ORIGINAL ARTICLE

Reconstitution of huPBL-NSG Mice with Donor-Matched Dendritic Cells Enables Antigen-Specific T-cell Activation

Airi Harui · Sylvia M. Kiertscher · Michael D. Roth

Abstract Humanized mouse models provide a unique opportunity to study human immune cells in vivo, but traditional models have been limited to the evaluation of non-specific T-cell interactions due to the absence of antigen-presenting cells. In this study, immunodeficient NOD/SCID/IL2r-γ^{null} (NSG) mice were engrafted with human peripheral blood lymphocytes alone or in combination with donor-matched monocyte-derived dendritic cells (DC) to determine whether antigen-specific T-cell activation could be reconstituted. Over a period of 3 weeks, transferred peripheral blood lymphocytes reconstituted the spleen and peripheral blood of recipient mice with predominantly human CD45-positive lymphocytes. Animals exhibited a relatively normal CD4/CD8 ratio (average 1.63:1) as well as reconstitution of CD3/CD56 (averaging 17.8%) and CD20 subsets (averaging 4.0%). Animals reconstituted with donor-matched CD11c+ DC also demonstrated a CD11c+ population within their spleen, representing 0.27% to 0.43% of the recovered human cells with concurrent expression of HLA-DR, CD40, and CD86. When immunized with adenovirus, either as free replication-incompetent vector (AdV) or as vector-transduced DC (DC/AdV), there was activation and expansion of AdV-specific T-cells, an increase in Th1 cytokines in serum, and skewing of T-cells toward an effector/memory phenotype. T-cells recovered from animals challenged with AdV in vivo proliferated and secreted a Th1-profile of cytokines in response to DC/AdV challenge in vitro. Our results suggest that engrafting NSG mice with a combination of lymphocytes and donor-matched DC can reconstitute antigen responsiveness and allow the in vivo assessment of human immune response to viruses, vaccines, and other immune challenges.

Keywords dendritic cell · adenovirus · vaccination · xenotransplant · cytokine · humanized mouse

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Introduction

While inbred strains of conventional and genetically modified mice have provided an unparalleled opportunity to examine the process of antigen presentation and the characteristics of adaptive T-cell immunity, translating these findings into new approaches for human immunotherapy has proven problematic (Mestas and Hughes 2004; Ju et al. 2010). Responses to cytokine therapy, vaccines, gene therapy, adjuvants, and adoptive cell transfers that are reproducible and robust in the mouse are often rare when translated into human clinical trials. This is not surprising given that human dendritic cells (DC) are distinctively

different from those of the mouse, as are the antigens of interest, the major histocompatibility complex (MHC) repertoire, toll-like receptor expression, cytokine responses, and many other features. Genetic diversity is also a major factor, as are the susceptibility and prior exposure to many human pathogens and immune challenges (Mestas and Hughes 2004; Zaiss et al. 2009). Even transgenic animals that express a human MHC haplotype or spontaneously develop organ-specific tumors have not proven to be predictive of integrated human immune responses (Pascolo et al. 1997).

One approach for bridging the gap between mouse models and human clinical trials has been to develop humanized mice—immunodeficient strains which permit the engraftment and expansion of human immune cells in vivo (Shultz et al. 2007; Pearson et al. 2008). A variety of different humanized mouse models have been developed and fall into two distinct categories: (1) those with a slow but more extensive immune reconstitution resulting from stem cell transfer (Lapidot et al. 1992; Greiner et al. 1998; Ito et al. 2002) or (2) those with rapid reconstitution based on the transfer of fully differentiated human peripheral blood cells (Mosier et al. 1988; Hesselton et al. 1995; May et al. 2005). We have focused on the latter, not only for the speed of the reconstitution but for the prospect that the reconstituted immune system would reflect the immunologic memory of the human donor. For these studies, immunodeficient NOD/SCID-IL2rγ^{null} (NSG) mice, which lack the γ-chain of the IL-2 receptor in addition to the NOD/SCID background, were chosen (Ishikawa et al. 2005; Shultz et al. 2005). NSG mice lack high affinity receptors for interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21. As a result of this ineffective cytokine signaling, these mice completely lack functional T-cells, B-cells, NK cells, macrophages, and DC and allow nearly complete immune reconstitution by engrafted human cells (Ishikawa et al. 2005; Shultz et al. 2005). We hypothesized that humanized NSG animals, when reconstituted with both peripheral blood lymphocytes (PBL) and with donor-matched antigen presenting DC, would allow us to examine the process of human antigen presentation and the activation of antigen-specific T-cells as it occurs in vivo, in the context of the immunologic repertoire of the human donor. In these studies, we have used a replication-incompetent serotype-5 adenoviral vector (AdV) as the antigen. Human exposure to adenovirus and the development of anti-viral memory T-cell responses occurs with a high frequency in the adult population (Olive et al. 2001; Roth et al. 2002). As predicted, only human peripheral blood lymphocyte (huPBL)-NSG mice that were reconstituted with both PBL and DC demonstrated AdV-specific T-cell proliferation and cytokine production. Furthermore, the characteristics of the AdV-specific response recapitulated

those of the originally implanted PBL. This model should provide a rapid and highly flexible system for studying the activation of human antigen-specific immunity and provide a novel platform for vaccine development and testing, as well as for the study of interactions between antigen-specific immunity and a variety of host challenges such as viral exposures (including HIV), tumors, and systemic drug effects.

