

The Use of Flow Cytometry to Monitor T Cell Responses in Experimental Models of Graft-Versus-Host Disease

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1. Introduction

1.1 Introduction to the immune system and flow cytometry

The immune system is a collection of dynamic cells that work together and interact to perform a variety of bodily functions. The immune system is involved in many disease states and changes to the immune system can be very impactful in progression or amelioration of a disease. The ability to monitor immune responses during disease progression and resolution can help to elucidate the underlying mechanisms of immune-mediated diseases

Flow cytometry is a method of monitoring immune responses using antibodies labeled with fluorochromes either directly or indirectly through a fluoresceinated secondary reagent. Antibodies are proteins that can bind to a particular epitope with very high affinity. To monitor immune responses via flow cytometry, immune cells must be isolated and incubated with antibodies to markers of choice. During co-incubation, antibodies randomly encounter cells in suspension and bind to the epitopes for which they are specific for. Following co-incubation, excess antibody is washed off and the cell suspension can either be passed through a flow cytometer immediately or the cell suspension can be fixed and passed through a flow cytometer at a later time. The resulting data are then analyzed and interpreted.

1.2 Introduction to applications of flow cytometry in graft-versus-host disease

Graft-versus-host disease (GVHD) is an immune mediated disease that results following bone marrow transplant. GVHD is caused by donor T cells that react to alloantigens expressed on host tissues. GVHD pathology is generally confined to the epithelia of the skin, liver, and intestinal tract. Following transplantation, T cell responses go through several phases with each phase characterized by various T cell activities. Flow cytometry provides a powerful tool to determine the disease state at any given time, characterize T cell responses, and examine the effectiveness of therapeutic intervention.

In this chapter, we will discuss the application of flow cytometry to characterizing T cell responses during GVHD. We will discuss isolating T cells from relevant immune

compartments, applying flow cytometry to identifying disease states over time, defining the roles of T cell subsets during GVHD, identifying the effectiveness of specific T cell depletion in vivo during GVHD, and the use of bioluminescent imaging to integrate flow cytometric data with T cell trafficking properties.

To monitor T cell responses during GVHD, T cells can be isolated from several locations. In this section of the chapter, we will describe methods for isolating and purifying T cell subsets from the intestinal tract, peripheral blood, mesenteric lymph node, and spleen. By examining these specific locations, researchers can gain insight into pathogenic T cell development and maintenance. We will provide data regarding T cell yield and expected ratios of various T cell subsets in each location.

Flow cytometry can be a valuable asset in identifying disease progression. Flow cytometry allows for both cell surface and intracellular identification of T cells. This section of the chapter will focus on how to identify disease states during GVHD based on cell surface staining as well as identify intracellular cytokine production characteristic of pathogenic T cells. We will discuss typical surface staining of T cells during various phases of GVHD.

The roles of T cell subsets in GVHD can be explored using data that characterizes T cell responses during GVHD. In this section of the chapter, we will discuss how flow cytometric analysis can be used to generate hypotheses about the roles of T cell subsets during GVHD. We will instruct the reader how to analyze flow cytometry data, draw subsequent conclusions, and generate hypotheses to confirm their conclusions. We will provide examples of this practice in the context of GVHD.

The final section of this chapter will focus on using flow cytometry to determine the effectiveness of therapeutic interventions. Our lab has used immunotoxins and depleting antibodies as potential therapies for GVHD. Flow cytometry plays a large role in determining the mechanism of action of our therapies as well as determining if the therapy was successful. In this section, we will include data showing how flow cytometry can be used to determine the effectiveness of immunotherapies.

2. Graft-versus-host disease

2.1 Introduction to bone marrow transplantation

Bone marrow transplantation represents a curative therapy for a variety of hematopoietic deficiencies including blood cancers. Because of the potentially fatal side effects, transplants are often only used when standard therapies are ineffective. Immediately prior to the transplant, the host is given a conditioning regimen of radiation and/or chemotherapy to suppress or ablate the host immune system. In doing so, the host is cured of their blood disorder; however, they are also severely immunocompromised and will not survive without a bone marrow transplant to restore immune function. Donor bone marrow can be isolated by injecting a syringe into a marrow containing bone and extracting marrow. This is a very laborious and painful process for the donor. Alternatively, a less invasive method of extracting hematopoietic stem cells involves treating the donor with granulocyte colony-stimulating factor (G-CSF) and pheresing stem cells out of the blood. Treatment with G-CSF mobilizes stem cells from the bone marrow into circulation, and stem cells can then be isolated from peripheral blood. Donor stem cells are isolated and injected into the immunosuppressed host.

2.2 Graft-versus-host disease

Acute Graft-versus-host disease is the most common detrimental outcome following bone marrow or hematopoietic stem cell transplants. In 1966, Billingham described three criteria for GVHD: 1) the graft must contain a sufficient number of immunologically competent cells, 2) the host must contain important isoantigens lacking in the graft, and 3) the host must be incapable of mounting an immune response against the graft (Billingham, 1966). There are four stages of GVHD that classify the severity of the disease with stage I being the least severe and stage IV being the most severe. Various stages are defined by the degree of pathology associated with each target organ (Table 1). There are several genetic risk factors that can negatively impact a recipient's probability of getting severe GVHD. These risk factors include, but are not limited to polymorphisms in proinflammatory cytokines such as TNF α and INF γ as well as polymorphisms in anti-inflammatory cytokines such as IL-10 and TGF β (Ball & Egeler, 2008). Other risk factors include age/sex of the donor and recipient, donor stem cell source, and degree of HLA mismatch (Koreth & Antin, 2008). It is of great importance to match the donor and recipient as closely as possible. The degree of HLA mismatch between the donor and recipient positively correlates with the severity of GVHD (Park et al., 2011). GVHD is caused by mature lymphocytes that are unintentionally isolated during graft procurement that generate an immune response to host antigens. It is not uncommon for the number of mature lymphocytes to vastly outnumber the stem cells in the transplant inoculum (Korbling & Anderlini, 2001). T cells represent the highest single cell type population (Korbling & Anderlini, 2001), and are the primary mediators of GVHD. Mature donor antigen presenting cells (APCs) and B cells contribute to the pathogenesis of GVHD by priming T cells against host tissue. However, donor T cells alone are sufficient to cause GVHD (Shlomchik et al. 1999). Interestingly, depletion of donor CD4+ T cells is ineffective in reducing GVHD severity, but selective depletion of CD8+ T cells ameliorates GVHD severity (Nagler et al., 1998; Nimer et al., 1994). These data suggest that CD8+ T cells play a dominant role in promoting GVHD pathology. Patients that develop acute GVHD often develop chronic GVHD. Chronic GVHD is characterized by the delay in onset as well as the breadth of target organ involvement. Acute GVHD is diagnosed if onset occurs within 100 days of transplant; whereas, clinical manifestations occurring after 100 days post

