor-ligands involved in adhesion, allorecognition and costimulation (*see* Table 1) T cell activation and proliferation was significantly attenuated (*see* Figures 7-8). However, these findings did not elucidate if there was a differential proliferation effect between T cell subsets. Hence, flow cytometric T cell subset phenotyping was used to further investigate the effects of immunocamouflage on T cell differentiation both *in vitro* and *in vivo*. [Afzali *et al.*, 2007, Hanidziar & Koulmanda, 2010, Heidt *et al.*, 2010, Mitchell *et al.*, 2009, Weaver & Hatton, 2009] For example, as shown in Figure 9, *in vivo* challenge with unmodified allogeneic cells results in the upregulation of proinflammatory Th17 cells and a downregulation of immunosuppressive Treg cells within the spleen as determined by flow cytometry. [Wang *et al.*, 2011] In contrast, the

immunocamouflage of the allogeneic cells resulted in increased Treg cells significantly above that of naïve mice and a virtually complete abrogation of the expected Th17 increase. Importantly, soluble polymer as well as unmodified or mPEG-modified syngeneic cells demonstrated no effects on the *in vivo* differentiation of Treg or Th17 cells. Similar finding in the lymph nodes and blood were observed. Indeed, as summarized in Figure 10, the immunocamouflage of allogeneic human (*in vitro*) and mouse (*in vitro* and *in vivo*) leukocytes has been experimentally shown to dramatically influence T cell differentiation resulting in the induction and persistence of a tolerogeneic/anergic state. [Chen & Scott, 2003, Chen & Scott, 2006, Wang *et al.*, 2011]