Materials and methods

Animals

Six- to 8-week-old NSG mice were obtained from The Jackson Laboratories (Bar Harbor, ME, USA) and housed at the UCLA Mouse/Human Chimera Core Facility under laminar flow conditions with all food, water (acidified), caging, and bedding autoclaved before use. No antibiotics were administered. All procedures were approved by the UCLA Animal Research Committee.

Culture media and reagents

Cells were cultured in complete medium composed of RPMI 1640 (Mediatech, Manassas, VA, USA) supplemented with 10% heat-inactivated human AB serum (Omega Scientific, Tarzana, CA, USA), penicillin–streptomycin–fungizone (Invitrogen, Carlsbad, CA, USA), and 10 mM HEPES (Sigma Chemical, St. Louis, MO, USA). Monoclonal antibody (mAb) combinations used for the enrichment of T-and B-cells (anti-CD14, anti-CD16, and anti-CD25) and DC (anti-CD3, anti-CD16, and anti-CD19) by negative depletion were obtained from BD Biosciences (San Jose, CA, USA). Anti-mouse Ig-conjugated immunomagnetic beads were from Invitrogen (M450 Dynabeads; Carlsbad, CA, USA). Fluorochrome-conjugated mAbs against CD3, CD4, CD8, CD11c, CD86, and HLA-DR were obtained from BD Biosciences; anti-CD127 and anti-GITR from eBiosciences (San Diego, CA, USA); and anti-CD25 from BioLegend (San Diego, CA, USA). T-cell proliferation in vitro was evaluated using carboxyfluorescein diacetate, succinimidyl ester (CFSE; Vybrant CFDA SE Cell Tracer Kit, Invitrogen-Molecular Probes, Eugene, OR, USA).

Adenoviral vectors

A replication-deficient adenoviral vector (AdV) expressing green fluorescent protein (GFP) and modified to express an additional Arg-Gly-Asp peptide sequence in the HI loop region of their fiber knob was obtained from Mizuguchi et

al. (2001) (AdV-GFP). A replication-deficient adenoviral vector with a matching CMV promoter but without an inserted transgene sequence (AdV-RR5) was also used as previously described (Roth et al. 2002). Adenoviral vectors were propagated in 293 cells, purified by CsCI density centrifugation, dialyzed, and stored in −80°C. Viral particle titers were determined spectrophotometrically (Mittereder et al. 1996).

Generation and transduction of donor-matched DC

Peripheral blood mononuclear cells (PBMC) were obtained from healthy normal donors by density gradient centrifugation and DC prepared by culturing the adherent fraction with 800 IU/ml granulocyte-macrophage colony stimulating factor (GM-CSF; Bayer Healthcare, Seattle, WA, USA) and 63 ng/ml IL-4 (R&D Systems, Minneapolis, MN, USA) for 5 days at 37°C as previously described (Kiertscher and Roth 1996). DC were recovered on day 5 and purified by negative depletion using a mAb cocktail and anti-mouse Ig-conjugated immunomagnetic beads. DC were either used fresh or aliquoted and cryopreserved in medium containing 20% AB serum and 10% DMSO. Fresh or cryopreserved DC that had been thawed and cultured for 8–24 h in complete media containing GM-CSF and IL-4 were transduced with AdV as described previously (Harui et al. 2006a, b). Briefly, 5.0×10^5 DC suspended in 100 μl of complete medium were exposed to a suspension of AdV in phosphate-buffered saline (PBS) containing calcium and magnesium at a multiplicity of infection (MOI) of 4,000 viral particles/cell. Cultures were incubated for 2 h at 37°C in a humidified incubator and an additional 800 μl of complete media containing GM-CSF and IL-4 was then added. Human MegaCD40L (200 ng/ml, Alexis Biochemicals, Plymouth Meeting, PA, USA) and interferon gamma (IFN-γ; 100 U/ml, PeproTech, Rocky Hills, NJ, USA) were added into culture media 24 h later to stimulate a mature DC phenotype, and cells were incubated for an additional 24 h and harvested.

Reconstitution and vaccination of NSG mice

A combination of T-and B-cells were purified from PBMC by negative depletion using a mAb cocktail and anti-mouse Ig-conjugated immunomagnetic beads. Cells were resuspended in PBS and administered to recipient NSG mice (10^7 cells/mouse) by intraperitoneal (i.p.) injection on day 0 of the reconstitution. To adapt huPBL-NSG mice as a platform for assessing human antigen-specific T-cell activation, animals were also administered an i.p. injection on day 0 of 0.5×10^6 DC that had been generated from the same donor (donor-matched) (Fig. 1). Monocyte-derived DCs were adminis-

tered as either control DC (which had not been exposed to AdV in vitro) along with 1×10^9 or 1×10^{10} free AdV-GFP particles, or as DC that had previously been transduced with the AdV-GFP vector in vitro (DC/AdV-GFP). For some experiments, animals were administered a second injection of donor-matched DC that had been prepared from the same donor (either DC/AdV-GFP or control DC in combination with free viral particles) 7 days later using the same methods.

Cell recovery and analysis of human cells by flow cytometry

Single-cell suspensions were prepared from the spleen by mechanical disaggregation and from the peritoneal cavity by lavage followed by depletion of red blood cells by hypotonic shock. Cells were stained with fluorochrome-conjugated mAbs and analyzed by flow cytometry (FacsCalibur, Becton-Dickinson, San Jose, CA, USA) in combination with FCS Express analysis software (DeNovo Software, Los Angeles, CA, USA). Human cells were defined by expression of human CD45 and subsets identified by specific marker expression for T-cell subsets (CD3+/CD4+ or CD3+/CD8+ alone or in combination with GITR, CD127, and/or CD25), B-cells (CD20), NK cells (CD3-/CD56+), NK-T-like cells (CD3+/CD56+), or DC (CD11c, CD40, CD86, and/or HLA-DR).

Detection of antigen-specific T-cell responses

Recovered spleen cells were challenged in vitro with donor-matched control DC or with DC/AdV-RR5 to detect responses to adenoviral capsid antigens. Antigen-specific responses were assessed by intracellular cytokine stimulation (ICCS), proliferation, or cytokine secretion assays. For ICCS, 2×10^6 spleen cells were stimulated with either control DC or DC/AdV-RR5 at a ratio of 1:10 (DC/spleen cells ratio) in complete media in the presence of 3 μg/ml anti-CD28 Ab (BD Biosciences) and 1 ng/ml IL-12 (PeproTech) for 5 h at 37°C. Brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) was added at 10 μg/ml after the first hour. At the end of the culture period, cells were fixed and cryopreserved. Intracellular staining for IFN-γ and tumor necrosis factor alpha (TNF-α) was then carried out according to the manufacturer's protocol (BD Biosciences) in combination with counterstaining using fluorochrome-conjugated anti-CD3, anti-CD4, and anti-CD8 antibodies. A minimum of 150,000 CD3+ cell events were analyzed by multi-color flow cytometry. For proliferation assays, spleen cells were first labeled with CFSE (0.5 μM) and then 2×10^5 cells/well co-cultured with either control DC or DC/AdV-RR5 at a ratio of 1:20 (DC/spleen cells ratio) in the presence of low dose IL-2 (10 U/
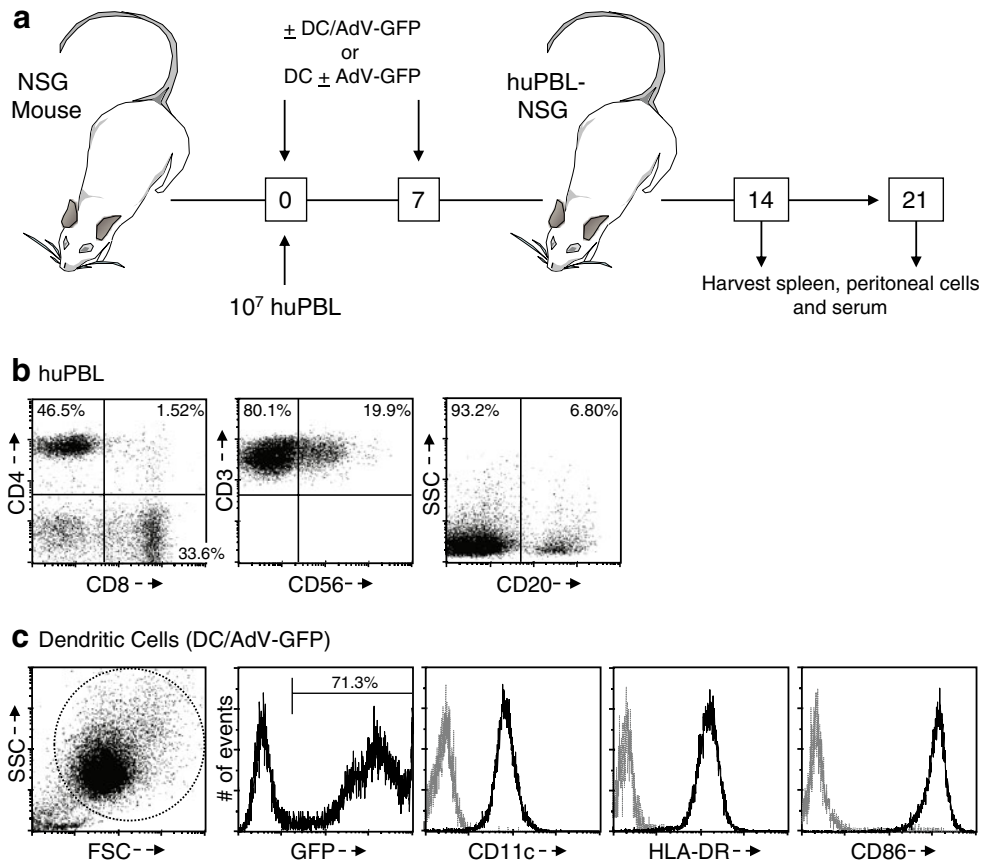
Fig. 1 Reconstitution of huPBL-NSG mice with both PBL and donor-matched dendritic cells. **a** Six- to 8-week-old NSG mice were implanted with 10^7 purified human T-and B-cells (*PBL*) by i.p. injection. In order to restore antigen-presenting activity, animals were also administered donor-matched monocyte-derived DC alone or in combination with adenoviral antigens (as either DC/AdV-GFP or control DC + free AdV-GFP particles) by i.p. injection. In some experiments, animals received a single administration of $5×10^5$ DC and in other experiments they received a second dose of DC 7 days later. Spleens, peritoneal cells, and serum were recovered on day 14 or 21 for assessment of antigen-specific immune responses. **b** Purified human PBL used for implantation into NSG mouse were composed of subsets of T-cells (CD3+/CD4+, CD3+/CD8+, and CD3+/CD56+) and B-cells. **c** Monocyte-derived DC generated from the same donor express high levels of antigen and maturation markers. DC were transduced with AdV-GFP at an MOI of 4,000 viral particles/cell and were stained with antibodies against DC phenotype/maturation markers including CD11c, HLA-DR, and CD86 48 h later. Flow cytometry demonstrated a uniform population of DC by forward scatter (*FSC*) and side scatter (*SSC*). The percentage of GFP-positive cells was used to measure the percentage of DC expressing AdV antigens after transduction. The fluorescent profile of unstained DC is presented in *gray* and fluorescent staining for specific markers are depicted by *black histogram curves*. Representative experiment, *n*=5