Stage	Skin	Liver	GI Tract
0	No rash due to GVHD	Normal serum bilirubin	None
I	Mild maculopapular rash over less than 25% of the body	Bilirubin from 2 to <3 mg/dl	Diarrhea >500-1000 ml/day
II	Moderate maculopapular rash over 25%-50% of the body	Bilirubin from 3 to <6 mg/dl	Diarrhea >1000-1500 ml/day
III	Severe maculopapular rash covering greater than 50% of the body	Bilirubin from 6 to < 15mg/dl	Diarrhea >1500 ml/day
IV	Severe maculopapular rash covering greater than 50% of the body.	Bilirubin > 15mg/dl	Diarrhea >1500 ml/day; abdominal pain or ileus

Adapted from Ball & Egeler, 2008, Bone Marrow Transplantation.

Table 1. Description of each clinical manifestation for each target organ at each stage of GVHD

transplant are characterized as chronic GVHD. Target organs affected by acute GVHD are largely limited to the skin, liver, and gut. In addition to the skin, liver, and gut, chronic GVHD can affect mucous membranes, lung, and musculoskeletal system (Koreth & Antin, 2008).

In addition to their ability to cause GVHD, donor T cells provide protective effects to transplant recipients. Donor T cells promote bone marrow engraftment, immunity to opportunistic infections, and eliminate residual malignant cells resistant to the host conditioning regimen. Elimination of residual tumor cells is seen in several solid tumors and a variety of hematological malignancies with chronic myeloid leukemia (CML) being the most sensitive (Morris et al., 2006). Immunity to residual tumor cells is termed the graft-versus tumor (GVT) effect and immunity to hematopoietic malignancy is termed the graft-versus-leukemia (GVL). Transplant recipients receive a variety of immunosuppressive drugs, many of which target T cells. Because T cells are a major defense against viral infections, reactivation and primary cytomegalovirus (CMV) infections are problematic post transplant (Bautista et al., 2008). Intense myeloablative condition regimens destroy host defense responsible for maintaining CMV latency. It has been established that total body irradiation (TBI) causes reactivation of latent murine CMV (mCMV) infections (Kurz et al. 1999). Virus-specific CTLs have been generated from *in vitro* expanded CD8+ T cells and adoptively transferred into recipients (Riddell et al., 1992). Identifying a target to reduce GVHD and maintain immunity to a broad spectrum of pathogens would be advantageous to advancing our understanding of the immune system after bone marrow transplantation.

In some transplants, T cells are depleted prior to transplant because the severity of GVHD can be predicted through analysis of the degree of mismatch between donor and host. Recipients of T cell depleted grafts do not develop GVHD, but they also do not benefit from the protective effects of T cells post transplant. It was shown that leukemic relapse and graft rejection were increased in patients who received T cell depleted grafts and concluded that global T cell depletion is not a viable treatment strategy (Horowitz et al., 1990). Therefore, GVHD research is focused on identifying a method to prevent GVHD while maintaining the beneficial T cell properties post transplant.

2.3 Animal models of GVHD

Animal GVHD models are often used in order to test hypotheses aimed to improve outcomes following bone marrow transplantation. GVHD is induced by irradiating recipient mice and adoptively transferring donor bone marrow cells. Unlike the clinical scenario, there are not enough donor T cells isolated during bone marrow procurement to induce GVHD, so exogenous T cells must be added to induce GVHD. The most common source of T cells used to induce GVHD is the spleen and/or lymph nodes. Whole splenocytes or various subsets of T cells can be purified and adoptively transferred with the bone marrow to induce GVHD.

Advances in clinical practice have been driven by breakthroughs discovered using small animal models of GVHD. Rat models of GVHD are widely used and provide researchers with a small animal model to test hypotheses to improve outcomes following bone marrow transplants. Given the additional size provided in rat models compared to murine models,

transplant studies in which GVHD is a complication of solid organ allografts can be done (Wakely et al., 1990; Muramatsu et al., 2010). However, murine GVHD models remain the most prominent transplant models. The vast number of MHC combinations available and the array of transgenic mice create an ideal situation for testing hypotheses aimed to discover immunological mechanisms governing GVHD. Murine GVHD models can be divided into two main categories: MHC-matched and MHC disparate. The disease course following GVHD induction in MHC-disparate models is much more rapid than the disease course following GVHD induction in MHC-matched models, and can generally be induced by CD4+ or CD8+ T cells alone. Donor CD4+ and CD8+ T cells in an MHC-disparate model are directed against differences in major histocompatibility antigens; whereas, T cells in an MHC-matched model are directed against differences in the minor histocompatibility antigens (miHAs). miHAs are polymorphic proteins that vary between individuals. miHAs are sufficient to cause GVHD despite the lower frequency of donor T cells specific for miHA differences (Goulmy et al., 1996). An MHC-matched model that closely resembles the clinical scenario is the C57Bl/6 (B6) into BALB.B strain combination. Both strains are of the H-2b haplotype, but are disparate for multiple miHAs. This model was well characterized in the Korngold laboratory, and it was established that GVHD was principally caused by CD4-dependent CD8+ T cells (Berger et al., 1994; Friedman et al., 1998). Furthermore, the immunodominant hierarchy has been established and it is known that the H60 miHA is immunodominant (Choi et al., 2001). The delayed mortality in MHC-matched models allows for disease progression that is more consistent with clinical disease progressions. The similarity of the relative roles of T cell subsets between the clinical scenario and the B6 into BALB.B strain combination and the delayed disease course make this model useful to study underlying mechanisms of GVHD.