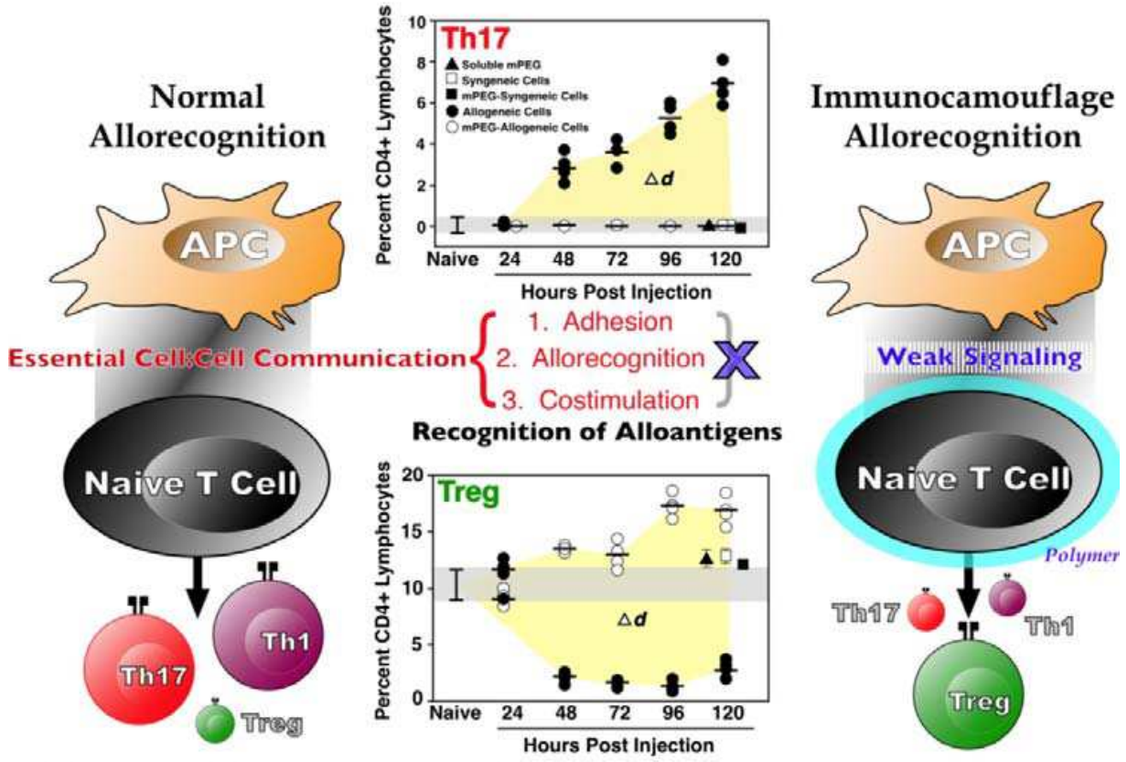


Fig. 9. Flow cytometric studies demonstrate that immunocamouflage of allogeneic murine splencoytes alters the *in vivo* systemic differentiation of Th17 and Treg lymphocytes. Shown are the percent Th17 and Treg CD4+ lymphocytes within the spleen of animals transfused with control or mPEG allogeneic splencocytes. Similar finding in the lymph nodes and blood were observed. Grey bars denote baseline levels of Th17 and Treg cells in naïve animals. The Δ*d* (yellow area) denotes the absolute difference between unmodified and mPEG-modified allogeneic splenocytes *Derived from Wang, Chen and Scott (2011)*

T cell subset differentiation is driven by variable intracellular signaling cascades triggered by the adhesion, allorecognition, costimulatory and cytokine receptor-ligand interactions occurring at the membrane of the naïve T cell. In the case of immunosuppressive Treg cells and the proinflammatory Th17 cells, the key nuclear transcription factors FoxP3+ and RORγ+, respectively, are used to detect these cell types via flow cytometry. Moreover, these markers can be combined with Phosflow™ assays to provide additional activation

information. [Krutzik *et al.*, 2004] This technique combines flow cytometric analysis of phosphospecific antibodies with, for example, lymphocyte subtyping allowing for more specific identification of activated cell types. Phosphospecific antibodies are specific to tyrosine or serine phosphorylated signaling intermediates, thus antibody will only bind to a protein in its phosphorylated state. Since phosphorylation is a key mechanism of regulation in signaling pathways, this methodology enables the studies of a vast number of pathways in a variety of cells. This technology provides significantly enhanced resolution relative to western blots or ELISAs that only assess the overall activity of an entire population and that do not allow for easy isolation or phenotypic identification of the alloreactive cells involved.