ml) and IL-7 (1 ng/ml). Cells were harvested 5 days later, counterstained with fluorescent-conjugated mAb to indicated markers and analyzed by flow cytometry. Antigen-specific cytokine secretion was assessed by collecting supernatants from the same wells used for proliferation assays and the levels of human IFN-γ and TNF-α measured by SearchLight multiplex assay (Thermo Scientific, Rockford, IL, USA).

In vivo cytokine production

Serum samples were collected from animals at the time of spleen recovery and assayed for the presence of human IFN-γ and TNF-α to determine whether reconstitution with control DC versus DC/AdV-GFP resulted in detectable differences in systemic cytokine levels, reflecting the in vivo activation of antigen-specific T-cells.

Statistical analysis

Comparisons between groups were performed using Student's *t* test, with *p*<0.05 accepted as a significant difference between groups. All experiments were performed with replicates and results from at least three separate experiments were pooled for statistical analysis.

Results

Reconstitution of huPBL-NSG mice with both PBL and donor-matched dendritic cells

To adapt huPBL-NSG mice as a platform for assessing human antigen-specific T-cell activation, animals were implanted with an i.p. injection of 10^7 human PBL and then supplemented with $0.5–1.0×10^6$ DC that had been generated from the same donor (donor-matched) as detailed in Fig. 1. Donor PBL were composed primarily of CD3+ T-cells with a normal complement of CD4 and CD8 subsets, a population of CD3+/CD56+ cells, and CD20+ B-cells (Fig. 1b). Culture of adherent PBMC from the same donor with GM-CSF and IL-4, followed by immunomagnetic depletion of contaminating lymphocytes, resulted in a relatively pure and homogenous population of DC (Fig. 1c). DC prepared in this manner were highly susceptible to transduction by AdV and expressed high levels of the characteristic CD11c marker, MHC molecules (HLA-DR), and costimulatory molecules (such as CD86) required for efficient T-cell activation.

Immune reconstitution of NSG mice with these two populations was associated with a marked migration to, and expansion of, human cells populating the spleen over the first 2–3 weeks. Splenic repopulation was associated with a concomitant decrease in the number of intraperitoneal cells (Fig. 2a). By the end of 3 weeks, the average yield of human cells from the spleen was $19.4±6.7×10^6$ CD45+ cells and the number of residual CD45+ cells in the peritoneum had decreased to $1.9±0.6×10^6$. After 14 days, 78.1±3.0% ($n=3$) of recovered spleen cells expressed human CD45 and the percentage of human cells remained relatively constant through 21 days (Fig. 2b).

Recovered spleen cells contained a distribution of lymphocyte subsets similar to that of normal human blood including CD3+/CD4+, CD3+/CD8+, and CD3+/CD56+ T-cells along with a small population of B-cells (CD20+) (Fig. 3a). The CD4/CD8 ratio averaged 1.63:1 ($n=15$) and there was a direct relationship between the frequency of CD3+/CD56+ T-cells in the PBL used for implantation and those covered from the spleen 2–3 weeks later ($r^2=0.85$, $p<0.01$). There was little evidence for T-cell activation as measured by the expression of either CD25 or CD69 (data not shown). The i.p. administration of mature monocyte-derived DC into huPBL-NSG mice also resulted in their migration and repopulation of the spleen by the end of 2 weeks. Similar to the mature phenotype of these cells at the time of administration, the majority of recovered CD11c+ splenic DC expressed high levels of HLA-DR and CD86 (>70%) (Fig. 3b). huPBL-NSG mice reconstituted in this manner exhibited no physical signs of graft versus host disease.