2.4 Effects of the conditioning regimen

GVHD is classified into three different phases. The first phase of GVHD comes as a result of the conditioning regimen the recipient receives to reduce or ablate the native immune system. The conditioning regimen is aimed to destroy rapidly dividing cells, so in addition to destroying lymphocytes, the conditioning regimen also attacks epithelial cells in the skin, the liver, and the gut. Destruction of epithelium in these compartments is thought to cause release of proinflammatory cytokines including, but not limited to IL-1 and TNF α . Increased cytokine production causes the upregulation of costimulatory molecules, adhesion molecules, and MHC antigens (Chang & See, 1986; Pober et al., 1996). This process is critical to the activation of host antigen presenting cells (APCs). Epithelial damage in the gut is particularly important in the initiation of GVHD. Damage to the intestinal epithelium causes systemic release of LPS, which further amplifies GVHD induction (Reddy, 2003). Released LPS is taken up by local APCs and an immune response is mounted against LPS, which, in turn further exacerbates GVHD progression and pathology.

2.5 Donor T cell activation

GVHD pathology is mediated by donor T cells directed against major and minor histocompatibility antigens in the host. Donor T cell trafficking to host secondary lymphoid tissue immediately following hematopoietic stem cell transplantation (HSCT) is a complex processes that displays extreme diversity between organs. Naïve T cells cause more severe

GVHD than memory T cells (Dutt et al., 2007); therefore, naïve T cell trafficking is an area of intense research. T cell priming in intestinal inductive sites (Peyer's patches, mesenteric lymph nodes) plays a major role in gut associated GVHD. In the gut, T cells enter secondary lymphoid tissue by T cell rolling initiated by the L-selectin (CD62L) expressed on all naïve T cells interacting with the peripheral node addressin (PNAd) on high endothelial venules (HEVs). Further tethering of the T cell to the HEV is achieved by the CCL21/CCR7 interaction. This interaction mediates upregulation of leukocyte function-associated antigen type 1 (LFA-1) on T cells, which firmly bind the T cell and HEV (Johansson-Lindbom & Agace, 2007). Peyer's patches and mesenteric lymph node HEVs express low levels of PNAd; however, mucosal addressin cell-adhesion molecule-1 (MAdCAM-1) serves as an additional ligand for CD62L in gut priming sites (Johansson-Lindbom & Agace, 2007). Another important ligand for MAdCAM-1 is the $\alpha 4\beta 7$ integrin. This integrin is expressed on naïve T cells and plays a critical role in directing naïve T cells to the gut, causing intestinal GVHD (Campbell & Butcher, 2002; Johansson-Lindbom & Agace, 2007; Mora et al., 2003; Stagg et al., 2002). Once in host lymphoid tissue, antigen presentation is primarily done by host APCs presenting self peptides. Donor derived dendritic cells can cross present host antigens; however, severe GVHD is dependent on host APCs presenting to alloreactive T cells (Shlomchik, 2007). Activated T cells leave through the efferent lymphatic system and return to circulation via the thoracic duct. When activated T cells return to sites of inflammation they migrate through blood vessel endothelium and into epithelial compartments.

2.6 Effector phase of GVHD

In the activation phase, donor T cells traffic to host secondary lymphoid tissue hours after transplant and expand within 2-3 days (Wysocki et al., 2005). In the lymphoid tissue, donor T cells are primarily primed by host antigen presenting cells (APCs) to mature into T helper 1 (Th1) CD4+ T cells or cytotoxic CD8+ T cells. Recently, it has been described that T helper 17 (Th17) cells can also mediate lethal GVHD in murine models (Carlson et al., 2009). Th17 cells are characterized by their ability to produce the cytokine IL-17, and have been shown to be proinflammatory (Gran et al., 2002; Gutmacher et al., 2006; Krakowski & Owens, 1996; Zhang et al., 2003). Th1 CD4+ T cells are characterized by their production of proinflammatory cytokines such as IFN γ , TNF α , and IL-2. 3-7 days post transplant, activated T cells traffic to and expand in GVHD target organs (Wysocki et al., 2005). The effector phase of GVHD consists of activated T cells migrating to epithelial compartments of GVHD target organs and destroying host tissues. Th1 CD4+ T cells mediate GVHD primarily through Fas/FasL mediated apoptosis, but also mediate epithelial damage via IL-1 and TNF α (Teshima et al., 2002). CD8+ T cells kill target tissues by direct contact to target cell or by release of cytotoxic soluble mediators (Ferrara et al., 1999). Contact dependent CD8+ T cell killing mechanisms include the Fas/FasL interaction and perforin/granzyme mediated cytotoxicity (Ferrara et al., 1999). In murine models where appropriate genetic differences exist, CD4+ T cells can induce GVHD by responding to MHC Class II differences; whereas, MHC Class I differences will elicit CD8+ T cell driven GVHD responses (Sprent et al., 1990). However, differences in miHAs elicit GVHD reactions from either CD4+ or CD8+ T cells following HLA-matched bone marrow transplantation.

3. Characterization of T cells during GVHD

3.1 T cell phenotypes involved in GVHD

Initially upon transfer from donor to host, donor T cells are in a naive state. Naive T cells characteristically express the lymphocyte homing receptor CD62L (L-selectin). The ligands for CD62L are GlyCAM-1, CD34, MadCAM-1, and PSGL-1. Each of these ligands are expressed on endothelial cells or high endothelial venules in the lymph node. Naive T cells home to host secondary lymphoid compartments following transplant. Naive T cells can be activated directly by antigen presentation by host APCs or indirectly by antigen presentation by donor APCs (Shlomchik et al., 1999; Teshima et al., 2002). Following activation, donor T cells down regulate CD62L, thereby freeing them from lymphoid recirculation, and upregulate integrins such as LFA-1 (CD11a/CD18) and VLA-4 (CD49d/CD29) which enable extravasation into host tissues (Figure 1). Activated T cells also upregulate the C-Type lectin protein, CD69, and the high affinity alpha chain of the IL-2 receptor (CD25).

Effector memory T cells can be classified by their expression of the hyaluronic acid receptor, CD44. CD44 is expressed at low levels on naive cells, but is highly upregulated upon activation. Naive T cells can be isolated from donor mice and tested for the expression of naive or activation markers to predict the GVHD inducing potential of a given population of T cells. Furthermore, naive CD4⁺ T cells can be cultured to polarization to Th phenotype (Swain et al., 1991). Polarized CD4⁺ T cells can be transferred to irradiated recipients to induce GVHD (Fowler et al., 1996).