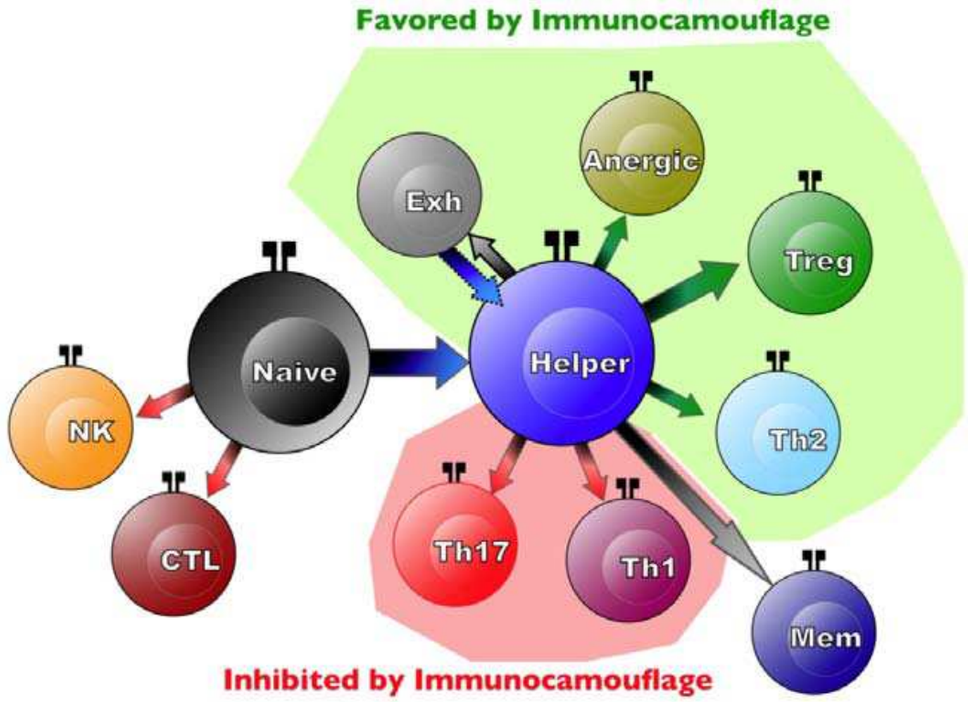


Fig. 10. Flow cytometric analysis of T cell differentiation both in vitro and in vivo demonstrates the polymer grafting to allogeneic leukocytes significantly influences the differentiation of naïve T cells. As shown diagrammatically above, immunocamouflage of allogeneic cells favors tolerogeneic/anergic T cell subsets and significantly inhibits proinflammatory T cell populations. Lymphocyte abbreviations: NK, natural killer; CTL, cytotoxic; Mem, memory; and Exh, exhausted (TCR-) T cells

For example, as illustrated in Figure 11, initiation of differentiation of the Th17 T cell lineage is controlled by the master regulatory transcription factor RORγ. This transcription factor is upregulated in response to IL-6 and TGF-β that, together with the T cell receptor (TCR) signal, initiate differentiation of Th17 from naive T cells via the Gp130-STAT3 pathways. [Kitabayashi *et al.*, 2010, Nishihara *et al.*, 2007] Il-6 acts directly to promote the development of Th17 by binding to the membrane IL-6 receptor of T cells and its signal transducer protein Gp130. Binding of the cytokine induces activation (phosphorylation) of Jaks which serve as a docking site for STAT3. STAT3 is then phosphorylated by Jak inducing its dimerization, nuclear translocation and DNA binding. To assess the activation of this pathway consequent

to mitogen or allostimulation, cells can be fixed, permeabilized and stained for phosphospecific (Tyr705) STAT3 and analyzed via flow cytometry.