Activation of antigen-specific T-cells in vivo

The capacity for human DC derived from the same donor to interact with and stimulate antigen-specific T-cells in vivo in huPBL-NSG animals was evaluated under two different conditions. In the first approach, animals were administered DC in combination with free AdV-GFP particles. In the second approach, animals were administered DC that had been loaded 48 h earlier with AdV capsid antigens in vitro. In both settings, the stimulation of memory T-cell responses to AdV capsid antigens was assessed by a standard 5-h ICCS assay in which nonspecific T-cell activation was assessed by challenging recovered cells with control DC (containing no antigen) while AdV-specific T-cell immunization was assessed by challenging with DC that had been loaded with the AdV/RR5 vector. Figure 4 demonstrates the results from a typical experiment. The baseline frequency of AdV-
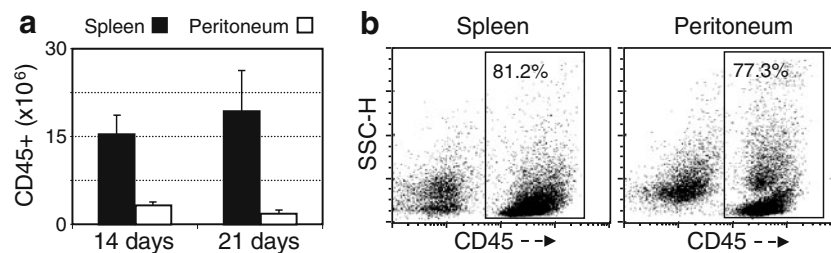

Fig. 2 Engraftment of NSG mice with human PBL. Six- to 8-week-old NSG mice were implanted with 10^7 purified human T-and B-cells (*PBL*) by i.p. injection. Spleen and peritoneal cells were recovered after 14 or 21 days, counted, and stained with anti-human CD45 mAb for analysis by flow cytometry. **a** The increase in human CD45+ leukocytes within the spleen from 14 to 21 days was associated with a concomitant decrease in peritoneal cell number, suggesting a combined redistribution and expansion of human cells following i.p. implantation. **b** Representative dot-plots demonstrating the percentage of recovered cells from the spleen and peritoneum that expressed human CD45 at day 14. Average percentage of human CD45+ cells in the spleen at day 14 was 78.1±3.0%, $n=3$
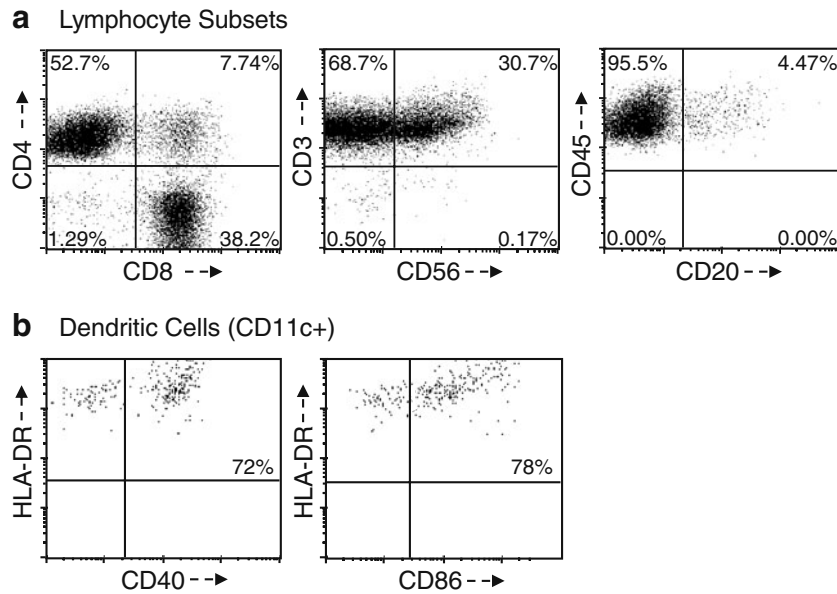
Fig. 3 Lymphocyte and DC phenotypes reconstituting the spleens of huPBL-NSG mice. **a** Six- to 8-week-old NSG mice were implanted with 10^7 purified human PBL at day 0 in combination with 5×10^5 donor-matched monocyte-derived DC by i.p injection at day 0 and day 7. Spleens were recovered 14 days later and cells expressing human CD45 analyzed by flow cytometry. CD3+ T-cells exhibited a normal distribution of CD4+ and CD8+ subsets (*left*); a small population of B-cells was identified by their expression of CD20 (*right*); and NK-T-like cells were identified by their expression of CD56 alone or in combination with CD3, respectively (*center*). Representative experiment, $n=15$. **b** Six- to 8-week-old NSG mice were implanted with 10^7 human PBL at day 0 and 5×10^5 donor-matched monocyte-derived DC by i.p injection at day 7. Spleens were recovered 14 days later and DC were identified by gating on the population of cells expressing CD11c and analyzing the CD11c+ subset for their expression of both HLA-DR and CD40 (*left*, gated for CD45+/CD11c+ cells) and both HLA-DR and CD86 (*right*, gated for CD45+/CD11c+ cells). Representative experiment, $n=5$

specific T-cells capable of producing IFN-γ and TNF-α before implantation was measured at 0.10% of CD4+ T-cells (all reported frequencies adjusted for the response to control DC) and 0.03% of CD8+ T-cells (Fig. 4a). When