It is well documented that adoptive transfer of naive T cells causes lethal GVD across either major or minor histocompatibility differences (OKunewick et al., 1990; Sprent et al., 1990); however, T cells of effector or memory phenotype are not as effective as naive T cells in inducing GVHD following adoptive transfer into allogeneic recipients (Anderson et al., 2003). Interestingly, central memory, effector memory, and effector CD8⁺ T cells have been shown to induce GVHD in a fully allogeneic strain combination (Zhang et al., 2005a); whereas, memory (characterized by the lack of CD62L expression) CD4⁺ T cells do not induce GVHD in either MHC-matched or MHC-disparate murine models (Anderson et al., 2003; Chen et al., 2004). The mechanisms governing the lack of GVHD induction by memory CD4⁺ T cells remains unclear. However, it has been hypothesized by Sondel and colleagues that the T cells transferred by Anderson et al., were of the terminally differentiated, CD4⁺CD44^{hi}CD62L⁻CD25⁻ effector memory variety (Sondel et al., 2003). Effector memory differ from activated memory cells by their lack of CD69L and CD25 expression and lack the ability to home to central lymphoid tissue (Figure 1) (Sondel et al., 2003). The inability to home to lymphoid compartments following transfer in to host circulation presumably prevents donor T cells from being effectively primed against host antigens, thus leaving them unable to induce GVHD. The specificity that exists in choosing the appropriate cell type to induce GVHD provides an opportunity to utilize flow cytometry to aid in the induction of GVHD. Flow cytometry can be used to confirm or test the purity of a cultured cell population that has been induced into a certain phenotype. Flow cytometry can also identify the composition of donor splenocytes or lymph node cells. Historical data from Jackson Laboratories shows that the spleen of female B6 mice is comprised of roughly 16% CD4⁺ T cells and 10% CD8⁺ T cells (Jackson Laboratories). When testing hypotheses, it is of great importance to optimize experiments in order to obtain the most accurate results. Using

flow cytometry to understand the composition of a given cell population allows researchers to accurately perform and analyze experiments.

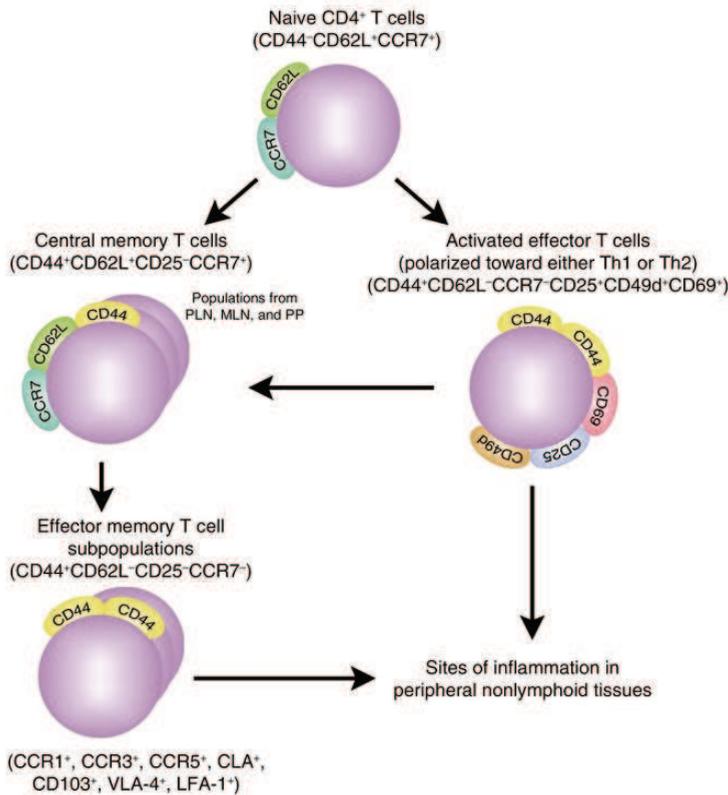


Fig. 1. Naïve CD4⁺ T cells develop into effector or memory CD4⁺ T cells. When naïve CD4⁺ T cells (initially CD44⁺CD62L⁺CCR7⁺) are activated they either develop into central memory CD4⁺ T cells (CD44⁺CD62L⁺CD25⁻CCR7⁺) or effector CD4⁺ T cells (CD44⁺CD62L⁻CCR7⁻CD25⁺CD49d⁺CD69⁺). Effector memory CD4⁺ T cells can extravasate into sites of inflammation or transition into central memory CD4⁺ T cells. Central memory CD4⁺ T cells can transition into effector memory CD4⁺ T cells (CCR1⁺CCR3⁺CCR5⁺CLA⁺CD103⁺VLA-4⁺LFA-1⁺). Adapted from Sondel et al., 2003, JCI

3.2 The role of regulatory T cells during GVHD

Regulatory T cells (Tregs) are a population of T cells that have been shown to suppress GVHD in murine models (Johnson et al., 2002). Tregs are characterized and controlled by several phenotypic markers with the most universal being expression of the transcription factor forkhead box P3 (Foxp3) (Hori et al., 2003). Despite being the universal Treg regulator, Foxp3 is also transiently upregulated following activation of CD4⁺ T cells (Esposito et al., 2010). Because of this, the function of Foxp3 expressing cells can be ambiguous. Transient Foxp3 expression by recently activated Th1 CD4⁺ T cells is

substantially lower than that in Foxp3 expressing Tregs (Esposito et al., 2010). Therefore, to accurately determine whether Foxp3 expression signifies a regulatory CD4⁺ T cell, protein quantification should be done. Protein quantification can be done through Western blotting and densitometry analysis. Moreover, CD25 is often used to classify CD4⁺ Tregs, but CD25 is also expressed on recently activated Th1, Th2, or Th17 T cells as well. Therefore, confirmation of a flow cytometric determination of a cellular phenotype may be supplemented using alternative methods.

CD4⁺ Tregs elicit several effector mechanisms, but they are widely regarded as immunosuppressive. Their immunosuppressive action can be carried out via cell-cell contact or secretion of anti-inflammatory cytokines including TGF β and IL-10. Tregs can be further characterized by surface expression of several other proteins, including the α E β 7 integrin, CD103, which is broadly expressed by a variety of leukocyte subsets including activated CD8⁺ T cells, dendritic cells, and regulatory CD4⁺ and CD8⁺ T cells (Cepak et al., 1994; Cerf-Bensussan et al. 1987; Huehn et al. 2004). CD103⁺ Tregs have been shown to have immunosuppressive properties reaching or exceeding those of their CD103⁻ counterparts (Lehman et al., 2002). There is a population of CD25-CD103⁺ Tregs that express CTLA-4, suppress T cell proliferation *in vitro*, and prevent severe colitis in the SCID mouse (Lehmann et al., 2002). CD25-CD103⁺ Tregs also produce a distinct cytokine profile. This subset of Tregs typically produces IL-4, IL-5, and IL-13 to a similar extent as Th2 CD4⁺ T cells; however, this cytokine profile is largely absent in their CD25⁺ counterparts (Lehmann et al., 2002).

CD4⁺CD25⁺CD103⁺ Tregs exhibit immunosuppressive properties *in vivo* as well as *in vitro*. Chronic GVHD frequently occurs in patients that develop acute GVHD, and *in vivo* transfer of CD4⁺CD25⁺CD103⁺ Tregs in mice has been shown to suppress ongoing chronic GVHD, and has been shown to reduce the number of alloantibody producing plasma cells and pathogenic T cells in GVHD target organs (Zhao et al., 2008). CD103 is also present on the surface of a population of CD8⁺ Tregs. CD8⁺ Tregs can acquire their antigen specificity peripherally and promote systemic tolerance. Antigen specific CD8⁺ Tregs can be induced by antigen injection into the anterior chamber of the eye. CD103 has been shown to be essential for the development and function of the CD8⁺ Tregs (Keino et al., 2006). Koch et al. characterized CD103⁺CD8⁺ Tregs as phenotypically different from other CD8⁺ suppressor T cell populations. CD103⁺CD8⁺ Tregs express CD28, but lack Foxp3, CD25, LAG-3, CTLA-4, and GITR (Koch et al., 2008).

3.3 Cell specific depletion during GVHD

Depletion of specific cell types can be an effective means to prevent GVHD. The most obvious clinical example of this is patients who receive T cell depleted grafts incur GVHD less frequently. However, this is not an effective treatment because globally depleting T cells results in increased rate of graft rejection, increased susceptibility to opportunistic infections, and increased rates of leukemic relapse. However, rare instances occur where the risk for severe GVHD is so great that the potential benefits outweigh the risks and T cells are selectively removed from the graft. In such instances, removal is done through the addition antibodies targeted to either CD4⁺ T cells, CD8⁺ T cells, or both. The antibodies are conjugated to magnetic beads and the graft-antibody mix is passed through a magnetic column. The magnetic beads bind to the magnetic column, so any CD4⁺ or CD8⁺ T cells

bound by antibody are retained in the column while the remainder of the graft passes through freely. Flow cytometry can be used to confirm the presence or absence of T cells. Flow cytometric analysis can be done not only on the T cell depleted graft to confirm the absence of the pan T cell marker, CD3, but analysis can be done on the cells retained in the column to confirm a pure population of T cells. Despite the dogma regarding T cells as the central mediators of GVHD pathology, researchers are now focusing their efforts on the role of B cells during GVHD. Rituximab is a monoclonal antibody directed to CD20, a pan mature B cell marker, and causes B cell depletion by antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), and direct arrest of cellular growth. Studies have shown an amelioration of chronic GVHD in patients who received Rituximab suggesting a prominent role for B cells in the progression of chronic GVHD (Alousi et al., 2010).

4. Analyzing T cell responses during GVHD

4.1 Isolating T cells

To induce murine GVHD, T cells are infused via the tail vein and are immediately propelled into host circulation. Donor lymphocytes rapidly accumulate in host lymphoid tissue and expand within 2-3 days post transplant (Wysocki et al., 2005). At this point, donor lymphocytes can be isolated from either the secondary lymphoid tissue (spleen or lymph nodes) or peripheral blood. Peripheral blood is advantageous because it is not a terminal procedure; however, the lymphocyte yield is lower than that from the spleen or lymph nodes. Lymphocyte counts in the spleen of a naïve mouse can exceed 100 million cells. However, following lethal irradiation and GVHD induction, splenic lymphocyte counts may be limited to as low as 5 million total lymphocytes.

Peripheral blood should be collected using a submandibular bleed. This can be done using a lancet to stick the submandibular vein just before it opens to the jugular vein (Golde et al., 2005). The volume of blood collected should be less than 0.3 mL (Golde et al., 2005). Lymphocyte yield from peripheral blood will likely not exceed 1 million cells; however, yield will vary greatly with the strain combination used, disease state at which the blood was collected, and the volume of blood collected. Blood should be collected in a tube containing heparin (or any other anti-coagulation reagent) and the red blood cells should be lysed. Alternatively, the spleen can be removed following euthanization. The spleen should be minced and made into a single cell suspension and red blood cells should be lysed. Once the red blood cells are lysed, count the remaining lymphocytes and resuspend in FACS Buffer (10% FBS, 0.2% Sodium Azide in PBS).

4.2 Setting up a flow cytometry experiment

For each experiment that will be analyzed using flow cytometry, appropriate controls will need to be included. Those controls consist of a tube containing cells alone and a tube with each antibody alone. The cells alone control tube will allow the flow cytometer to calibrate the size and granularity of the cell population without antibody present. Additional tubes containing lymphocytes and each fluorochrome to be used in the experiment conjugated to an antibody that will positively bind the lymphocyte population (i.e. a positive control) should be added separately. The purpose of these tubes is to calibrate the cytometer to recognize each fluorochrome independently.

Lastly, isotype controls must be included and set up in individual tubes with lymphocytes. Isotypes are antibodies that are specific for an antigen that is not likely to be present on the cell population of interest. The isotype controls account for any non-specific binding of the antibodies of interest and can be helpful in accurately analyzing the data. If the experiment is designed to analyze cell populations of low frequency, it will be helpful to add each non-isotype antibody in addition to adding the isotype control (see example). It is preferable to use the lymphocytes to be used in the experimental tubes for all of the control tubes; however, if this is logistically impossible, alternative lymphocytes can be used.