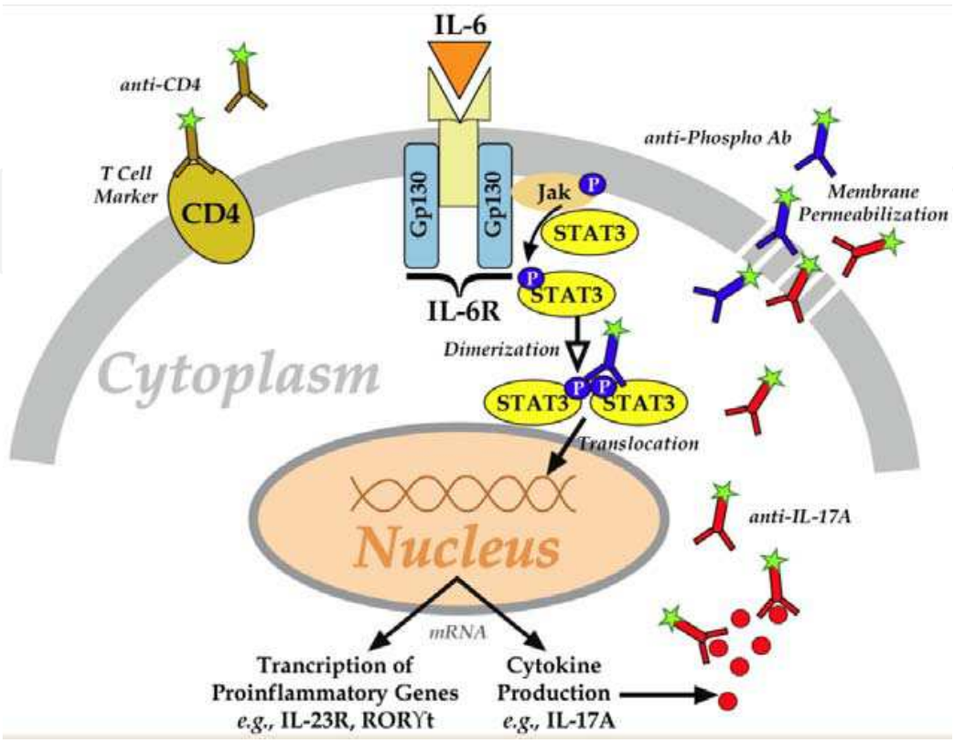


Fig. 11. Phosphospecific flow cytometry can measure the phosphorylation state of intracellular proteins at the single cell level. Many different phosphorylation states can be measured simultaneously in each cell, along with other intracellular (*e.g.*, IL-17A) and/or surface (e.*g.*, CD4) markers, enabling complex signaling networks to be resolved. Phosflow assays are performed by stimulating cells, fixing and permeabilizing before staining with phosphospecific fluorophore conjugated antibody for flow cytometric analysis

Thus, by using the wide variety of flow cytometric tools available, it is possible to detect changes within a small subpopulation of a heterogenous cell population without need to go through expensive cell sorting or purification steps. Finally, powerful analysis can be done using multiple parameter flow cytometer staining. However, timing of phosphorylation events with membrane surface phenotype expression, gene transcription and cytokine release still pose a significant challenge in the utilization of the Phosflow™ technology.

5. Conclusion

Flow cytometry has assumed an increasingly important role in the diagnosis and monitoring of disease progression or regression in modern medicine.[Chesney *et al.*, 2011, Dieterlen *et al.*, 2011, Hedley *et al.*, 2011, Hernandez-Fuentes & Salama, 2006, Mittag & Tarnok, 2011, Panzer & Jilma, 2011, Tung *et al.*, 2007, Venet *et al.*, 2011] Indeed, flow cytometry has proved to be an exceptionally powerful tool in assessing the rejection or acceptance of allogeneic tissues. Flow cytometry is essential in characterizing both *in vitro* and *in vivo* immunological response including cell proliferation (CSFE staining and murine

H2 determination), cytokine expression (cytometric bead array), intra-cellular signaling cascades, lymphocyte differentiation (*e.g.,* Tregs and Th17 cells) and *in vivo* cell trafficking (*e.g.,* thymus, spleen, lymph node and blood). Moreover, the flow cytometry tools available for immunological studies continue to expand thereby enhancing our ability to decipher the immune response to non-self antigens. By using the unique tools provided by flow cytometry, our own studies have been better able to elucidate the mechanisms by which the polymer-mediated immunocamouflage of allogeneic cells influences T cell differentiation (both *in vitro* and *in vivo*) to produce a stable tolerogeneic/anergic state. [Chen & Scott, 2003, Chen & Scott, 2006, Murad *et al.*, 1999a, Wang *et al.*, 2011]

6. Acknowledgment

This work was supported by grants from the Canadian Blood Services, Canadian Blood Services-Canadian Institutes of Health Research (CBS-CIHR) Partnership Fund and Health Canada. The views expressed herein do not necessarily represent the view of the federal government of Canada. We thank the Canada Foundation for Innovation and the Michael Smith Foundation for Health Research for infrastructure funding at the University of British Columbia Centre for Blood Research.

7. References

Abadja, F., Videcoq, C., Alamartine, E., Berthoux, F., Mariat, C. (2009) Differential effect of cyclosporine and mycophenolic acid on the human regulatory T cells and TH-17 cells balance. *Transplant Proc,* 41(8), 3367–3370.

Afzali, B., Lombardi, G., Lechler, R. I., Lord, G. M. (2007) The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. *Clin Exp Immunol,* 148(1), 32–46.

Allison, A. C. (2000) Immunosuppressive drugs: the first 50 years and a glance forward. *Immunopharmacology,* 47(2-3), 63–83.

Battaglia, M. (2010) Potential T regulatory cell therapy in transplantation: how far have we come and how far can we go? *Transpl Int,* 23(8), 761–770.

