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

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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, these 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
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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]
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 allore cognition 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 (Rg; 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). Consequently 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 transudation of immunocamouflaged allogeneic murine splenocytes prevents alloreognition 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 alloreognition 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, alloreognition 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) alloreognition; and 3) costimulation. [Chen & Scott, 2001, Murad et al., 1999a] Blockade or attenuation of any (or all) of these events will reduce alloreognition 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, alloreognition 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, allore cognition 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.

**Table 1.** The covalent grafting of mPEG to membrane proteins results in the global camouflage of multiple proteins crucial for effective allore cognition 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

| 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*  |

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 quantitative 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 Biosciences, San Diego, CA and Soft Flow Inc, St. Louis Park, MN) to provide a quantitative value for the selected cytokines.

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 acquired using a BD FACSCalibur™ 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 Biosciences, 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 analyte 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, ³H-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 background 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 3H-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 allore cognition 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 percent 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 tolerogenic 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, allore cognition 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 & Koummanda, 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 tolerogenic/anergic state. [Chen & Scott, 2003, Chen & Scott, 2006, Wang et al., 2011]

![Flow Cytometric Studies](image)

**Fig. 9.** Flow cytometric studies demonstrate that immunocamouflage of allogeneic murine splenocytes 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 splenocytes. Similar finding in the lymph nodes and blood were observed. Grey bars denote baseline levels of Th17 and Treg cells in naïve animals. The ∆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, allore cognition, 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 RORγt+, 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 tolerogenic/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]

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7. References

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