For flow cytometry, incubate 1 million cells in separate test tubes that are compatible with the flow cytometer to be used with each antibody to be included in the experiment for 30 minutes in the dark at 4 degrees Celsius. During this incubation period, incubate lymphocytes with each positive control tube and isotype controls. After the incubation period, wash off excess antibody with 3 mL of FACS Buffer and resuspend with 300 uL of a fixative solution such as FACS Fix Solution (FACS Buffer with 10% Neutral Buffered Formalin). The cells should be analyzed on a flow cytometer as soon as possible, but can be delayed for up to several days.

Example Experiment: The goal in this example experiment is to identify the percent of naive CD8+ T cells in the spleen 7 days post transplant of 3 mice with GVHD.

- Each mouse is to be euthanized and the spleens are to be removed.
- Mince each spleen and create three single-celled suspensions by passing the spleen through a 40 uM nylon mesh filter.
- Collect each suspension, pellet the cells and lyse the red blood cells.
- Resuspend cells in FACS Buffer, count the cells, and transfer ~1 million cells (volume ~100 uL) into 3 tubes. These tubes will contain the antibodies to analyze the percent of naive CD8+ T cells.
- Combine all remaining cells and transfer ~1 million cells (volume ~100 uL) to 7 tubes. These tubes will make up the positive control and isotype tubes.
- Because we want to know the frequency of naive CD8+ T cells, we will need to stain the cells with 3 different antibodies: 1) CD3e (to identify T cells), 2) CD8a (to identify the CD8 population of T cells), and 3) CD62L (To identify the naive population of CD8+ T cells. Because these are three relatively common antigens, they should be available on a variety of fluorochromes. For this example, we will use CD3e-FITC, CD8a-PE, CD62L-APC. We will also need an isotype matched negative control for each antibody (Hamster IgG1-FITC, Rat IgG2a-PE, Rat IgG2a-APC respectively).
- See Table 2 for detailed experimental set up.
- Three mice were used (Tubes 5-7) so statistical analysis can be done to the flow cytometry data obtained.

4.3 Analyzing T cells from the gut during intestinal GVHD

During the conditioning regimen, the gut is heavily damaged and is a target organ of acute GVHD. Analyses of early T cell trafficking events indicate that T cells are primed against host antigens and migrate to the gut (Wysocki et al., 2005). The degree to which T cells are primed in the Peyer's patches is a controversial matter; however, it is clear that secondary lymphoid tissue in the gut contributes to the perpetuation of donor T cell pathology (Murai

	<i>FITC</i>	<i>PE</i>	<i>APC</i>
Tube 1	-	-	-
Tube 2	CD4		
Tube 3		CD4	
Tube 4			CD4
Tube 5	CD3e	CD8a	CD62L
Tube 6	CD3e	CD8a	CD62L
Tube 7	CD3e	CD8a	CD62L
Tube 8	Isotype CD3 (Hamp. IgG1)	CD8a	CD62L
Tube 9	CD3e	Isotype CD8a (Rat IgG2a)	CD62L
Tube 10	CD3e	CD8a	Isotype CD62L (Rat IgG2a)

Table 2. Sample of a table which describes the antibodies to be added to each tube

et al., 2003; Welniak et al., 2006). We and others have modified the protocol Isolation of Mouse Small Intestine Intraepithelial Lymphocytes, Peyer's Patch, and Lamina Propria Cells from the *Current Protocols in Immunology* series to isolate and analyze T cells that infiltrate the gut during GVHD. Briefly, the small intestine is removed and flushed with PBS. The Peyer's Patches are removed and the small intestine is cut longitudinally and into ~5 mm sections. Intraepithelial lymphocytes (IELs) and, during GVHD, gut infiltrating lymphocytes (GILs) are isolated from the intestinal sections.

Elegant flow cytometric experiments have been performed using GILs to test hypotheses regarding the role of T cell subsets during GVHD. El-Asady et al. used a competition based mixing experiment to show a role for CD103 in promoting CD8 T cell accumulation in the intestinal epithelium during GVHD. In this experiment, equal numbers of CD8+ T cells from CD90.1 (Thy1.1) congenic mice were mixed with CD8 T cells from a CD103-/- CD90.2 (Thy1.2) mouse and transferred into irradiated recipients. At various time points, GILs were isolated and analyzed for the proportion of CD103-/- CD8+ T cells in the gut compared to the spleen. Their data show that the proportion of CD103-/- CD8+ T cells is lower at day 28 than in earlier time points. This indicates that CD103-/- CD8+ T cells are less efficient in their ability to accumulate in the gut during GVHD suggesting that CD103 plays a significant role in promoting CD8+ T cell accumulation in the gut during GVHD (El-Asady et al., 2005). Furthermore, the T cell receptors on each set of CD8+ T cells is transgenic so that they only recognize an antigen expressed by host cells, thus adding a higher level of sophistication to the experiment and the conclusions that can be drawn from the results.

1. 1B2 is the antibody that binds to the transgenic T cell receptor. 1B2 positive cells indicate they are of donor origin and are specific for host antigens.
2. Thy1.1 (also known as CD90.1) positive cells represent the CD8 T cells that are able to express CD103.
3. The proportion of Thy1.1 cells increases compared to Thy1.1 negative (Thy1.2/CD90.2) cells indicating that CD103 deficient cells are unable to effectively accumulate in the gut during GVHD.
4. CD103-/- CD8 T cells are retained in the spleen with similar efficiency as wild type CD8 T cells.

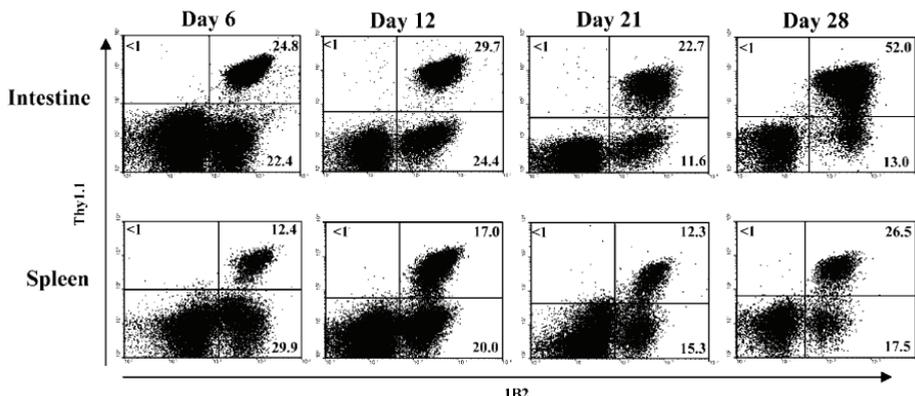


Fig. 2. CD103 promotes retention of CD8⁺ T cells in the gut during GVHD. Lethally irradiated BALB/c recipients were adoptively transferred with equal numbers (0.5×10^6) of donor (2C) CD103^{-/-} (Thy1.2) or wild-type (Thy1.1) CD8⁺ T cells. Dot plots of 1B2 positive cells plotted against Thy1.1 positive cells show proportion of CD103^{-/-} CD8⁺ T cells compared with wild-type CD8⁺ T cells at day 6, 12, 21, and 28. Adapted from El Asady et al., 2005, JEM