Bonnotte, B., Pardoux, C., Bourhis, J. H., Caignard, A., Burdiles, A. M., Chehimi, J., Mami-Chouaib, F., Chouaib, S. (1996) Inhibition of the human allogeneic mixed lymphocyte response by cyclosporin A: relationship with the IL-12 pathway. *Tissue Antigens,* 48(4 Pt 1), 265–270.

Bradley, A. J., Murad, K. L., Regan, K. L., Scott, M. D. (2002) Biophysical consequences of linker chemistry and polymer size on stealth erythrocytes: size does matter. *Biochim Biophys Acta,* 1561(2), 147–158.

Bradley, A. J., Scott, M. D. (2004) Separation and purification of methoxypoly(ethylene glycol) grafted red blood cells via two-phase partitioning. *J Chromatogr B Analyt Technol Biomed Life Sci,* 807(1), 163–168.

Chen, A. M., Scott, M. D. (2001) Current and future applications of immunological attenuation via pegylation of cells and tissue. *BioDrugs,* 15(12), 833–847.

Chen, A. M., Scott, M. D. (2003) Immunocamouflage: prevention of transfusion-induced graft-versus-host disease via polymer grafting of donor cells. *J Biomed Mater Res A,* 67(2), 626–636.

Chen, A. M., Scott, M. D. (2006) Comparative analysis of polymer and linker chemistries on the efficacy of immunocamouflage of murine leukocytes. *Artif Cells Blood Substit Immobil Biotechnol,* 34(3), 305–322.

Chesney, A., Good, D., Reis, M. (2011) Clinical utility of flow cytometry in the study of erythropoiesis and nonclonal red cell disorders. *Methods Cell Biol,* 103, 311–332.

Cote, I., Rogers, N. J., Lechler, R. I. (2001) Allorecognition. *Transfus Clin Biol,* 8(3), 318–323.

Dallman, M. J. (2001) Immunobiology of graft rejection. In *Pathology and immunology of transplantation and rejection* (S. Thiru, H. Waldmann, eds.), Blackwell Science Ltd, Oxford, UK, 1–19.

Devetten, M. P., Vose, J. M. (2004) Graft-versus-host disease: how to translate new insights into new therapeutic strategies. *Biol Blood Marrow Transplant,* 10(12), 815–825.

Dieterlen, M. T., Eberhardt, K., Tarnok, A., Bittner, H. B., Barten, M. J. (2011) Flow cytometry-based pharmacodynamic monitoring after organ transplantation. *Methods Cell Biol,* 103, 267–284.

Goldstein, D. R. (2011) Inflammation and transplantation tolerance. *Semin Immunopathol,* 33(2), 111–115.

Hanidziar, D., Koulmanda, M. (2010) Inflammation and the balance of Treg and Th17 cells in transplant rejection and tolerance. *Curr Opin Organ Transplant,* 15(4), 411–415.

Hedley, D. W., Chow, S., Shankey, T. V. (2011) Cytometry of intracellular signaling: from laboratory bench to clinical application. *Methods Cell Biol,* 103, 203–220.

Heidt, S., Segundo, D. S., Chadha, R., Wood, K. J. (2010) The impact of Th17 cells on transplant rejection and the induction of tolerance. *Curr Opin Organ Transplant,* 15(4), 456–461.

Hernandez-Fuentes, M. P., Salama, A. (2006) In vitro assays for immune monitoring in transplantation. *Methods Mol Biol,* 333, 269–290.

Kitabayashi, C., Fukada, T., Kanamoto, M., Ohashi, W., Hojyo, S., Atsumi, T., Ueda, N., Azuma, I., Hirota, H., Murakami, M., Hirano, T. (2010) Zinc suppresses Th17 development via inhibition of STAT3 activation. *Int Immunol,* 22(5), 375–386.

Kleinman, S., Chan, P., Robillard, P. (2003) Risks associated with transfusion of cellular blood components in Canada. *Transfus Med Rev,* 17(2), 120–162.

