Biol Blood Marrow Transplant 19 (2013) S44–S51

Section IX: PEDS–Immunology

Viewing Transplantation Immunology Through Today's Lens: New Models, New Imaging, and New Insights

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INTRODUCTION

The last several decades have brought significant immunologic advances in the fields of inflammation, infection, and transplantation tolerance. Heretofore, our understanding of how complex immune interactions occur has been limited to static in situ tissue analysis and in vitro dynamic studies using isolated cells devoid of stromal elements typically present in vivo. Recent advances in molecular, flow cytometry, and intravital imaging have provided new insight into the dynamic interactions occurring among a variety of cells within the bone marrow (BM) and immune systems, ranging from undifferentiated hematopoietic progenitors to fully committed effector memory cells, which will likely have direct clinical and translational implications. In this review we highlight how the application of these cutting-edge technologies will sculpt the landscape of the next generation of immunologic advances.

NEW IMAGING

Real-Time Interrogation of Cellular Homing and Tissue Response Dynamics in the BM and Brain

Dynamic nature of hematopoietic lineage cells

The blood and immune systems are derived from hematopoietic stem cells (HSCs), rare multipotent cells with self-renewing capacity. The BM provides the microenvironment in which HSCs reside, allowing the development of their closest progeny, hematopoietic progenitor cells (HPCs). Together, hematopoietic stem and progenitor cells (HSPCs) produce, maintain, and regenerate lineage-restricted blood and immune progenitor cells [1]. HSPC activities within the BM niche can be modulated through communications with BM-resident stromal cells and mature immune cells. Along with their differentiated leukocyte progeny, HSPCs have the ability to migrate between BM and other tissue sites and to provide reconstitution after BMT [2]. Thus, understanding the control of recruitment, migration, and interaction dynamics of these cells has direct clinical and translational implications. In this regard, more is known about the migratory behavior of mature immune cells, whose intrinsic mobility constitutes a unique feature of the vertebrate immune system. Trafficking and recruitment of immune cells among various tissue compartments has profound effects on these cells' overall functional outcome. For instance, effector cells of the innate immune system are rapidly mobilized from BM and enter inflamed tissues from the blood, whereas sentinel antigen-presenting cells (APCs), such as dendritic cells, mobilize from peripheral tissues and transit to local draining lymph nodes [3]. These orchestrated series of interactions result in changes in gene expression, alterations in surface receptor repertoire, and production of effector molecules to ensure the quality and magnitude of immune responses against foreign challenges [4].

Intravital microscopy shedding new light

Until a decade ago, evidence for immune cell trafficking and stem cell homing was largely inferred from static tissue analysis, as well as in vitro dynamic studies of isolated cells devoid of stromal elements typically present in vivo [5]. Similarly, early imaging studies were limited to low-resolution leukocyte behavior in assessable anatomic sites, such as blood vessels [6]. The development of intravital 2-photon laser scanning microscopy (2P-LSM) overcome these technical limitations, and 2P-LSM has become a tool of choice for detailed assessment of in vivo cellular migration and interactions [7]. To date, data generated with 2P-LSM—whose advantages include increased visual depth (>200 μm), high spatial resolution (<1 μm), superb signal-to-noise ratio, reduced photobleaching, prolonged acquisition (minutes to hours), and a multiplex dataset (*xyzt* with multispectral detection)—have yielded new perspectives and insights into how, where, and when immune cells interact in vivo [8]. More recently, application of 2P-LSM has enabled detailed, real-time assessment of cellular migration and interactions within the intact BM cavity—functions critical to the homing and early engraftment of HSPCs [9].

Visualizing the BM niche in situ

Using a combination of confocal and intravital 2P-LSM imaging methods, Lo Celso et al. [9] tracked individual hematopoietic cells within the calvarium BM of mice. This study was designed to examine the relationships between HSPCs and blood vessels, osteoblasts, and endosteal surfaces as they home and engraft in irradiated, c-Kit receptor–deficient recipient mice. Their analysis showed that

Financial disclosure: See Acknowledgments on page S50.
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1083-8791/$ – see front matter
http://dx.doi.org/10.1016/j.bbmt.2012.10.020

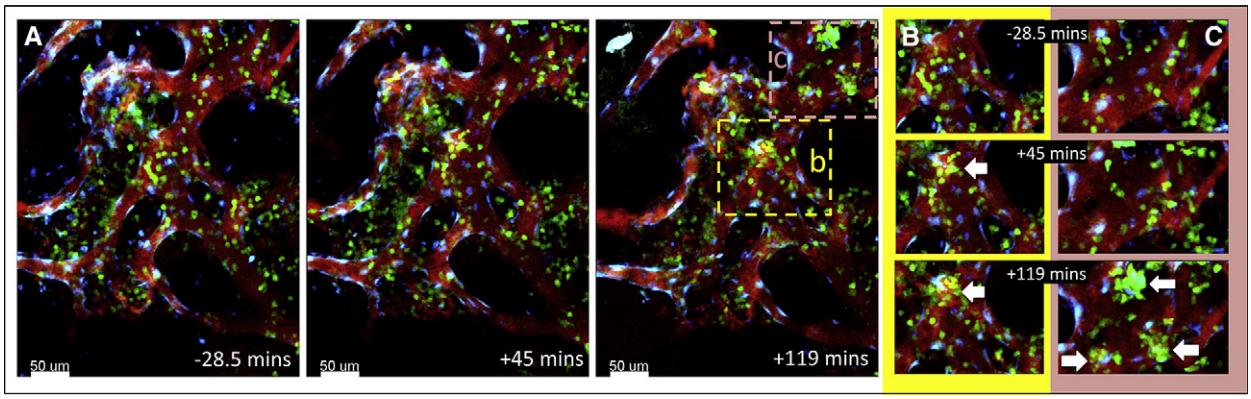

Figure 1. Dynamic mobilization of neutrophils in the BM after systemic LPS challenge. LPS (100 ng) was administered i.v. into a LysM-GFP$^{+/-}$→ubiquitin-CFP chimera mouse during an intravital 2P-LSM imaging session on the calvarium BM cavity. (A) Representative snapshots showing the dynamic nature of neutrophil (green) mobilization in the BM. Time stamp = min:sec relative to LPS administration. (Scale bar: 50 μm.) Vessels are highlighted by TRITC-dextran (red). Note the fenestrated endothelium (blue-white). (B and C) Two zoomed-in views of (A) at the same 3 time points, showing rapid "swarming" behavior by BM-resident neutrophils (white arrows) in response to LPS.

HSPCs reside in the BM within a complex, nonrandom tissue architecture comprising osteoblasts and microvessels. Furthermore, HSPC subsets localize to distinct BM sublocations during differentiation in both a cell-autonomous and cell-nonautonomous manner. Our laboratory also used 2P-LSM to demonstrate the effect of differential Notch glycosylation of HSPCs on BM niche occupancy [10]. Mendez-Ferrer et al. [11] subsequently demonstrated that mesenchymal stem cells exhibit a symbiotic relationship with HSPCs as heterotypic stem cell pairs within a unique BM niche.

To further advance our understanding of cellular dynamics in the BM space, we studied one of the most abundant cellular components in the BM, the polymorphonuclear neutrophils (PMNs). We applied 2P-LSM to the calvarium BM of LysM-eGFP$^{+/-}$ knockin mice in which one allele of lysozyme M (an abundant enzyme in developing and mature PMNs) is replaced by enhanced green fluorescent protein (eGFP) to facilitate the study of early PMN mobilization in a model of systemic sepsis. As early as 30 minutes after i.v. injection of lipopolysaccharide (LPS), the BM-resident PMNs appear to "swarm" and rapidly mobilize within the BM cavity (Figure 1 and Supplemental Movie 1), presumably in response to local and systemic signals for release of PMNs into the general circulation. Using BM chimeras, we are beginning to tease apart BM-derived versus radioresistant stromal and endothelial cells within the complex BM architecture (Figure 1). In the future, this multicolor imaging approach will afford the opportunity to interrogate the positioning, function, and interaction of HSPCs and their progeny with surrounding stroma. Similarly, methods used for single-cell tracking of BM-resident HSPCs provide an opportunity to study how mature immune cells in the BM interact with other BM-resident or circulating cells. For example, central memory T cells have been shown to engage circulating dendritic cells in the BM [12], and regulatory T cells (Tregs) have been observed to co-occupy with HSPCs in the same BM niche, possibly providing an immune sanctuary site for the persistence and survival of allogeneic HSPCs in the BM [13]. Our imaging data also suggest that under inflammatory conditions, BM-resident T cells migrate at a markedly slower speed compared with T cells found in an inflamed lymph node (Supplemental Movie 2). Additional imaging studies have shed light onto how other cell types migrate to the BM niche, including circulating leukemic cells, which use specialized BM endothelium to enter the BM in an E-selectin– and stromal cell derived factor 1 (SDF-1)–dependent manner [14]. Future imaging experiments promise to further highlight cellular and molecular determinants responsible for observed stem cell and immune cell behaviors within the live BM space.

Tracking hematopoietic-derived cells in the central nervous system

In addition to studying cellular dynamics in the previously inaccessible BM cavity, investigators have increasing interest in imaging immune responses in the central nervous system (CNS). Questions regarding the role of donor-derived versus host-derived immune cell components in clinical settings such as BM transplantation (BMT) and graft-versus-host disease (GVHD) can now be studied using this high-resolution intravital imaging approach. McGavern et al. [15] have led the field in applying 2P-LSM to study CNS immune response to an infectious challenge. Other imaging studies have revealed immune cell behavior during viral meningitis, including cytotoxic T cell activity and cellular division on interaction with perivascular and parenchymal APCs displaying cognate viral antigens, as well as infection-associated vascular injury in the CNS. Using BM chimeras, we were recently able to differentiate donor BM-derived versus host tissue-derived APCs during immune reactivity in the CNS (Figure 2 and Supplemental Movie 3), opening the door for multicellular analyses of CNS immunity in the future. In performing these sophisticated intravital imaging experiments, McGavern et al. [15] compared the craniotomy and skull-thinning procedures commonly used for intravital CNS imaging experiments, and found that the craniotomy approach resulted in increased astrocyte and meningeal activation. In their hands, the thin-skull technique resulted in less meningeal irritation, because the procedure does not breach bone integrity. However, because the integrity of the cranial BM cavity is breached in the thin-skull approach, the procedure carries a high risk of systemic inflammation, which may lead to local and global changes in CNS tissues. Furthermore, we have found that with careful tissue handling, microglia activation is not significantly affected with either technique, and that craniotomy can produce results that

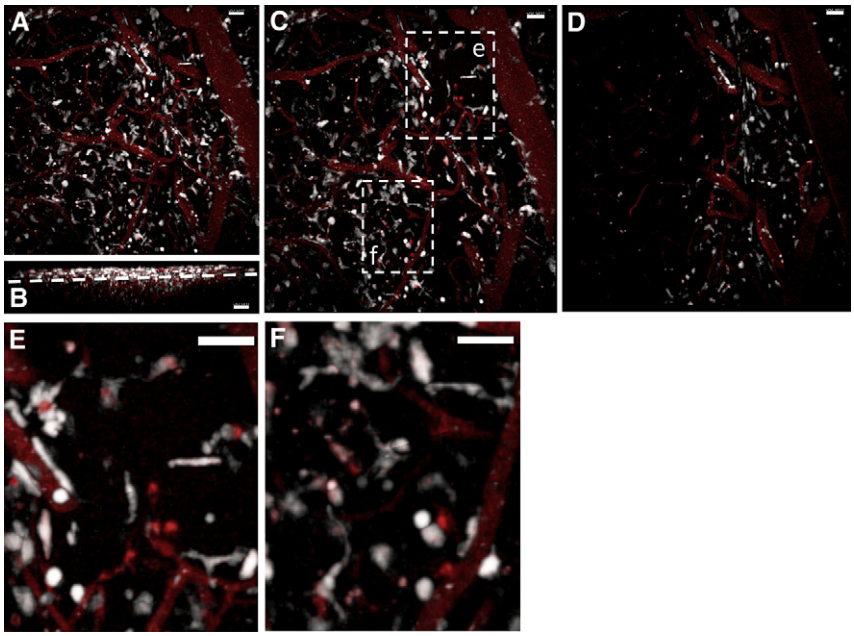

Figure 2. Replacement of CNS APCs by BM-derived hematopoietic cells after BMT. A lethally irradiated C57BL/6 mouse was rescued with BM cells isolated from an ubiquitin-CFP (white) donor mouse. (A) Static intravital 2P-LSM image captured through an implanted cranial observation window after LPS challenge showing partial reconstitution of CNS APCs from BM-derived origin (white). (B) An *xz* view of the same imaged volume in (A). Dashed lines denote the arachnoid space, delineating the meningeal layer (C) and the parenchymal layer (D). (E and F) Two zoomed-in views of (C). Note that both BM-derived (white and red) and CNS-resident (red only) cell populations exhibit phagocytic capacity (intracellular red TRITC-dextran signals). Vessels are highlighted by TRITC-dextran (red). (Scale bar: 50 μm.).

may yield novel insights when carefully applied (data not shown). In summary, intravital CNS imaging techniques, combined with cell lineage–specific fluorescent reporter mouse models, should enable detailed studies of immune cell–vessel–parenchyma communication in the settings of BMT, infection, autoimmunity, and malignancy.

Summary

In vivo imaging is a rapidly advancing field, with increasing sophistication in imaging equipment, experimental models, functional reporter reagents, and high-throughput multiparameter data collection and analysis. Indeed, the future of intravital imaging is dynamic and bright. With proper appreciation of the promises and potential pitfalls inherent in the2P-LSM technique [8], investigators now have an exciting tool to probe biological processes and mechanisms that were previously difficult to ascertain. Insights gained through 2P-LSM will be further enhanced by interdisciplinary efforts in biology, physics, mathematics, and computer sciences, which will be required to examine fainter cells, smaller molecular structures, thicker and denser tissues, and faster and more complex biochemical events.

NEW INSIGHTS

Defining the Molecular Phenotype of Virus-Specific T Cells

Immunodeficiency is a hallmark feature of the period after BMT [16]. Although the degree of immunodeficiency varies among individuals and is affected by numerous clinical factors, a common feature of myeloablative preparative regimens is lymphopenia. However, the numeric loss of immune cells is not the sole factor explaining the susceptibility of transplant recipients to infection. In the case of the T cell compartment, both qualitative and quantitative defects in T cell immunity occur. Although enumerating the frequency of T cells is relatively straightforward, assessing the qualitative, phenotypic aspects of T cell immunity is more complex. In this review we discuss recent advances in the tools used by immunologists to identify phenotypes of pathogen-specific T cells.

During a prototypical immune response to a viral pathogen, antigen-specific $CD8^+$ T cells become activated, proliferate, and gain effector functions. This initial burst of proliferation yields a large number of effector cells, most of which die during the contraction phase, leaving a smaller population of antigen-specific cells that form the memory pool. However, the population of antigen-specific $CD8^+$ T cells formed in response to a viral infection is highly heterogeneous in terms of function and phenotype [17]. Thus, T lymphocytes specific for pathogens represent a diverse collection of T cell states with a range of abilities to exert control over infection. This diversity poses a particular challenge for immunologists attempting to identify the qualitative aspects of T cells correlated with protective immunity [18].

Some of the most important advances in immunology have come from studies of this heterogeneity in the T cell compartment. Differential expression of surface antigens first identified the existence of helper T cell and cytotoxic T cell lineages before an antigen encounter [19]. Subsequently, isoforms of CD45 were used to differentiate T cells that had experienced antigen stimulation from naive cells [20]. Sallusto et al. [21] further divided antigen-experienced T cells into those that express CCR7 and are found in lymphoid tissues (central memory cells) and those that do not express CCR7 and circulate in the periphery (effector memory cells).

Along with their phenotypic heterogeneity, T cells are also functionally heterogeneous. Landmark studies by Mosmann et al. [22] identified T helper cell (Th) Th1 and Th2 lineages in the CD4 T cell compartment and paved the way for

identification of the large number of functionally distinct CD4 T cell lineages known today [23]. A high frequency of Th1-type CD4 cells that secrete several cytokines simultaneously is characteristic of T cell responses that confer immunity in some settings. The immunologic benefits of this trait, known as polyfunctionality, have subsequently been confirmed in HIV infection and vaccination [24,25], but its usefulness as a marker of immunocompetence in allogeneic BMT recipients is unclear.

The discovery of MHC tetramers provided a critical tool with which to dissect the heterogeneity of the pool of antigen-specific $CD8^+$ T cells in humans [26]. Tetramer staining allows antigen-specific T cells to be identified and isolated independent of their surface phenotype. However, although antigen-specific T cells can be assayed by costaining with antibodies specific for many differentiation markers, there is a limit to the number of labeled antibodies that can be used to costain a population of cells, given the overlap of excitation and emission spectra from closely related fluorochromes [27]. Because of this, it has become difficult to probe the phenotypic and functional complexity of the T cell compartment beyond the existing states already known.

Recent developments in flow cytometry technology have partially overcome this obstacle, however. The diversity of the CD8 T cell compartment in humans has been studied with a novel flow cytometry technique based on single-cell mass spectrometry known as cytometry by time-of-flight [28]. This platform allows resolution of many more antibody species (potentially hundreds), because there is essentially no overlap in the mass:charge ratio of the isotopes used to label the antibodies, unlike the emission spectra of fluorochromes. This technique has been used to profile the heterogeneity in the T cell compartment, and has suggested that this phenotypic heterogeneity is even greater than that already known. One important application of this approach is in studying the recovery of phenotypic heterogeneity after BMT, much as previous studies have done with TCR diversity.

In addition to probing the heterogeneity in the T cell compartment using flow cytometry, advances in nanofluidics enable examination of the functional diversity of a T cell population [25]. In this approach, the functional state of individual cells at a particular moment in time is studied by placing individual live cells in nanowells and sampling the cytokine content of the medium in each well repetitively after T cell stimulation. Studies have shown that polyfunctionality does not appear to be the preserve of a particular subset of the cells, but rather is a transient characteristic of many different cells that have produced and will produce only a single cytokine.

A third approach to defining the phenotypic features of antigen-specific T cells involves analysis by genome-wide gene expression profiling to create broad molecular phenotypes [29,30]. We have studied gene expression profiles of HIV-specific T cells from infected individuals with chronically elevated viral load in the absence of antiretroviral therapy (progressors) and compared them with those of individuals with spontaneous control of viral replication (controllers) [30]. We found that the global pattern of gene expression in HIV-specific T cells from progressors was very similar to that seen in mouse lymphocyte choriomeningitis–specific T cells responding to chronic versus acute viral infection. Moreover, analysis of the genes that distinguish between exhausted $CD8^+$ T cells and their more functional counterparts identified a new role for the transcription factor BATF in suppressing proliferation and cytokine secretion, possibly by modulating AP-1 target genes.

As higher-powered techniques to assay the phenotype of T cells are becoming available, several challenges have emerged. These include the difficulty in distinguishing true heritable differentiation states from transient activation phenotypes, and the challenge of isolating sufficient numbers of increasingly rare subpopulations of T cells to allow them to be studied. Finally, linking new T cell phenotypes to their ability to confer immunologic protection to individuals after BMT becomes more difficult as candidate phenotypes multiply.

NEW MODELS

Insights into the Immunology of Transplantation Tolerance Using a Nonhuman Primate Model

In the previous sections we described novel strategies to interrogate the immune response using both single-cell and systems biology approaches. The increasing sensitivity and flexibility of these techniques has enabled the interrogation of increasingly complex immunologic systems at a level of detail not previously feasible. These advances are facilitating a more detailed understanding of the biology of the immune response in murine models and in patients, as well as in novel transplant models, including those involving clinically relevant nonhuman primates (NHPs). Toward this end, our group and others have now developed NHP models able to incorporate the genetic, flow cytometry, and gene expression tools traditionally available only for in vitro and murine studies, such that the mechanisms controlling alloimmunity can be discerned in this complex but highly translational species.

Although historically NHP models have been rarely used to study allogeneic BMT, in the field of solid organ transplantation, NHP models have long been identified as critical to the most rapid introduction of new therapeutics into clinical practice [31]. However, until very recently, transplantation studies using NHPs have been severely limited by poor knowledge of the NHP MHC. Indeed, until the advances of the last 5 years, NHP transplantation studies were usually performed with minimal or no information on animal pedigree or MHC genetics. Given the critical impact of both MHC and minor histocompatibility antigen disparity on BMT outcomes, this severely limited the utility of NHP models of BMT. However, 2 recent technical advances have fundamentally improved the ability to perform MHC typing in macaques [32]: the application of DNA microsatellite-based typing to rhesus pedigree analysis and MHC typing [33], and the application of massively parallel pyrosequencing to allele-specific MHC typing [34]. These technical advances have allowed the creation of directed breeding colonies of rhesus macaques in which the pedigree and MHC disparity in all animals is known. Our group has used this resource to develop the first MHC-defined models of both rejection and GVHD in NHPs [32].

We have used both in vitro and in vivo analysis of alloreactivity to study the mechanisms controlling transplant rejection and GVHD using the new NHP models. Central to the in vitro studies was the flow cytometric mixed lymphocyte reaction (MLR), which is capable of distinguishing the degree of alloproliferation before and after transplantation, as well as identifying the detailed phenotype of the alloproliferating cells (Figure 3). In this assay, peripheral blood mononuclear cells (PBMCs) from transplant recipients are

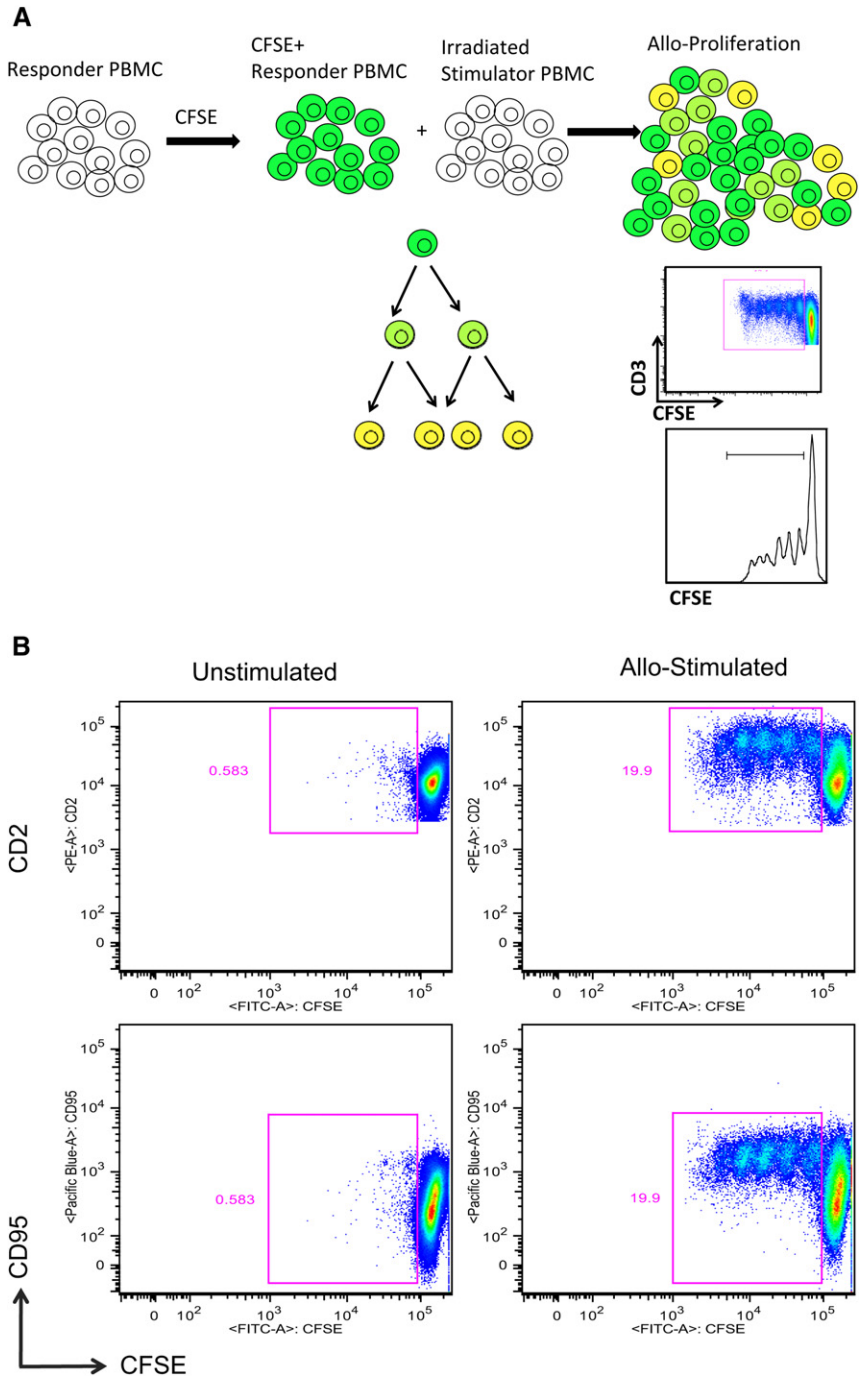

Figure 3. CFSE MLR to measure alloreactivity. (A) Experimental schema for the CFSE MLR assay. To perform a CFSE MLR assay, PBMCs are purified and labeled with CFSE as described previously [36]. The CFSE-labeled "responder" PBMCS are then mixed in culture with unlabeled "stimulator" PBMCs. As the T cells in the CFSE-labeled responder population proliferate in response to the allostimulus, the CFSE in each successive division decreases owing to dilution. Thus, proliferation can be measured by the decrease in CFSE fluorescence, and T cell alloproliferation can be calculated by determining the proportion of cells demonstrating reduced CFSE labeling. (B) T cells proliferating in response to alloantigens exhibit a $CD2^{High}/CD95^{+}$ memory phenotype. In the example shown here, CFSE-labeled responder PBMCs were allostimulated with MHC-disparate stimulator PBMCs for 5 days. Cells were stained with an antibody cocktail consisting of anti-CD2, -CD3, -CD4, -CD8, -CD28, and -CD95 antibodies, and data were acquired on an LSR II flow cytometer (BD Biosciences, San Jose CA) and then analyzed with BD FACSDiva Software (BD Biosciences, San Jose CA). Alloproliferation of T cells over the course of the assay was followed by CFSE dilution, and the expression of CD2 and CD95 was measured on the proliferating cells.

purified and labeled with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE). These cells are then mixed in vitro with either donor PBMCs (to study rejection) or cryopreserved pretransplantation recipient PBMCs (to study GVHD), and the degree of alloreactivity is inferred from the amount of proliferation measured in vitro (Figure 3A) [35]. These studies confirmed that alloactivation in NHPs is directly related to the degree of MHC disparity [35], and that cells proliferating in response to alloantigens exhibit a $CD2^{+}/CD95^{+}$ memory phenotype (Figure 3B). CFSE MLR has been subsequently used to predict the onset of rejection in an NHP model of reduced-intensity conditioning and HSCT, in which

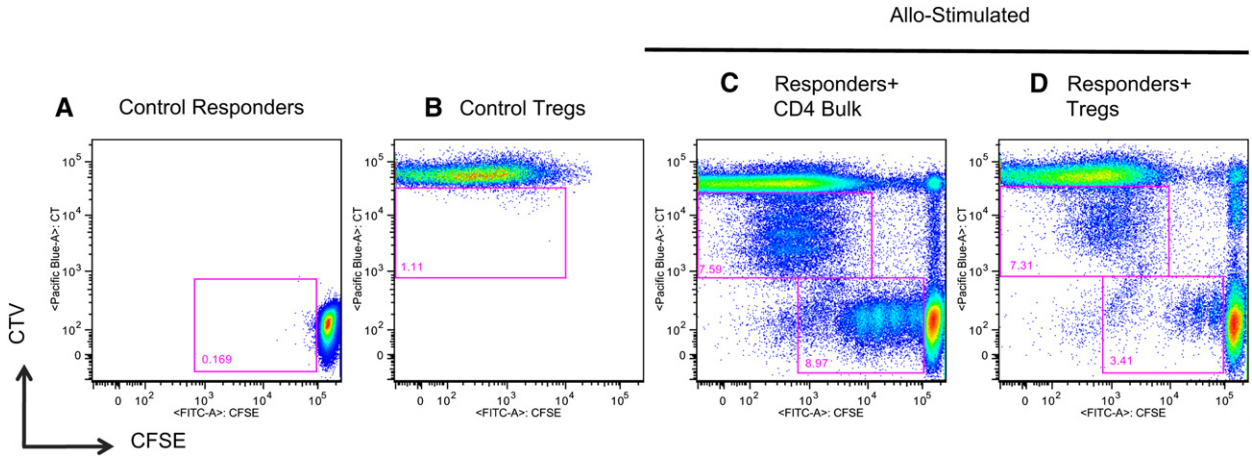

Figure 4. Tandem MLR to study the alloreactivity of effector T cells and Tregs in the same flow cytometric reaction. Both $CD4^+$ Tregs and T effectors were individually stained with the proliferation marker CellTrace Violet (CTV), and then added to responder PBMCs stained with CFSE. Cultures were allostimulated with unlabeled PBMCs for 5 days and then stained for CD3, CD4, and CD8. (A) Control MLR, with CFSE-labeled responders demonstrating no proliferation in the absence of stimulator PBMCs. (B) Control MLR, with CTV-labeled Tregs demonstrating no proliferation in the absence of stimulator PBMCs. (C) MLR showing significant alloproliferation of both CFSE-labeled responder T cells and CTV-labeled effector T cells. (D) Ability of Tregs to inhibit the alloproliferation of CFSE-labeled responder cells (compare the CFSE proliferation in D and C) while simultaneously undergoing alloproliferation themselves (compare the CTV proliferation in D and B).

mixed hematopoietic chimerism can be induced but is then rejected by recipient T cells that are resistant to tolerance-induction strategies [35]. We have recently refined the alloproliferation assay and developed a "tandem MLR" in which the alloreactivity of both effector T cells (labeled with CFSE) and of ex vivo expanded Tregs labeled with a second trackable dye (Cell-Trace Violet; Invitrogen, Carlsbad, CA) can be determined in the same flow cytometry experiment (Figure 4) [36]. Through this dual-label approach, the impact of both positive and negative signals (and their reciprocal interactions with both APCs and with one another) can be sensitively measured in vitro.

In addition to in vitro assays of alloreactivity, our group and others have used multiparameter flow cytometry analysis to identify in vivo markers of both rejection and GVHD in NHPs. We have developed a series of flow cytometry panels (Figure 5) that allow us to sensitively query the peripheral blood, BM, and lymph nodes longitudinally and the spleen,

	Laser	488 nm Blue					633 nm Red			407 nm Violet		
	filter/photo-multiplier	530/30	575/26	670/14	695/40	780/60	660/20	710/50	780/60	450/50	525/50	660/40
Phenotype/Functional Parameters Measured	Tube #	FITC, Alexa488	PE	PerCP	PerCP-Cy5.5	PE-Cy7	APC Alexa 647	Alexa 700	APC-Cy7	V450	V500	Qdot 655/ EF650
Flow Cytometry Compensation	1	CD20	CD127	CD8	CD14	CD28	CD27	CD4	CD8	CD20	IgG1	CD279/ PD1
Stem Cells from Bone Marrow or Apheresis Products	2	CD38	CD34	CD45		CD14	CD3	CD4	CD16	CD20	CD8	
CFSE MLR	3	CFSE			CD3	CD28	CD2		CD4	CD95	CD8	
T Cell, B cell and NK Cell Enumeration	4	CD20	NKG2a		CD3	CD56	NKp80	CD4	CD16	CD8		
T cell Naïve/Memory Subpopulations	5	CD28	CD127		CD14/ CD20	CD45RA	CCR7	CD3	CD4	CD95	CD8	
T cell Memory and Exhaustion	6		CD2		CD127	CD45RA	CD28	CD3	CD4	CD95	CD8	CD279 (PD1)
Memory and Adhesion Molecule Expression	7	CXCR4	CD127		CCR5	CD45RA	CD28	CD3	CD4	CD95	CD8	
T cell Proliferation and Activation	8	Ki67	BCL2	HLA DR		CD28	CD38	CD4	CD8	CD95	CD3	
T cell Cytotoxicity and Activation	9	Perforin	Granzyme B	HLA DR		CD28	CD38	CD4	CD8	CD95	CD3	
Proliferation/ Cytotoxicty Co-Expression	10	Ki67	Granzyme B			CD28	CD127	CD4	CD8	CD95	CD3	
Tregs	11	FOXP3	CD25		CD4	CD127				CD3		
Th17 Enumeration	12	IFN-gamma	IL-17A		CD3			CD4			CD8	

Figure 5. Phenotyping Panel for Multiparameter Flow Cytometry in NHPs.

liver, lungs, and colon at terminal analysis for a large number of phenotypic and functional markers. The majority of the antibodies used in these panels are highly cross-reactive with human targets and thus also can be used to build similar panels to analyze clinical trial samples longitudinally. The cross-reactivity of the analytic reagents used for post-transplantation immune monitoring represents a key strength of the NHP studies, in that direct comparisons can be made between immune analysis in NHPs and in patients. Indeed, the multiparameter flow cytometry (MFC) analysis that we and others have performed in primates has led to several observations that are now being tested in clinical trials. The first observation concerns the expansion of memory phenotype cells that occurs before rejection of both BM and solid organ allografts [35,37]. In NHPs, the expansion of CD95 (Fas)$^+$/CD28$^+$ central memory (CD4$^+$) and CD95$^+$/CD28$^-$ effector memory (CD8$^+$) cells was strongly correlated with impending rejection. This linkage between memory cell expansion and rejection is now being studied in clinical trials of both solid organ transplantation and HSCT. The memory phenotype T cells that were implicated in rejection in NHPs also demonstrate increased expression of the adhesion molecules CD2 and LFA-1 [38,39]. This observation has led to the development of monitoring strategies for both CD2 and LFA-1 expression (to test their predictive power for the risk of alloactivation), as well as several intervention trials targeting both CD2 and LFA-1 to prolong allograft acceptance.