4.4 The use of intracellular flow cytometry to monitor T cell responses during GVHD

Intracellular staining for flow cytometry is a valuable technique for testing hypotheses regarding the function of various cell populations in that T cell function can be gleaned from the cytokines produced. To advance experiments in which surface markers are analyzed, cytokines can be probed for and analyzed via flow cytometry. Liu et al. exemplify the use of intracellular flow cytometry to help draw conclusions regarding the function of two T cell populations. In this study, intracellular flow cytometry was performed to compare the cytokine profile of wild type and CD103^{-/-} CD8⁺ T cells. CD103^{-/-} CD8⁺ T cells were shown to be equally effective in their ability to clear solid tumors as wild type CD8⁺ T cells following murine bone marrow transplantation. To confirm that CD103^{-/-} CD8⁺ T cells were mediating tumor clearance, those cells were isolated and the profile of cytokines produced queried (Liu et al., 2011). It was found that in fact CD103^{-/-} CD8⁺ T cells produce the same levels of various proinflammatory cytokines as wild type CD8⁺ T cells. Coupling this finding with the finding that CD103^{-/-} CD8⁺ T cell recipients are able to clear tumor with similar efficacy as wild type CD8⁺ T cell recipients leads to the logical conclusion that CD103^{-/-} CD8⁺ T cells are functionally similar with regard to tumor fighting ability as compared to wild type CD8⁺ T cells.

4.5 Supplementing flow cytometry with bioluminescent imaging

Bioluminescent imaging (BLI) is a technique that utilizes the light as a product of the chemical reaction between the enzyme luciferase and its substrate luciferin. Luciferase is a gene naturally expressed by fireflies and is responsible for their characteristic green glow. The luciferase gene has been inserted into the murine genome on the B6 background generating a mouse that constitutively expresses luciferase (Cao et al., 2005). T cell populations have been purified and transferred with luciferase negative cell populations for

induction of GVHD. Immediately following the administration of luciferin to GVHD recipients, recipients can be imaged in a charged coupled device (CCD) camera and a pseudo-colored image can be generated (Figure 3).

The non-invasive nature of BLI allows its use to supplement data generated via flow cytometry. Determining the cell source for lymphocyte isolation and analysis by flow cytometry is driven by hypothesis testing and historical results. By utilizing BLI, researchers can determine the anatomical location of T cell accumulation in real time and can isolate T cells based on their accumulation patterns rather than being restricted to analyzing experimental animals at specific time points and specific compartments.

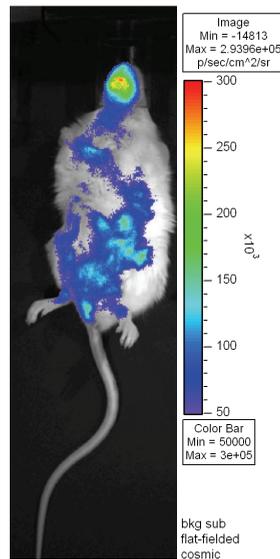


Fig. 3. Bioluminescent imaging as a supplement to flow cytometry: Lethally irradiated BALB/c recipients were adoptively transferred with 10^7 wild-type splenocytes and 2×10^6 luciferase positive CD8⁺ T cells. A representative mouse is shown following 4 mg D-luciferin injection and imaging in Xenogen IVIS CCD camera for 5 minutes

5. GHVD treatments

5.1 Prophylactic treatment

In the absence of GVHD prophylaxis, the incidence of acute GVHD is nearly 100% (Sullivan et al., 1986). Prophylactic treatment with methotrexate results in a substantial decrease in the incidence of acute GVHD (Storb et al., 1974). Methotrexate - originally used as a chemotherapeutic agent - acts to inhibit folic acid metabolism and the cellular result is the inability to synthesize DNA; thus, resulting in inhibition of cellular proliferation. Treatments with calcineurin inhibitors (cyclosporine and tacrolimus) are more efficacious in preventing GVHD. Calcineurin inhibitors act to block the action of the transcription factor nuclear factor of activated T cells (NFAT) (Crabtree, 1989; Shaw et al., 1988). Prophylactic use of

calcineurin inhibitors with low dose methotrexate results in as low as 20% GVHD incidence following bone marrow transplant (Nash et al., 1996; Storb et al., 1986). Flow cytometry can be used to monitor T cell responses following preventative treatment with methotrexate and/or calcineurin inhibitors. In murine models, carboxyfluorescein succinimidyl ester (CFSE) is used to monitor T cell proliferation by flow cytometry. CFSE is a fluorescent dye that is able to traverse cell membranes. CFSE is taken up by cells prior to adoptive transfer. As cells divide, CFSE is equally divided between daughter cells, so when cells are monitored by flow cytometry, cells that have gone through several rounds of divisions have markedly less CFSE than cells that have not proliferated.

5.2 Treatment of established GVHD

The primary treatment for established GVHD is the use of corticosteroids (Koreth & Antin, 2008). Binding of corticosteroids to their receptors on immune cells causes the upregulation of anti-inflammatory transcription factors and a suppression of the immune system. Although corticosteroids are ineffective prophylactically, their use for treatment of ongoing GVHD is widely established and effective (Chao et al., 2000). Intracellular flow cytometry can be used to track the production of pro- or anti-inflammatory cytokines following corticosteroid treatment. Other treatments for established GVHD include mycophenolate mofetil (MMF) and sirolimus (also called rapamycin), but have yet to supplant corticosteroids as the gold standard for treatment of established GVHD. Patients with disease progression after three days of corticosteroid treatment or patients that do not show improvement after seven days of corticosteroid treatment are considered to be steroid-refractory and are treated with a more intense regimen. Such therapies include the use of monoclonal antibodies aimed to deplete T cell subsets. Monoclonal antibodies and/or fusion proteins can be used to block the action of proinflammatory cytokines. The efficacy of treatments for steroid-refractory GVHD is limited by lack of effectiveness or the high incidence of severe side effects.

5.3 Prospective therapeutic approach

Current prophylactic therapies of ongoing GVHD target a broad spectrum of cell types, and thus substantially inhibit post transplant immunity. Immunotherapies specifically targeted to GVHD-causing T cells are desirable. In mice, blockade of integrins expressed on T cells has been shown to be effective in preventing GVHD, while maintaining the beneficial properties of T cells post transplant (El-Asady et al., 2005; Liu et al., 2011). Waldman et al. has shown that the absence of the B7 integrin family on donor T cells results in reduced GVHD morbidity and mortality without compromising GVT effects (Waldman et al., 2006). Similarly, CD8⁺ T cells deficient in their ability to express CD103 are unable to induce GVHD, but maintain immunity to solid tumors (Lui et al., 2011). Flow cytometric sorting techniques can be used to selectively deplete T cells that express certain markers prior to transplantation. Cells are sorted based on fluorescence, so unbound cells are retained separately from cells bound by antibodies. This technique creates highly pure populations of cells of a given phenotype. Because proteins are expressed on T cell surfaces transiently and their expression is a dynamic process, cell sorting is limited to sorting cells based on their phenotype when analyzed. However, despite this limitation, the applications for flow cytometric sorting are vast due to the high throughput and accuracy with which cells are sorted.

Graft engineering is another therapeutic approach that has the potential to maintain post transplant immunity while preventing GVHD. Graft engineering is the idea of selectively depleting the mature donor T cell population of potentially pathogenic cells. The non-pathogenic cells are retained in the graft and are able to promote engraftment, post-transplant immunity, and GVT/GVL effects. Alternatively, T cells can be broadly depleted and repopulated with ex vivo expanded T cells that are specific for a particular tumor or microbial antigen. Several studies have demonstrated the ability to deplete alloreactive T cells or expand and adoptively transfer T cells specific for tumor (Verneris et al., 2001; Amrolia et al., 2004). Flow cytometry plays an important role in differentiating between tumor/pathogen reactive and alloreactive T cell populations. Because the sequences of many antigenic epitopes are known, synthetic peptides can be developed and conjugated to fluorochromes to be used for flow cytometry. T cells specific for the synthetic peptide bind their cognate antigen-fluorochrome complex and their frequency can be identified. Moreover, flow cytometric sorting techniques are able to remove these antigen-specific T cells from the graft. For ex vivo expansion of pathogen or tumor specific antigens, this application of flow cytometry is used to test the purity of the ex vivo expanded T cell population.

To broadly suppress GVHD, Tregs can be expanded ex vivo and adoptively transferred into bone marrow transplant recipients. Until recently, adoptive transfer of Tregs was confined to murine models; however, in 2011, Tregs were expanded ex vivo and adoptively transferred into bone marrow transplant recipients (Brunstein et al., 2011). Suppression of the ex vivo expanded Treg population was confirmed in vitro and following adoptive transfer the incidence rate of acute GVHD was significantly lower in patients who received Tregs (Brunstein et al., 2011). In mice, several groups have reported that adoptive transfer of Tregs can reduce GVHD incidence and severity (Edinger et al., 2003; Taylor et al., 2002). Tregs act to reduce GVHD by inhibiting proinflammatory effector responses by donor T cells, but interestingly, addition of exogenous Tregs does not inhibit GVL effects in mice (Edinger et al., 2003).

5.4 Concluding remarks

Current prophylactic and first-line therapies for GVHD are limited due to the breadth of immune suppression. Global immunosuppressive approaches limit the beneficial properties of T cells post transplant. The use of engineered grafts is an exciting therapeutic approach as it has the potential to separate GVHD from the beneficial GVL effects. Adoptive transfer of ex vivo expanded Tregs also has the potential to separate GVHD from GVL and has been shown to ameliorate GVHD in bone marrow transplant recipients. Innovative advances in the ability to modify T cell subsets have opened the door to novel therapeutic approaches to preventing GVHD without attenuating GVL effects.

In addition to facilitating the determination of efficacy of GVHD therapies, flow cytometry plays a central role in GVHD research. Flow cytometry aids researchers by allowing accurate identification of cellular phenotypes and cytokine profiles of cell populations involved in disease. GVHD remains the limiting factor to the broad use of bone marrow transplants as a curative therapy for hematological disorders. Flow cytometry is a valuable tool with a variety of applications to help separate GVHD from the beneficial properties of T cells post transplant.

6. References

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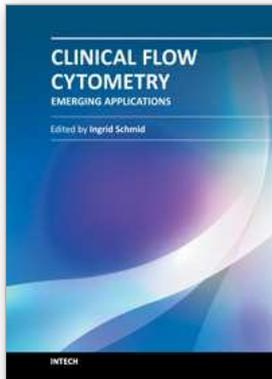
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Clinical Flow Cytometry - Emerging Applications

Edited by M.Sc. Ingrid Schmid

ISBN 978-953-51-0575-6

Hard cover, 204 pages

Publisher InTech

Published online 16, May, 2012

Published in print edition May, 2012

"Clinical Flow Cytometry - Emerging Applications" contains a collection of reviews and original papers that illustrate the relevance of flow cytometry for the study of specific diseases and clinical evaluations. The chapters have been contributed by authors from a wide variety of countries showing the broad application and importance of this technology in medicine. Examples include chapters on autoimmune disease, cancer, and the evaluation of new drugs. The book is intended to give newcomers a helpful introduction, but also to provide experienced flow cytometrists with novel insights and a better understanding of clinical cytometry.

How to reference

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

Bryan A. Anthony and Gregg A. Hadley (2012). The Use of Flow Cytometry to Monitor T cell Responses in Experimental Models of Graft-Versus-Host Disease, *Clinical Flow Cytometry - Emerging Applications*, M.Sc. Ingrid Schmid (Ed.), ISBN: 978-953-51-0575-6, InTech, Available from:
<http://www.intechopen.com/books/clinical-flow-cytometry-emerging-applications/the-use-of-flow-cytometry-to-monitor-t-cell-responses-during-graft-versus-host-disease>

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