Krutzik, P. O., Irish, J. M., Nolan, G. P., Perez, O. D. (2004) Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. *Clin Immunol,* 110(3), 206–221.

Le, Y., Scott, M. D. (2010) Immunocamouflage: the biophysical basis of immunoprotection by grafted methoxypoly(ethylene glycol) [mpeg]. *Acta Biomater,* 6, 2631–2641.

Li, J., Lai, X., Liao, W., He, Y., Liu, Y., Gong, J. (2011) The dynamic changes of Th17/Treg cytokines in rat liver transplant rejection and tolerance. *Int Immunopharmacol,* 11, 962–967.

McCoy, L. L., Scott, M. D. (2005) Broad spectrum antiviral prophylaxis: Inhibition of viral infection by polymer grafting with methoxypoly(ethylene glycol). In *Antiviral Drug Discovery for Emerging Diseases and Bioterrorism Threats* (T. PF, ed.), Wiley & Sons, Hoboken, NJ, *379–395*.

Mitchell, P., Afzali, B., Lombardi, G., Lechler, R. I. (2009) The T helper 17-regulatory T cell axis in transplant rejection and tolerance. *Curr Opin Organ Transplant,* 14(4), 326–331.

Mittag, A., Tarnok, A. (2011) Recent advances in cytometry applications: preclinical, clinical, and cell biology. *Methods Cell Biol,* 103, 1–20.

Muller, Y. D., Seebach, J. D., Buhler, L. H., Pascual, M., Golshayan, D. (2011) Transplantation tolerance: Clinical potential of regulatory T cells. *Self Nonself,* 2(1), 26–34.

Murad, K. L., Gosselin, E. J., Eaton, J. W., Scott, M. D. (1999a) Stealth cells: prevention of major histocompatibility complex class II-mediated T-cell activation by cell surface modification. *Blood,* 94(6), 2135–2141.

Murad, K. L., Mahany, K. L., Brugnara, C., Kuypers, F. A., Eaton, J. W., Scott, M. D. (1999b) Structural and functional consequences of antigenic modulation of red blood cells with methoxypoly(ethylene glycol). *Blood,* 93(6), 2121–2127.

Nishihara, M., Ogura, H., Ueda, N., Tsuruoka, M., Kitabayashi, C., Tsuji, F., Aono, H., Ishihara, K., Huseby, E., Betz, U. A., Murakami, M., Hirano, T. (2007) IL-6-gp130-STAT3 in T cells directs the development of IL-17+ Th with a minimum effect on that of Treg in the steady state. *Int Immunol,* 19(6), 695–702.

Nistala, K., Wedderburn, L. R. (2009) Th17 and regulatory T cells: rebalancing pro- and anti-inflammatory forces in autoimmune arthritis. *Rheumatology (Oxford),* 48(6), 602–606.

Noris, M., Casiraghi, F., Todeschini, M., Cravedi, P., Cugini, D., Monteferrante, G., Aiello, S., Cassis, L., Gotti, E., Gaspari, F., Cattaneo, D., Perico, N., Remuzzi, G. (2007) Regulatory T cells and T cell depletion: role of immunosuppressive drugs. *J Am Soc Nephrol,* 18(3), 1007–1018.

O'Gorman, W. E., Dooms, H., Thorne, S. H., Kuswanto, W. F., Simonds, E. F., Krutzik, P. O., Nolan, G. P., Abbas, A. K. (2009) The initial phase of an immune response functions to activate regulatory T cells. *J Immunol,* 183(1), 332–339.

Oukka, M. (2007) Interplay between pathogenic Th17 and regulatory T cells. *Ann Rheum Dis,* 66 Suppl 3iii87–90.

Panzer, S., Jilma, P. (2011) Methods for testing platelet function for transfusion medicine. *Vox Sang,* 101(1), 1–9.

Rossi, N. A., Constantinescu, I., Brooks, D. E., Scott, M. D., Kizhakkedathu, J. N. (2010a) Enhanced cell surface polymer grafting in concentrated and nonreactive aqueous polymer solutions. *J Am Chem Soc,* 132(10), 3423–3430.