Along with using MFC to monitor and predict transplant rejection, our group also has developed a rhesus macaque model of GVHD in which alloactivation is measured through a series of cell-based and serum-based multiplex assays [40]. Studies using the NHP GVHD model have established the capability of MFC to monitor CD4$^+$ and CD8$^+$ T cell proliferation (using Ki-67), as well as the acquisition of markers of memory (CD45RO, CCR7, CD95, CD28, CD2), activation (CD38, HLA-DR, BCl-2, perforin, granzyme), and regulation (FoxP3, CD25 in CD127$^-$ CD4$^+$ cells) during GVHD or its prevention with both standard-of-care and novel therapies. This work identified the expression of both Ki-67 and granzyme B on peripheral blood CD8$^+$ and CD4$^+$ T cells as sensitive markers of both GVHD (Ki-67^{high}, granzyme B^{high}) and its prevention (Ki-67^{low}, granzyme B^{low}), suggesting a novel approach to GVHD diagnosis and monitoring in the blood, which is currently under clinical validation.

One of the key advantages of using the NHP model is that, unlike other preclinical models, most of the novel biological therapies currently being developed for patients are cross-reactive with NHP targets and thus can undergo efficient preclinical efficacy evaluation in NHPs. Our first proof of concept of this close link between NHP models and the clinic was found with the costimulation blockade agent CTLA4-Ig (abatacept). Our NHP studies supported the efficacy of an abatacept-containing regimen for the prevention of acute GVHD. These results rapidly led to a first-in-disease trial of abatacept for GVHD prevention (ClinicalTrials.gov; NCT 01012492). This pilot study has shown encouraging pharmacokinetic, biological, and clinical results and has led to the initiation of a multicenter randomized Phase II study of abatacept for GVHD prevention.

The development of detailed, multiplexed analysis tools has transformed our ability to explore the mechanisms controlling alloreactivity in both simple and complex preclinical models. These technical advances have greatly expanded our ability to use the clinically relevant NHP model to understand 2 of the most critical complications of BMT: rejection and GVHD. These studies are able to offer new mechanistic insights into the cause and control of these posttransplantation complications, as well as provide key preclinical data to rapidly translate new diagnostic and therapeutic modalities to the clinic.

CONCLUSION

Dynamic cellular imaging is a rapidly advancing field. Armed with increasingly sophisticated imaging equipment, along with a proper appreciation of the promises and potential pitfalls inherent to individual techniques and of the new challenges that will inevitably emerge, investigators now have an exciting opportunity to fully interrogate biological processes and mechanisms that were previously difficult to fully ascertain. Visualization and molecular characterization of interactions involving a spectrum of immune cells, ranging from undifferentiated blood stem cell progenitors to terminally differentiated central and effector memory cells, is now possible at a level of detail not previously realized. Application of these techniques in relevant mouse, NHP, and human systems is yielding new perspectives with respect to how, where, and when immune cells interact in vivo during inflammation, infection, and alloimmunity and promises to adjust the lens through which the next generation of immunologic advances will be observed, appreciated, and enjoyed.

ACKNOWLEDGMENTS

Karnail Singh was supported by the National Institute of Allergy and Infectious Diseases (grant 2U19 AI051731).

Financial disclosure: Alex Y. Huang was supported by the National Cancer Institute (grant R01 CA171523), National Institute of Allergy and Infectious Diseases (grant R21A1092299), St Baldrick's Foundation, Hyundai "Hope-on-Wheels" Program, Alex's Lemonade Stand, Gabrielle's Angel Foundation, Dana Foundation, and Cancer Research Institute. Agne Petrosiute was supported by the National Institutes of Health (grant K12HD057581), St Baldrick's Foundation, and Hyundai "Hope-on-Wheels" Program. Deborah S. Barkauskas was supported by the National Institutes of Health (grant T32EB7509). W. Nicholas Haining was supported by the National Institutes of Health (grants AI082630, AI057266, and AI090023). Kenneth R. Cooke was supported by the Burroughs Welcome Fund, Leukemia and Lymphoma Society, and National Center for Regenerative Medicine at Case Western Reserve University. Leslie S. Kean was supported by the National Institute of Allergy and Infectious Diseases (grant 2U19 AI051731), National Heart, Lung and Blood Institute (grants 1R01 HL095791 and 2U24 RR018109), and a Burroughs Wellcome Fund Career Award in the Biomedical Sciences.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2012.10.020.

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