PBL were administered to NSG mice in the absence of DC, the frequency of AdV-specific CD4+ T-cells recovered from day 21 spleen cells was always less than 0.02% (data not shown). However, huPBL-NSG mice that were
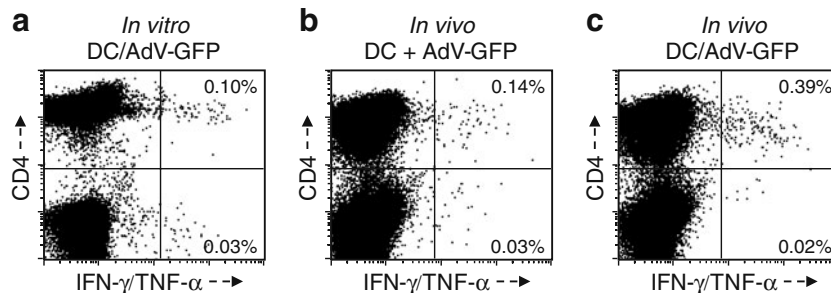

Fig. 4 Donor-matched DC loaded with AdV antigens stimulate antigen-specific T-cell responses in vitro and in vivo in huPBL-NSG mice. Adherent PBMC were cultured with GM-CSF and IL-4 for 5 days, transduced with 4,000 MOI AdV-GFP (DC/AdV-GFP), with 4,000 MOI AdV-RR5 (DC/AdV-RR5), or cultured in transduction medium alone (control DC), and then matured with MegaCD40L and IFN-γ. **a** The capacity to stimulate antigen-specific T-cells in vitro was assessed by co-culturing DC/AdV-GFP with purified donor-matched T-cells (DC/T ratio=1:20) for 7 days followed by challenge with either control DC or DC/AdV-RR5 to detect AdV-specific T-cell frequency in a standard 5-h ICCS assay. Frequencies of antigen-specific CD4+ and CD8+ T-cells expressing IFN-γ and/or TNF-α are noted, respectively (all frequencies are adjusted for the background response to control DC in the absence of antigen). Representative experiment, $n=3$. **b, c** The capacity for DC to stimulate antigen-specific T-cells in vivo in huPBL-NSG mice was similarly assessed by immunizing animals with either **b** 5×10^5 DC/AdV-GFP (on day 7) or with **c** 5×10^5 control DC (on day 7) in combination with 2×10^9 free AdV-GFP particles. Spleen cells were harvested 14 days later and challenged in vitro with either control DC or DC/AdV-RR5 to detect AdV-specific T-cell frequency in a standard 5-h ICCS assay. Frequencies of antigen-specific CD4+ and CD8+ T-cells expressing IFN-γ and/or TNF-α are noted, respectively. Representative experiment, $n=4$

immunized in vivo with the combination of DC and free AdV particles exhibited an AdV-specific CD4+ T-cell frequency of 0.14% (Fig. 4b). Furthermore, when huPBL-NSG mice were immunized in vivo with DC/AdV-GFP, the frequency of AdV-specific CD4+ T-cells increased markedly to 0.39% (Fig. 4c). In replicate experiments, the AdV-specific CD4+ T-cell responses in animals that were immunized with DC/AdV-GFP were always greater than the responses in animals that received control DC in combination with free AdV-GFP. When administering free AdV-GFP, the response to 10^{10} viral particles (shown in Fig. 4b) was generally greater than the response to 10^9 viral particles (data not shown).

Antigen-specific cytokine production induced by DC-based immunization

Sera from immunized huPBL-NSG mice were tested for the presence of human IFN-γ and TNF-α (Fig. 5). Compared to serum from control animals that were immunized with DC alone (no AdV exposure), serum from animals immunized with DC/AdV-GFP demonstrated consistently increased levels of both cytokines consistent with the responses measured in the ICCS assay.

Antigen-specific T-cells generated by immunization of huPBL-NSG mice respond to a secondary in vitro challenge

To further evaluate the function of antigen-specific T-cells that were activated in vivo, spleen cells were recovered from huPBL-NSG mice that were immunized with DC/AdV-GFP. The cells were labeled with CFSE and re-challenged in vitro for 5 days with either control DC (containing no antigens) or with DC/AdV-RR5 (Fig. 6). Although some background proliferation was observed in response to control DC, there was a clear antigen-specific increase in the percentage of proliferating CD4+ and CD4− T-cells in response to challenge with DC/AdV-RR5
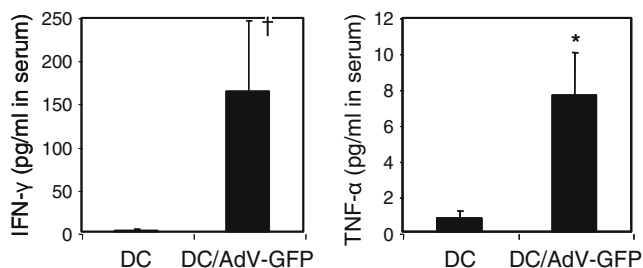
Fig. 5 In vivo cytokine response to DC/AdV-GFP. Sera from huPBL-NSG mice were collected 14 days after immunization with either control DC or DC/AdV-GFP and assayed for human IFN-γ and TNF-α by SearchLight multiplex assay. Representative experiment, $n=3$. †$p<0.10$; *$p\le0.05$
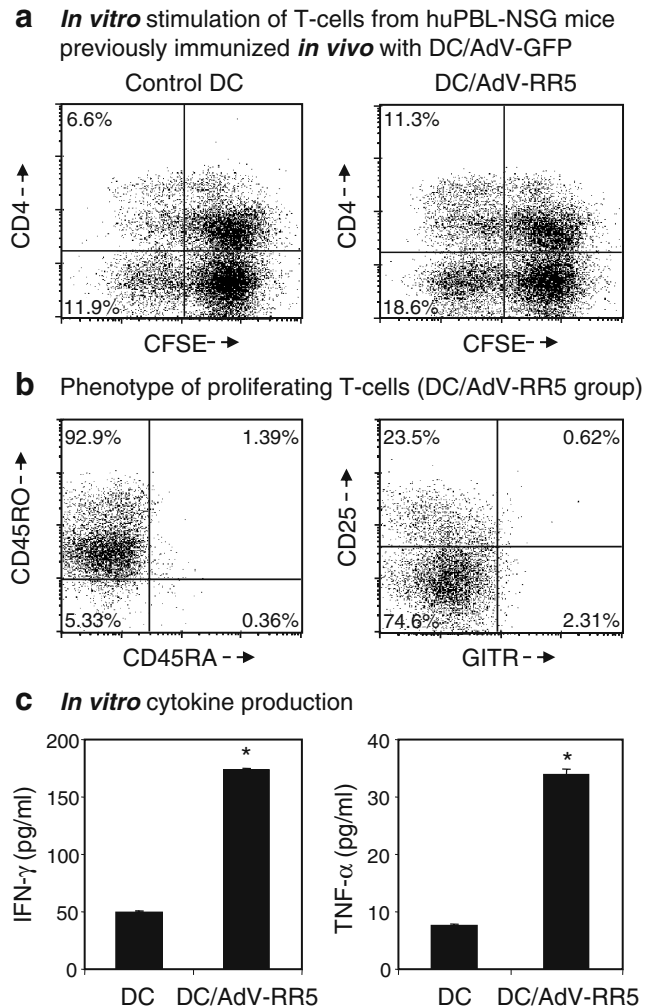
Fig. 6 T-cells recovered from immunized huPBL-NSG mice respond to antigen-specific stimulation in vitro. Spleen cells recovered from huPBL-NSG mice that were previously immunized with DC/AdV-GFP in vivo were labeled with CFSE and co-cultured with either control DC or DC/AdV-RR5 (DC/T ratio=1:20) for 5 days in vitro. **a** Cells were stained with anti-CD3 and anti-CD4 and the CD3+ T-cells analyzed for adenoviral-specific proliferative responses by flow cytometry. Representative experiment, $n=5$. **b** The sub-population of cells proliferating in response to stimulation with DC/AdV-RR5 (CFSE-dim) were further analyzed for their expression of effector/memory and regulatory T-cell markers. **c** Supernatants from the same wells were harvested and assessed for the presence of human IFN-γ and TNF-α by SearchLight multiplex assay. Representative experiment, $n=3$. *$p<0.01$

(Fig. 6a). Further phenotyping of the proliferating cells revealed that they expressed CD45RO, a memory cell marker, and that a significant percentage expressed CD25, an activation marker, in the absence of GITR, suggesting that they were not suppressor cells (Fig. 6b). Consistent with the expansion of effector/memory T-cells, supernatant collected from these co-cultures demonstrated antigen-specific induction of IFN-γ and TNF-α (Fig. 6c).

Discussion

Fundamental differences between the mouse and human immune systems, differences in genetic diversity, as well as inherent differences in the susceptibility and exposure to human disease, often limit the translational potential of mouse models for developing effective treatments for human disease. A variety of "humanized" animal models have been developed to address these issues by either expressing human genes/traits in animals (Pascolo et al. 1997; Mangalam et al. 2008; Conforti et al. 2009) or by engrafting human immune cells into immunodeficient mice (Mosier et al. 1988; Ito et al. 2002; Ishikawa et al. 2005). However, one significant limitation has been the study of adaptive immunity. The huPBL-NSG model provides a unique opportunity to study the interaction of various immune challenges with human immune cells (Kumar et al. 2008; Strowig et al. 2009; King et al. 2009). We herein demonstrate that the addition of donor-matched monocyte-derived DC to the reconstitution of huPBL-NSG animals enabled us to rapidly assess and stimulate the donor's antigen-specific memory in vivo, in the intact animal, as well as ex vivo, after recovery of spleen cells from immunized animals.

Our results confirm that rapid reconstitution of the spleen and peripheral blood occurs following a single i.p. injection of human PBL. Within a few weeks, 15–25 million human cells can be recovered from the spleen with the CD3+/CD4+, CD3+/CD8+, CD3+/CD56+, and CD20+ populations all well represented in appropriate proportions. Regardless of whether administered only once (at day 0 or 7) or twice (at both day 0 and 7), implanted DC were detected at low levels in the spleen and expressed the phenotypic markers of functional antigen-presenting cells. Importantly, reconstitution with DC did not lead to physical signs of GVHD or to changes in the level of acute activation markers (CD25 and CD69; data not shown). However, reconstitution with DC did enable the huPBL-NSG animals to respond to antigen challenge. Adenovirus was chosen as the target antigen for this work due to its well-characterized immunogenicity in humans and the presence of highly sensitive assays for assessing AdV-specific T-cell responses (Roth et al. 2002). Similar to the response pattern observed in vivo when humans are immunized with AdV (Gahéry-Ségard et al. 1997), fully reconstituted huPBL-NSG animals rapidly expanded the population of AdV-specific T-cells following exposure to AdV. In the absence of an antigen challenge, essentially no detectable AdV-specific T-cells were detected in freshly harvested splenocytes. However, following active immunization with DC/AdV-GFP, there was a dramatic upregulation of AdV-specific T-cells that far exceeded the level detected when the original donor PBL were stimulated in vitro for a week with IL-2, IL-7, and DC/AdV-GFP. As

might be expected when healthy patients are immunized with a vaccine, immunization of the intact animal was more effective than standard in vitro stimulation. While a cell-based vaccination (DC/AdV-GFP) elicited the greatest in vivo response, administration of free AdV-GFP vector also stimulated an AdV-specific T-cell response. In this case, the magnitude of the response appeared to increase with the quantity of antigen administered.

In addition to the in vivo expansion of antigen-specific T-cells, immunization of huPBL-NSG animals with DC/AdV-GFP induced the systemic release of IFN-γ and TNF-α into the serum. The detection of these circulating cytokines was a robust indicator of the AdV-specific T-cell response and consistent with reports that IFN-γ and TNF-α represent the predominant T-cell cytokines produced upon AdV challenge (Hutnick et al. 2010). It is noteworthy that resting cytokine levels for IFN-γ and TNF-α were low in unchallenged animals, arguing against the presence of spontaneous responses to endogenous mouse antigens.

One advantage of the huPBL-NSG mouse compared to its NOD/SCID precursors is the robust repopulation of the spleen with human cells. Harvesting spleens from these animals provides an abundant source of T-cells for in vitro analysis. As occurs when human T-cells are recovered from the blood of healthy subjects, the T-cells recovered from the spleens of immunized animals were able to undergo further expansion and testing when stimulated with AdV for another 5–7 days in vitro. Labeling recovered spleen cells with CFSE allowed us to track the proliferative response to a secondary in vitro challenge with DC/AdV-RR5 and to characterize the phenotype of responding cells. Interestingly, this was the one situation in which there was a significant background response when the cells were stimulated with control DC that had not been loaded with AdV. As the co-cultures contained up to 30% mouse cells recovered from the spleen, it seems likely that uptake and processing of these cells by the added DC lead to an in vitro anti-mouse response. However, despite this higher background proliferation, challenging in vitro with DC/AdV-RR5 still led to a significantly greater response than did challenging with control DC alone. T-cells proliferating in response to DC/AdV-RR5 were mostly CD45RO+ cells and approximately 25% of them expressed moderate levels of CD25 without expression of GITR, suggesting the presence of memory/effector T-cells, but not T-regulatory cells. This would be expected given our use of mature DC to immunize the animals in vivo (Roth et al. 2002; Harui et al. 2004). As detected in serum collected from immunized animals, IFN-γ and TNF-α were secreted into the culture supernatant during the in vitro challenge.

In summary, these findings support our hypothesis that reconstitution of NSG mice with a combination of human

PBL and mature donor-matched human DC results in a huPBL-NSG model in which adaptive T-cell responses can be stimulated and measured in the intact animal. There are several potential advantages of this model over others that have been reported in the literature. Gorantla et al. (2005) reported that NOD/SCID animals can be treated with asialo-GM1 and anti-CD122 antibodies to aid human engraftment and then vaccinated with antigen-loaded DC. While providing an important proof of concept, the use of NOD/SCID animals for this purpose has been replaced by newer animal strains due to the large number of cells required and the very limited engraftment that is obtained (Shultz et al. 2000; Shultz et al. 2003). Conventional engraftment of NSG mice with CD34+ stem cells is limited by the failure of normal T-cell development and the lack of pre-existing immune memory (Ishikawa et al. 2005). Yu et al. (2008) overcame these limitations by transplanting NOD/SCID β2 microglobulin$^{−/−}$ mice with peripheral blood CD34+ stem cells (3×10^6/animal) to promote DC and B-cell repopulation followed by supplementation 4–8 weeks later with donor-matched PBL as the source of T-cell responders. While requiring a leukopheresis to obtain sufficient CD34 donor cells, whole-body irradiation in combination with a 10-day course of human FLT3 ligand to promote engraftment, and then a protracted period for reconstitution, the resulting humanized animals responded well to both live influenza virus and inactivated influenza vaccine. As in our model, they observed a significant in vivo expansion of memory/effector T-cells with defined antigen specificity and cytokine production. Also similar to our work, they demonstrated that DC were crucial for the generation of an antigen-specific response. While based on similar principals, our model allows reconstitution from a single peripheral blood draw, and animals are ready for immunologic testing in as little as 2 weeks without further manipulation. In addition, these animals are stable for at least 6 weeks after implantation of human PBL. The large number of human cells that can be recovered from the spleen facilitates the extensive in vitro analysis that is often required when mapping out T-cell responses. Altogether, this model provides a highly adaptable platform to study the interaction of antigen presentation and antigen-specific T-cell responses in vivo. Immunologic responses to a variety of viruses can likely be studied using these approaches, new vaccines tested without the need to administer them to human subjects, and a variety of drugs and immune modulators evaluated for their impact on various stages of antigen presentation and T-cell function.

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