Rossi, N. A., Constantinescu, I., Kainthan, R. K., Brooks, D. E., Scott, M. D., Kizhakkedathu, J. N. (2010b) Red blood cell membrane grafting of multi-functional hyperbranched polyglycerols. *Biomaterials,* 31(14), 4167–4178.

Scott, M. D., Bradley, A. J., Murad, K. L. (2003) Stealth erythrocytes: effects of polymer grafting on biophysical, biological and immunological parameters. *Blood Transfusion,* 1, 244–265.

Scott, M. D., Murad, K. L., Koumpouras, F., Talbot, M., Eaton, J. W. (1997) Chemical camouflage of antigenic determinants: stealth erythrocytes. *Proc Natl Acad Sci U S A,* 94(14), 7566–7571.

Suthanthiran, M., Strom, T. B. (1995) Immunobiology and Immunopharmacology of Organ Allograft Rejection. *Journal of Clinical Immunology,* 15, 161–171.

Sutton, T. C., Scott, M. D. (2010) The effect of grafted methoxypoly(ethylene glycol) chain length on the inhibition of respiratory syncytial virus (RSV) infection and proliferation. *Biomaterials,* 31(14), 4223–4230.

Tung, J. W., Heydari, K., Tirouvanziam, R., Sahaf, B., Parks, D. R., Herzenberg, L. A., Herzenberg, L. A. (2007) Modern flow cytometry: a practical approach. *Clin Lab Med,* 27(3), 453–68, v.

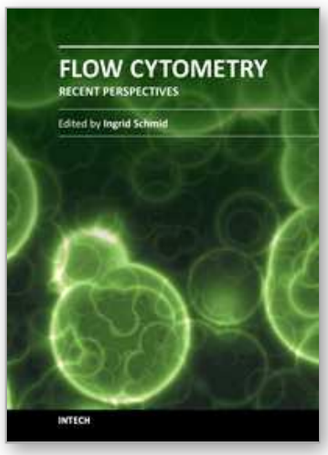
Venet, F., Guignant, C., Monneret, G. (2011) Flow cytometry developments and perspectives in clinical studies: examples in ICU patients. *Methods Mol Biol,* 761, 261–275.

Wang, D., Toyofuku, W. M., Chen, A. M., Scott, M. D. (2011) Induction of immunotolerance via mPEG grafting to allogeneic leukocytes. *Biomaterials,* 32, 9494–9503.

Weaver, C. T., Hatton, R. D. (2009) Interplay between the TH17 and TReg cell lineages: a (co-)evolutionary perspective. *Nat Rev Immunol,* 9(12), 883–889.

Weigt, S. S., Wallace, W. D., Derhovanessian, A., Saggar, R., Saggar, R., Lynch, J. P., Belperio, J. A. (2010) Chronic allograft rejection: epidemiology, diagnosis, pathogenesis, and treatment. *Semin Respir Crit Care Med,* 31(2), 189–207.

Weston, S. A., Parish, C. R. (1990) New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. *J Immunol Methods,* 133(1), 87–97.



Flow Cytometry - Recent Perspectives

Edited by M.Sc. Ingrid Schmid

ISBN 978-953-51-0626-5
Hard cover, 500 pages
Publisher InTech
Published online 13, June, 2012
Published in print edition June, 2012

"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Duncheng Wang, Wendy M. Toyofuku, Dana L. Kyluik and Mark D. Scott (2012). Use of Flow Cytometry in the In Vitro and In Vivo Analysis of Tolerance/Anergy Induction by Immunocamouflage, Flow Cytometry - Recent Perspectives, M.Sc. Ingrid Schmid (Ed.), ISBN: 978-953-51-0626-5, InTech, Available from: http://www.intechopen.com/books/flow-cytometry-recent-perspectives/use-of-flow-cytometry-in-the-in-vitro-and-in-vivo-analysis-of-tolerance-anergy-induction-by-immu

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen