ELSEVIER

Experimental Hematology

Experimental Hematology 2008;36:1496–1506

Compartmentalization of allogeneic T-cell responses in the bone marrow and spleen of humanized NOD/SCID mice containing activated human resident myeloid dendritic cells

Slavica Vuckovic[a], Fadilah S. Abdul Wahid[b], Alison Rice[a], Masato Kato[a], Dalia Khalil[a], Robyn Rodwell[c], and Derek N.J. Hart[a]

[a]*Mater Medical Research Institute, Dendritic Cell Program, South Brisbane, Queensland, Australia;* [b]*Cell Therapy Center, Hospital University Kebangsaan Malaysia, Kuala Lumpur, Malaysia;* [c]*Queensland Cord Blood Bank, Mater Health Services, South Brisbane, Queensland, Australia*

(Received 14 January 2008; revised 27 May 2008; accepted 18 June 2008)

Objective. **Human allogeneic (allo)–T-cell responses within recipient lymphoid tissues and the degree to which they are altered in the presence of activated tissue-resident dendritic cells (DC) remain unknown. This study examined allo–T-cell recruitment and the early allo–T-cell responses that occur in the bone marrow (BM) and spleen (SP) of humanized (hu) nonobese diabetic (NOD)/severe combined immunodeficient (SCID) recipients containing activated human tissue-resident myeloid DC (MDC).**

Materials and Methods. **Human naïve allo–T cells were transferred into polyinosinic:polycytidylic acid [poly(I:C)]–treated or untreated huNOD/SCID recipients containing human tissue-resident DC derived from transplanted $CD34^+$ cells. Activation of human tissue-resident MDC mediated by poly(I:C) treatment, recruitment, proliferation, and effector differentiation of allo–T cells in the BM and SP of huNOD/SCID recipients were analyzed in vivo by flow cytometry.**

Results. **Poly(I:C) treatment induced transient activation of human MDC within a maximum of 8 hours, as evidenced in the BM by an increased proportion of MDC-expressing CD86 while in the SP by MDC expressing CD86 and producing interleukin-12. Poly(I:C)-pretreated huNOD/SCID recipients showed changes in the recruitment of allo–T cells in the BM and SP and developed different allo–T cell responses within the BM and SP compartments. In the BM, allo–T cells underwent multiple divisions and increased numbers of interferon-γ^+ and tumor necrosis factor–α^+ effector cells, while the majority of splenic allo–T cells underwent a single division and had fewer effector allo–T cells.**

Conclusions. **Our experimental transplantation model demonstrates that early allo–T-cell responses are regulated by compartmentalization in the BM and secondary lymphoid tissues; events potentially occurring after allotransplantation in human recipients.** © **2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

In vascularized lymphoid tissues like bone marrow (BM) and spleen (SP), tissue-resident dendritic cells (DC) recognize blood-borne pathogens or their products via toll-like receptors (TLR) and initiate activation programs involving phenotypic and functional changes followed by anatomical redistribution within lymphoid tissues [1–3]. Subsequent to these changes, activated tissue-resident DC induce activation, proliferation, and effector differentiation of local T cells [4–6].

In allotransplantation settings, tissue-resident DC presenting host peptides within the context of major histocompatibility complex molecules became activated by pretransplantation conditioning regimens and pathogen products and initiate allo–T-cell responses (direct allorecognition). Experimental murine transplantation models suggest that tissue-resident DC and macrophages are essential for triggering activation and proliferation of allo–T cells [7,8] but, that they also recruit allo–T cells into their target tissue during the effector phase of acute graft-vs-host disease [9].

Offprint requests to: Slavica Vuckovic, Ph.D., Mater Medical Research Institute, Dendritic Cell Program, Aubigny Place, Raymond Tce, South Brisbane, QLD 4101, Australia; E-mail: slavica.vuckovic@mmri.mater.org.au

0301-472X/08 $–see front matter.
doi: 10.1016/j.exphem.2008.06.011

Human allo–T-cell responses within lymphoid tissues and how they are altered by tissue-resident DC are unknown because of restricted access to tissues from allotransplant recipients and lack of alternative experimental models.

Human lymphoid tissue-resident DC are now amenable to experimental analysis in immunodeficient mice "humanized" by the engraftment of human hematopoietic stem cells (HSC). The two major subsets of human DC, myeloid DC (MDC) ($CD11c^+$ DC) and plasmacytoid DC (PDC) ($CD303^+$ DC), develop in lymphoid tissues of humanized (hu) nonobese diabetic (NOD) severe combined immunodeficienc (SCID) mice transplanted with HSC obtained from either cord blood, fetal liver, or mobilized peripheral blood [10–12]. Human MDC and PDC also develop in the lymphoid tissues of NOD/SCID mice with a complete null mutation of the interleukin-2 receptor (IL-2R) γ chain (NOD/SCID/IL-2Rγc^{null} mice) and in BALB/c mice with both recombination-activating gene 2 (RAG2) and truncated γc mutations (BALB/cRag2$^{-/-}$γc$^{-/-}$ mice) when engrafted as newborns with cord blood $CD34^+$ cells [13,14]. The humanized NOD/SCID/IL-2Rγc^{null} and BALB/cRag2$^{-/-}$γc$^{-/-}$ mice also permit human T-cell development within their lymphoid tissues and, as such, allow a more complete human immune system to be developed from transplanted $CD34^+$ cells [13,14].

Based on these precedents, we reasoned that the adoptive transfer of human naïve allo–T cells into humanized mice containing human tissue-resident DC, derived from transplanted $CD34^+$ cells, could be used as an experimental strategy to study in vivo interactions between human tissue-resident DC and allo–T cells. In this study, we transferred human naïve allo–T cells into polyinosinic:polycytidylic acid [poly(I:C)]-treated or untreated huNOD/SCID recipients containing human tissue-resident DC and analyzed recruitment of allo–T cells and allo–T cell responses within the BM and SP of recipient mice. Evidence presented indicates that pretreatment of huNOD/SCID recipients with poly(I:C) activates tissue-resident MDC leading to changes in recruitment, activation, proliferation, and effector differentiation of allo–T cells in the BM and SP of recipient mice. Significantly, allo–T cells in the BM underwent multiple divisions and increased the numbers of interferon (IFN)-γ^+ and tumor necrosis factor (TNF)-α^+ effector cells, while the majority of splenic allo–T cells underwent a single division and had few effector cells, suggesting that early allo–T-cell responses are regulated by compartmentalization in the BM and secondary lymphoid tissues after allotransplantation.

Materials and methods

Cord blood $CD34^+$ cells isolation and generation of huNOD/SCID mice

Cord blood and peripheral adult blood were obtained after appropriate informed consent in accordance with the Mater Adult Hospital Human Ethics Committee Guidelines. $CD34^+$ cells were isolated from cord blood using the $CD34^+$ isolation kit (Miltenyi Biotec, Sydney, NSW, Australia) according to manufacturer's instructions. NOD/SCID mice were housed in a pathogen-free facility at the Mater Medical Research Institute animal facility. All experimental work involving animals was approved by the University of Queensland Animal Ethics Committee. Six-week-old female NOD/SCID mice were sublethally irradiated with 325 cGy using a gamma beam ^{137}Cs source and 48 hours later were transplanted intravenously (IV; tail vein) with cord blood $CD34^+$ cells (2×10^5 $CD34^+$ cells per mouse) in 200 μL Hank's balanced salt solution (Invitrogen, Sydney, NSW, Australia); thereafter referred to as humanized (hu)NOD/SCID mice.

Detection of human engraftment and human lineages in huNOD/SCID mice

To define human cell engraftment in the blood, huNOD/SCID mice were bled between weeks 4 and 10 post-transplantation. HuNOD/SCID mice with evidence of $huCD45^+$ cells in their blood were sacrificed 8 to 10 weeks post-transplantation and blood, BM, and SP collected for analysis by flow cytometry [15]. Lymph nodes are rudimentary in NOD/SCID and could not be analyzed [16]. The lack of cross-reactivity of anti–human monoclonal antibodies (mAb) with mouse cells was confirmed by staining cells from BM, blood, and SP of NOD/SCID mice. All mAb were purchased from BD Biosciences (Sydney, NSW) unless otherwise indicated. For detection of human engraftment, cells were labeled with anti–huCD45- peridinin-chlorophyll-protein (PerCP)-conjugated mAb combined with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)–conjugated mAb specific for huCD34, CD19, CD14, CD15, CD3, CD56 antigens. For detection of human MDC and PDC in the blood, BM and SP cells were labeled with FITC-conjugated mAb specific for lineage (Lin) human markers, huCD3, CD14, CD15, CD19, CD20, CD34, CD56, anti–mouse (m)CD45-FITC, anti–huCD45-PerCP, anti–huHLA-DR-PE combined with either anti–huCD11c-allophycocyanin (APC), for MDC detection, or anti–huCD303-APC (Miltenyi Biotech) for PDC detection. To further characterize the phenotype of human MDC in the BM and SP, cells from the BM and SP of huNOD/SCID mice were stained with anti–huLin-FITC, anti–mCD45-FITC, anti–huCD45-PerCP, anti–huCD11c-APC combined with either mIgG-PE, anti–huCD33-PE, anti–huCD1c-PE, anti–huCD141-PE, anti–huCD1a-PE, anti–huCD205-PE (anti–huCD205-PE mAb produced in house [17]), anti–huCD16-PE, anti–huCD4-PE, anti–huCD123-PE, anti–huHLA-DR-PE, anti–huCD86-PE, anti–huCD40-PE, anti–huCD83-PE or anti–huTLR3-PE.

Activation of human MDC in BM and SP of huNOD/SCID mice

HuNOD/SCID mice (at 8–10 weeks post-transplantation) were treated with poly(I:C) (Sigma, St Louis, MO, USA;100 μg poly(I:C) in 200 μL phosphate-buffered saline (PBS) per mouse, intraperitoneal injection) or with PBS (Invitrogen, Sydney, NSW, 200 μL per mouse, intraperitoneal injection). At the indicated time after poly(I:C) or PBS injection, mice were sacrificed and the human MDC in the BM and SP were analyzed for surface phenotype and cytokine production. To define activated human MDC, cells were prepared from the BM and SP of huNOD/SCID mice, stained with anti–huLin-FITC, anti–mCD45-FITC,

anti–huCD45-PerCP, anti–huCD11c-APC combined with either anti–huCD86-PE, anti–huCD40-PE, anti–huCMRF44-PE or anti–huCMRF56-PE (anti–huCMRF44-PE and anti–huCMRF56-PE mAb produced in house [18,19]). For cytokine production by activated human MDC, cells were prepared from the BM and SP of huNOD/SCID mice, stained with anti–huLin-FITC, anti–mCD45-FITC, anti–huCD45-PerCP, anti–huCD11c-APC, fixed/permeabilized (FIX and PERM kit; Caltag Laboratories, Sydney, NSW), labeled with either anti–huIL-6-PE, anti–huIL-10-PE, or anti–huIL-12p40/p70-PE (BD Bioscience, Sydney, NSW, Australia) and analyzed by flow cytometry.

Adoptive transfer of human naïve allo–T cells

Naïve $CD3^+$ T cells (hereafter referred to as allo–T cells), for adoptive transfer into NOD/SCID or huNOD/SCID recipients, were prepared by labeling peripheral blood mononuclear cells with unconjugated mAb-specific for huCD19, CD20, CD11c, CD14, CD34, CD56, HLA-DR, glycophorin-A, and CD45RO (Coulter Immunotech, Gladesville, NSW), followed by incubation with goat–anti-mouse IgG beads (Miltenyi Biotech) and magnetic depletion of the positive cell fraction using the AutoMACS DEPL025 protocol ($CD3^+CD45RA^+$ cells, >90% purity). Allo–T cells, unlabeled or labeled with 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) were adoptively transferred into NOD/SCID or huNOD/SCID recipients (20×10^6 allo–T cells in 200 μL PBS per mouse, IV injection).

Detection of allo–T-cell responses in the BM and SP of huNOD/SCID recipient mice

Allo–T-cell responses were analyzed in the BM and SP of recipient mice, at day 3 to 5 after adoptive allo–T-cell transfer. To detect total allo–$CD3^+$ T cells or allo–$CD4^+$ T and allo–$CD8^+$ T-cell subsets, cells were stained with anti–huCD45-PerCP, anti–huCD3-APC combined with either anti–huCD4-PE anti–huCD8-PE. To analyze the activation status of allo–T cells, cells were stained with anti–huCD45-PerCP, anti–huCD3-APC combined with either anti–huCD25-PE or anti–huCD69-PE. Proliferation kinetics of CFSE-labeled allo–T cells were assessed by CFSE dilution analysis [20]. To detect allo–T-effector cells, cells were stained with anti–huCD45-PerCP, anti–huCD3-APC, fixed/permeabilized, labeled with either anti–huIFN-γ, anti–huTNF-α, anti–huIL-2-PE, anti–huIL-4-PE, or anti–huIL-10-PE mAb (BD Bioscience, Sydney, NSW) and analyzed for cytokine production by flow cytometry [21].

Statistical analysis

Significant differences in human cell engraftment were calculated using an unpaired two-tailed *t*-test. *p* values for MDC activation and allo–T-cell response comparisons were calculated using unpaired or paired two-tailed *t*-test. Significance was defined as $p < 0.05$.

Results

Analysis of engrafted huCD45$^+$ cells in huNOD/SCID mice

$HuCD45^+$ cells were detectable in the blood of huNOD/SCID mice 6 weeks post-transplantation of cord blood $CD34^+$ cells ($CD45^+$ cells ≥1%; 65% mice; n = 10). However, huNOD/SCID mice exhibited higher engraftment frequency in the blood (>90% mice) 8 to 10 weeks post-transplantation. Analysis of engrafted $huCD45^+$ cells in the BM and SP of huNOD/SCID mice 8 to 10 weeks post-transplantation showed presence of $CD34^+$ precursors, $CD19^+$ B cells, $CD14^+$ monocytes, and $CD15^+$ granulocytes, but no evidence of $CD3^+$ T or $CD56^+$ natural Killer (NK) cells (Table 1). HuNOD/SCID mice had higher levels of human $CD45^+$, $CD34^+$, $CD14^+$, and $CD15^+$ cells in the BM as compared with the SP (Table 1).

In the BM and SP of huNOD/SCID mice, human MDC and PDC were defined by SSC and huCD45 gating (Fig. 1A, BM; upper dot plot, SP; lower dot plot), followed by gated acquisition of $huCD45^+CD11c^+$ and $huCD45^+CD303^+$ events (Fig. 1A, B, and Fig. 1A, C, respectively) and then MDC and PDC, within the gated population, were detected by $huHLA\text{-}DR^+Lin^{-/dim}$ $mCD45^-$ expression (Fig. 1B, C, respectively). Blood MDC and PDC were defined using the same gating strategy (not shown). As expected, MDC and PDC represented rare cells within $huCD45^+$ cells in the BM, blood, and SP of engrafted mice (Table 1). The level of human MDC in the BM was lower than that observed in the blood and SP. MDC were detected at lower levels in the BM and blood of huNOD/SCID mice than PDC ($p < 0.05$; Table 1).

All human MDC in the BM and SP strongly expressed CD33, CD1c, CD4, CD123, and CD205, whereas CD1a was weakly positive on most MDC (Fig. 1D). Most BM and splenic MDC (>70%; n = 3) were negative for CD141, whereas all BM and spleen MDC were negative for CD16. CD86 and CD40 molecules were expressed by MDC in the BM and SP, whereas CD83 was absent. In addition, human MDC in the BM and SP uniformly expressed TLR3, predicting their responsiveness to the TLR3 ligand, double-stranded RNA, or synthetic product poly(I:C). Human MDC in the BM were distinct from splenic MDC in that they expressed a higher level of CD11c and a lower level of HLA-DR (relative fluorescence intensity was, on average, 2.7 times higher for CD11c and 1.8 times lower for HLA-DR, Fig. 1B, D). In brief, human MDC engrafted in the BM and SP of huNOD/SCID mice had a phenotype similar to that of the $CD11c^+CD16^-$ MDC subset found in human blood and spleen [22,23].

Poly(I:C) treatment induces transient activation of human MDC in the BM and SP of huNOD/SCID mice

Given that BM and SP are vascularized, we predicted that resident MDC within these tissues recognize pathogen product spread by the local bloodstream via their TLR and ultimately became fully activated. To test this hypothesis, we analyzed activation of human MDC in the BM and SP of huNOD/SCID mice from 2 to 12 hours after poly(I:C) treatment. Two hours after poly(I:C) treatment, the proportion of human MDC expressing CD86 increased in the BM and SP of treated mice compared with PBS-treated mice

Table 1. Engrafted human cells in the BM, blood, and SP of huNOD/SCID mice

Human cells	BM	Blood	SP
CD45			
Mean ± SD	89.1 ± 2.7[a]	2.8 ± 0.6[a]	69.4 ± 5.7
Range	44.6–98.9	0.6–7.1	7.7–96.0
n	30	10	17
MDC			
Mean ± SD	1.4 ± 0.3[a,b,c]	2.9 ± 0.5[d]	2.3 ± 0.3
Range	0.3–5.0	0.9–7.7	0.4–4.4
n	15	15	16
PDC			
Mean ± SD	4.4 ± 0.5	4.7 ± 0.6	2.9 ± 0.3
Range	2.3–7.5	1.3–7.5	1.1–5.4
n	15	14	16
CD34		NA	
Mean ± SD	22.3 ± 2.6[a]		7.6 ± 1.1
Range	7.8–33.8		2.0–13.0
n	10		10
CD19		NA	
Mean ± SD	73.4 ± 3.7		85.3 ± 1.7
Range	57.8–90.0		78.0–93.0
n	10		10
CD14		NA	
Mean ± SD	4.6 ± 0.4[a]		1.2 ± 0.3
Range	2.0–6.0		0.2–3.2
n	10		10
CD15		NA	
Mean ± SD	2.7 ± 0.6[a]		0.3 ± 0.1
Range	0.9–7.3		0.0–1.0
n	10		10
CD3	ND	NA	ND
n	10		10
CD56	ND	NA	ND
n	10		10

Engrafted human cells in the bone marrow (BM), blood, and spleen (SP) of huNOD/SCID mice were examined at 8 to 10 weeks post-transplantation with cord blood $CD34^+$ cells. Human $CD45^+$ cells are represented as percentage of total live cells; human MDC, PDC, $CD34^+$ precursors, B cells, monocytes, granulocytes are represented as percentage of $huCD45^+$ cells. BM = bone marrow; MDC = myeloid dendritic cells; NA = not analyzed; ND = not detected; PDC = plasmacytoid dendritic cells; SP = spleen.
[a,b]Denote significant differences when compared with these cells in the spleen and blood.
[c,d]Denote significant differences when compared MDC with PDC in the BM and blood ($p < 0.05$).

(Fig. 2A). The proportion of human MDC expressing CD86 was maximal in the BM and SP after 2 hours of poly(I:C) treatment and no further increases occurred after 8 hours of poly(I:C) treatment (Fig. 2A). This increase in the proportion of human MDC expressing CD86 was transient and by 12 hours after poly(I:C) treatment, levels of $CD86^+$ MDC in the BM and SP of poly(I:C)-treated mice returned to levels similar to that in control mice (Fig. 2A). On the other hand, the proportion of human MDC expressing CD40, CD83, CMRF44, and CMRF56 was comparable in the BM and SP of poly(I:C)-treated and PBS-treated huNOD/SCID mice (not shown).

We further analyzed whether poly(I:C) treatment changed inflammatory cytokine production by MDC in the BM and SP of poly(I:C)-treated huNOD/SCID mice. Eight hours after poly(I:C) treatment, the proportion of human MDC producing IL-12 was increased in the SP of poly(I:C)-treated mice compared with PBS-treated mice (Fig. 2B). Further, at this same time point, a higher proportion of MDC producing IL-12 was detected in the SP as compared to the BM of poly(I:C)-treated mice (Fig. 2B). No changes in the proportion of MDC producing IL-6 and IL-10 were observed in the BM and SP of poly(I:C)-treated mice compared with control mice (not shown). Thus, poly(I:C) treatment induced rapid but transient activation of human tissue-resident MDC, similar to the transient activation of tissue-resident myeloid DC seen in mice [2]. In addition, these data suggest inherent differences between BM and splenic-activated MDC, whereby activated MDC in the BM provide a CD86 costimulatory signal, while splenic-activated MDC provide both CD86 and IL-12 costimulatory signals to induce T-cell responses.

Changes in recruitment of allo–T cells in the BM and SP of poly(I:C)-pretreated huNOD/SCID recipients

We investigated the possibility that poly(I:C) treatment and subsequent activation of resident MDC may have consequences for recruitment of adoptively transferred allo–T cells in the BM and SP of poly(I:C)-pretreated huNOD/SCID recipients. Human naïve allo–T cells were transferred into poly(I:C)-pretreated and PBS-pretreated huNOD/SCID recipients (2, 8, and 12 hours after poly(I:C) injection or 2 hours after PBS injection). To control for the contribution of human cells in huNOD/SCID recipients, allo–T cells were also transferred into poly(I:C)-pretreated or PBS-pretreated nonhumanized NOD/SCID recipients (8 hours after poly(I:C) injection; 2 hours after PBS injection). Cells were recovered from the BM and SP of recipient mice 3 days after allo–T cell transfer (average 20–30 $\times$ 10^6 cells from two femurs or 10–30 $\times$ 10^6 cells from SP) and analyzed for presence of allo–T cells (Fig. 3A). Few allo–T cells were detected in the BM and SP of either PBS- or poly(I:C)-pretreated nonhumanized NOD/SCID recipients (Fig. 3B, left panel). In contrast, allo–T cells were readily detected in huNOD/SCID recipients, but were, on average, 35- to 37-fold fewer in the BM than in the SP of PBS-pretreated huNOD/SCID recipients (Fig. 3B, right panel). These data suggest that in the absence of overt activation of human resident MDC, allo–T cells are recruited, albeit at different levels, to both the BM and SP of huNOD/SCID recipients.

Pretreatment of huNOD/SCID recipients with poly(I:C) 2 and 8 hours prior to allo–T-cell transfer, increased the number of allo–T cells three to fivefold in the BM and twofold in the SP of recipient mice, as compared with PBS-pretreated recipients (Fig. 3B, right panel). In contrast, there was a trend for lower numbers of allo–T cells in the

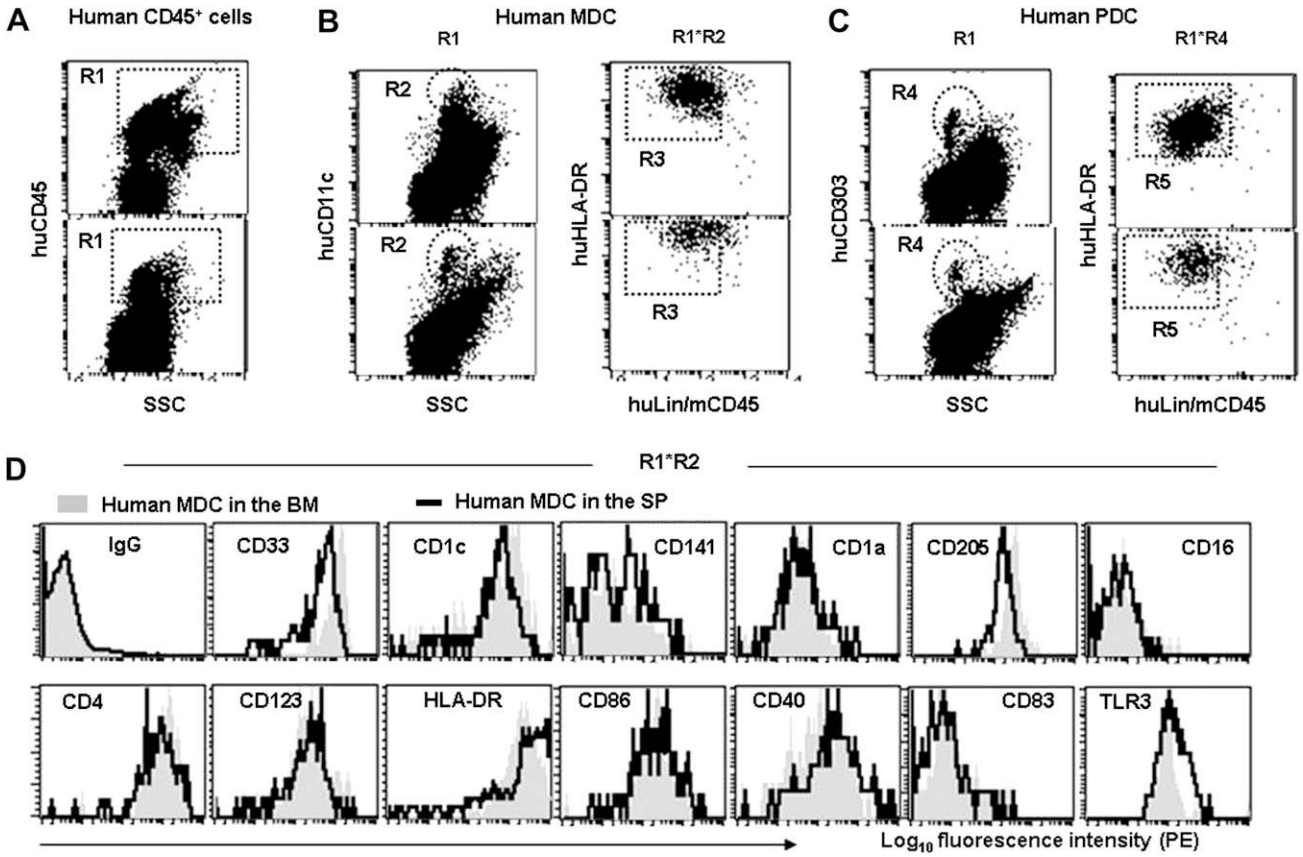

Figure 1. Human MDC and PDC engrafted in the BM and SP of huNOD/SCID mice. (**A**, **B**, **C**) Human MDC and PDC were defined in the BM and SP of huNOD/SCID mice by flow cytometry (BM, upper dot plots, SP lower dot plots). (**A, B**) To define human MDC, gates were defined as follows: R1, huCD45$^+$ events; R2, huCD11c$^+$ events; R3, huHLA-DR$^+$huLin$^-$mCD45$^-$ events. (**A**, **C**) To define human PDC, gates were defined as follows: R1, huCD45$^+$ events; R4, huCD303$^+$ events; R5, huHLA-DR$^+$huLin$^-$mCD45$^-$ events. (**D**) Expression of surface molecules on gated human MDC in the BM (gray fill) and SP (black line) of huNOD/SCID mice.

BM and SP of poly(I:C)-pretreated huNOD/SCID recipients that received allo–T cells 12 hours after poly(I:C) treatment, compared to other huNOD/SCID recipients (Fig. 3B, right panel). The changes we observed in the number of allo–T cells are suggestive of inherent differences within the BM and SP of poly(I:C)-pretreated huNOD/SCID recipients, which have direct consequences for allo–T-cell recruitment.

Activation and proliferation of allo–T cells occurs in the BM and SP of poly(I:C)-pretreated huNOD/SCID recipients

Next, we investigated the possibility that poly(I:C)-pretreated huNOD/SCID recipients are capable of mounting early allo–T-cell responses within BM and SP compartment. Activation and proliferation of allo–T cells were analyzed in the BM and SP of huNOD/SCID recipients 3 days after transfer of CFSE-labeled allo–T cells. This experimental approach necessitated different donor–recipient pairing and, consequently, considerable variation was observed between individual huNOD/SCID recipients, making it difficult to obtain sufficient evidence for significant differences between experimental groups. There was a trend for an increased proportion of activated allo–T cells expressing CD25 and CD69 in the BM and SP of huNOD/SCID recipients pretreated with poly(I:C) 2 to 8 hours prior to allo–T-cell transfer, as compared with PBS-pretreated recipients (Fig. 4A). However, a significantly higher proportion of activated allo–CD3$^+$CD25$^+$ T cells was only seen in the BM of huNOD/SCID recipients pretreated with poly(I:C) 2 hours prior to allo–T-cell transfer, compared with PBS-pretreated recipients (Fig. 4A).

In addition, there was a trend for a greater proportion of proliferating allo–T cells in the BM and SP of poly(I:C)-pretreated recipients, as compared with PBS-pretreated recipients (Fig. 4B). Significantly higher proportions of proliferating allo–CD3$^+$T and allo–CD4$^+$T cells were found in the BM and SP of allo–T-cell recipients pretreated with poly(I:C) 8 hours prior to allo–T-cell transfer compared to PBS-pretreated recipients (Fig. 4B, top and middle panel).

Next, we compared the division kinetics of allo–CD3$^+$T cells in the BM and SP of huNOD/SCID recipients pretreated with poly(I:C) 2 or 8 hours prior to allo–T-cell

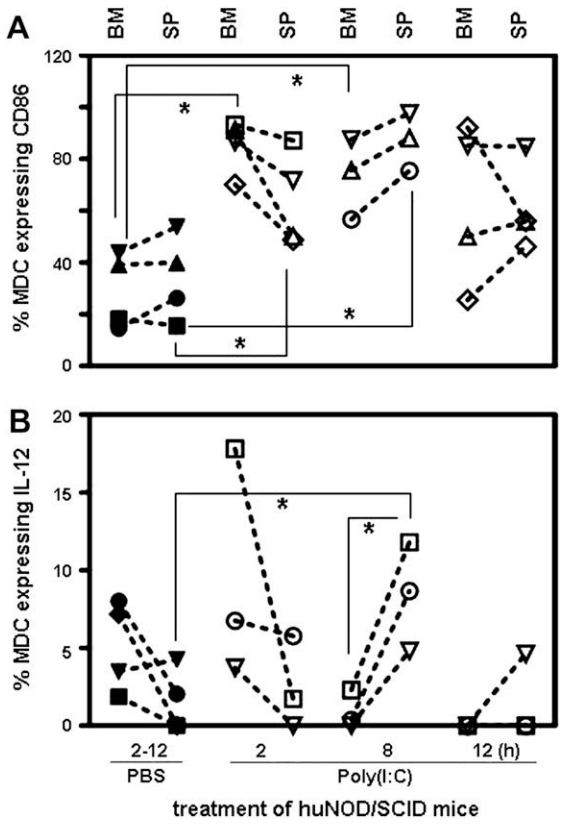

Figure 2. Activated human MDC in the BM and SP of poly(I:C)–treated huNOD/SCID mice. Proportion of human MDC expressing CD86 (**A**) or producing IL-12 (**B**) in the BM and SP of polyinosinic: polycytidylic acid [poly(I:C)] treated or control (PBS)–treated huNOD/SCID mice. Each symbol identifies individual mice; paired BM and SP from individual mice are connected by dotted lines; three to four mice per group; $*p < 0.05$.

transfer. It was impossible to acquire sufficient events to clearly define division kinetics of allo–T cells in huNOD/SCID recipients pretreated with poly(I:C) 12 hours prior to allo–T cell transfer or PBS-pretreated recipients. In the BM of huNOD/SCID recipients pretreated with poly(I:C) 8 hours prior to allo–T-cell transfer, we observed that allo–$CD3^+$ T cells underwent as many as four to eight divisions, whereas in the SP of these same recipients allo–T cells primarily accumulated in the first division, implying faster allo–T-cell proliferation kinetics in the BM compared to SP (Fig. 4C, D). Similarly, huNOD/SCID recipients pretreated with poly(I:C) 2 hours prior to allo–T-cell transfer also showed a trend for faster allo–T-cell proliferation kinetics in the BM as compared to the SP (not shown). This kinetic analysis was restricted to allo–$CD3^+$ T cells, as it was impossible to track the smaller number of division events within the $CD4^+$T- and $CD8^+$T-cell subsets. These results suggest that pretreatment with poly(I:C) of huNOD/SCID recipients is critical for activation and proliferation of allo–T cells, and that activated allo–T cells undergo more rapid division in the BM as compared to the SP of the same recipient mice.

Effector allo–T cells emerge in the BM of poly(I:C)-pretreated huNOD/SCID recipients

We next investigated whether the activation and proliferation of allo–T cells observed in the BM and SP of poly(I:C)-pretreated huNOD/SCID recipients was coupled with effector allo–T-cell generation. Three days after transfer of allo–T-cell transfer, effector allo–T-cells producing IFN-γ, TNF-α, IL-2, or IL-4, but not IL-10 were observed in the BM and SP of huNOD/SCID recipients that were pretreated with poly(I:C) 8 hours prior to allo–T-cell transfer (Fig. 5A, B). Unexpectedly, there were a significantly higher proportion of effector allo–T cells producing IFN-γ and TNF-α and a trend toward a greater proportion of effector allo–T cells producing IL-2 and IL-4 in the BM as compared to SP of these animals. Additionally, 5 days after allo–T cell transfer, these animals still had a greater proportion of effector allo–T cells in the BM compared to SP (not shown). There were few effector allo–T cells in the BM and SP of huNOD/SCID recipients pretreated with poly(I:C) 2 or 12 hours prior to allo–T-cell transfer. Effector allo–T cells were undetectable in control PBS-pretreated mice (not shown). These findings demonstrate that poly(I:C) treatment of huNOD/SCID recipients is required for effector allo–T-cell generation and the BM rather than the SP is the privileged site for effector allo–T-cell generation.

Discussion

In humans, in situ donor allo–T-cell responses induced by host-activated tissue-resident DC after allotransplantation are impossible to analyze. In the present work, adoptive transfer of human allo–T cells into huNOD/SCID recipients, containing human tissue-resident DC derived from transplanted $CD34^+$ cells was used as an experimental strategy to define donor allo–T-cell responses within the BM and SP of recipient mice. We showed that allo–T cells underwent activation, proliferation, and effector differentiation in the BM and SP of huNOD/SCID recipients in which activated tissue-resident human MDC were induced by poly(I:C) treatment. Faster allo–T-cell proliferation kinetics and increased numbers of effector allo–T cells were observed in the BM as compared to the SP of poly(I:C)-pretreated huNOD/SCID recipients, suggesting that early allo–T-cell responses are regulated by compartmentalization in the BM and secondary lymphoid tissues after allotransplantation.

Adoptive transfer of human allo–T-cells into PBS-pretreated huNOD/SCID recipients suggested that human allo–T cells were recruited to both the BM and SP of huNOD/SCID recipients and that this occurred regardless of the activation status of the resident MDC. However, the number of allo–T cells detected in the SP was 35- to 37-fold higher compared to the BM. There are several nonexclusive explanations for this finding. It is theoretically possible that splenic resident MDC or (other cells) attract

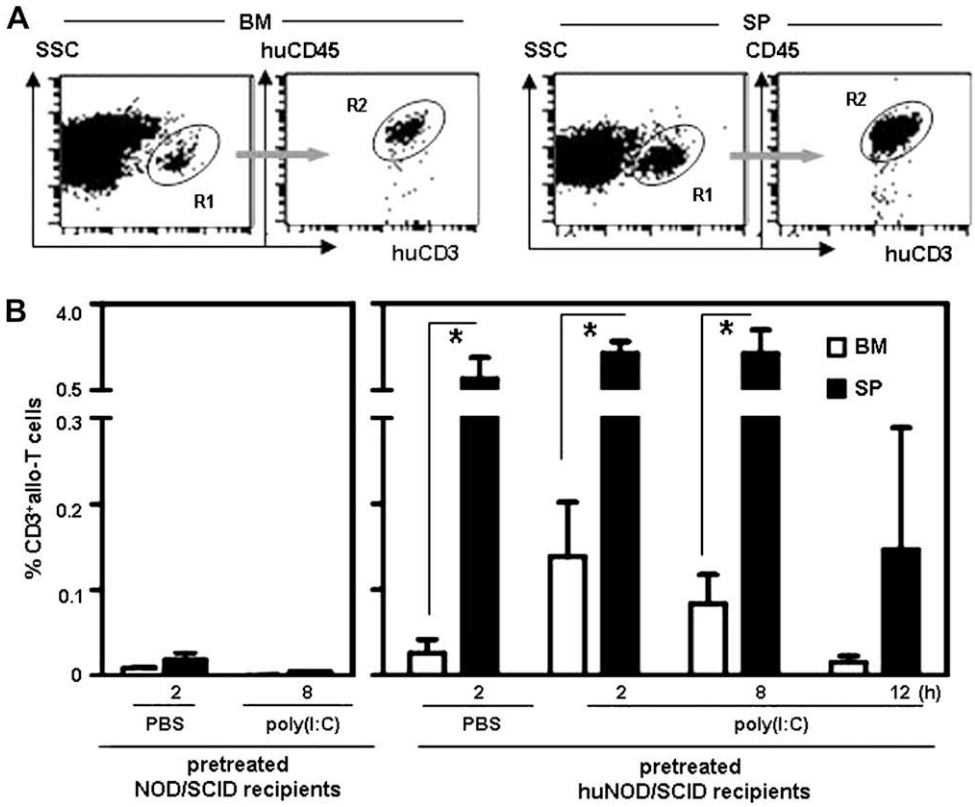

Figure 3. Human allo–T cells in the BM and SP of allo–T cell recipients. (**A**) Human allo–T cells in the BM and SP of recipient mice at day 3 after allo–T cell transfer were defined as follows: R1, huCD3$^+$ events; R2, huCD3$^+$CD45$^+$ events. (**B**) Proportions of allo–CD3$^+$ T cells within total live cells in the BM and SP of polyinosinic:polycytidylic acid [poly(I:C)]–pretreated and PBS–pretreated NOD/SCID or huNOD/SCID recipients (mean ± SEM, three to four mice/group). *$p < 0.05$.

allo–T cells via a chemokine gradient or provide an alternative survival signal to facilitate allo–T cells persistence. We found that human splenic MDC differed from their BM counterparts in that they displayed higher levels of surface HLA-DR and lower levels of CD11c molecules. Although these differences are likely to represent maturation changes that CD11c$^+$MDC must make during their journey from the BM to the SP, these distinct phenotypic properties of splenic-resident MDC may also dictate their capacity to attract and maintain allo–T cells. In human spleen, CD11c$^+$MDC are mostly found in the T-cell area and are postulated to be good candidates for interaction with allo–T cells [23]. Another possibility is that naïve allo–T cells with distinct homing and chemokine receptors preferentially traffic to the SP, unlike T memory cells, which preferentially home to the BM [4,24,25].

Compared to PBS-pretreated recipients, there was trend for increased numbers of allo–T cells in the BM and SP of poly(I:C)-pretreated recipients. These changes in allo–T-cell numbers in the BM and SP of poly(I:C)-pretreated recipients occurred in coordinated fashion with activation of resident MDC, implying that activated resident MDC may be involved in allo–T cell recruitment into the BM and SP of recipient mice, but further studies are required to resolve this issue.

We demonstrated that poly(I:C) pretreatment of allo–T-cell recipients was required to initiate allo–T-cell responses. The initial phase of the allo–T-cell response, which includes activation, proliferation, and effector differentiation, occurs within 3 days of allo–T-cell transfer. These data are compatible with allo–T-cell responses seen in murine transplantation models, where donor allo–T-cell activation and proliferation, induced by host-activated DC and macrophages, occurred within 24 hours of donor T-cell transplantation [7,26]. This also confirms that our experimental model, based on the adoptive transfer of allo–T-cells into huNOD/SCID recipients, has the potential to provide significant new insights into events associated with human allo–T-cell responses after allotransplantation that otherwise would not be possible to obtain.

Secondary lymphoid tissues are considered to be the major sites of allo–T-cell responses in allotransplant recipients [7,9]. We suggested some time ago that human BM cells can prime allo–T-cell responses in vitro [27], however, since then, the possibility that hematopoietic organs such as BM could also be a site for allo–T-cell responses has remained unexplored. In the present study, we detected allo–T-cell responses in the SP but also in the BM of poly(I:C)-pretreated huNOD/SCID recipients. We only monitored allo–T-cell responses in the BM and SP 3 days

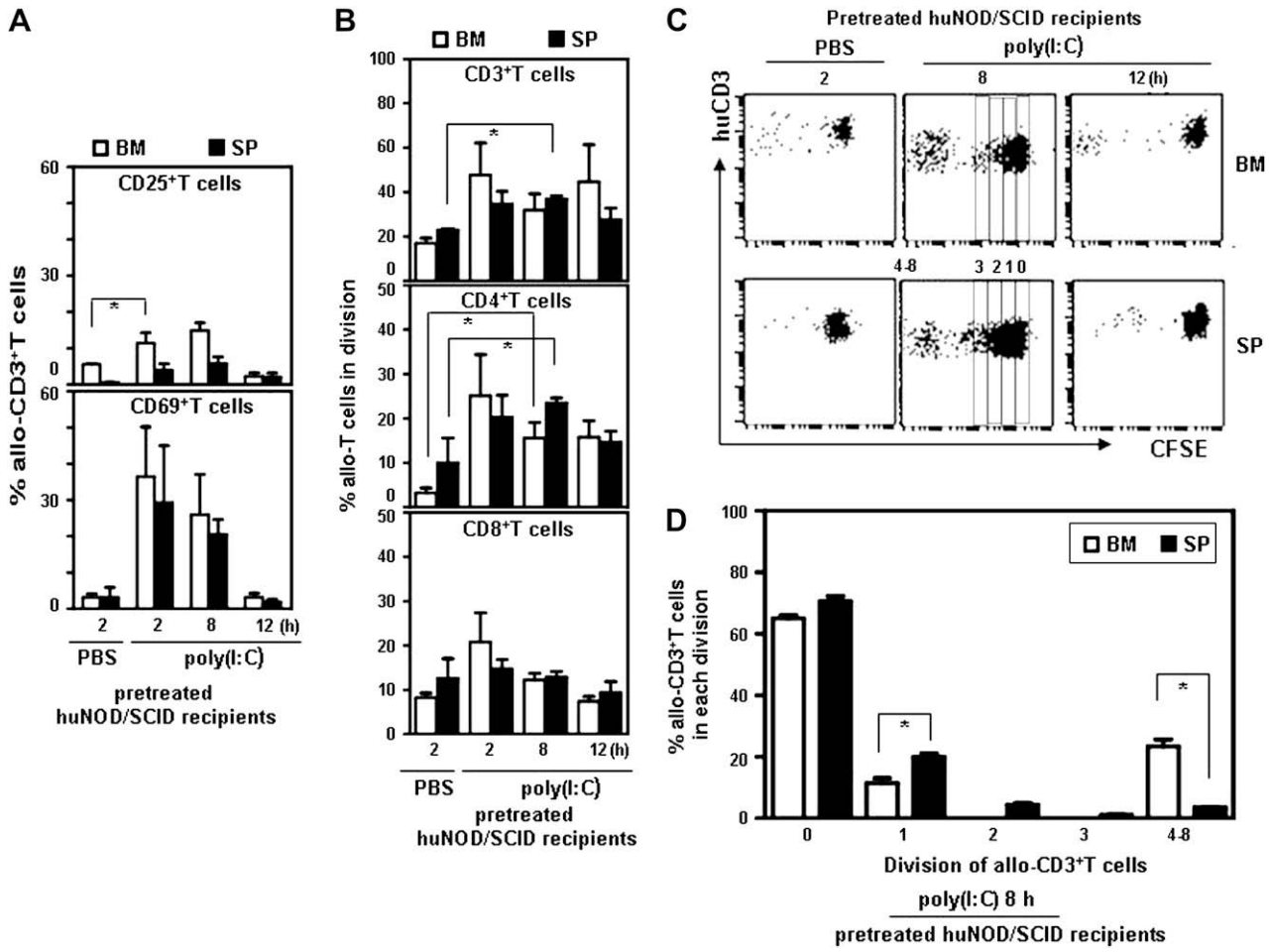

Figure 4. Activation and proliferation of allo–T cells in the BM and SP of huNOD/SCID recipients. (**A**) Proportions of activated allo–$CD3^+$ T cells expressing CD25 (upper panel) or CD69 (lower panel) in the BM and SP of polyinosinic: poly(I:C)–pretreated and control PBS-pretreated huNOD/SCID recipients at day 3 after allo–T cell transfer (mean ± SEM, three to four mice/group). (**B**) Proportions of dividing CFSE–labeled allo–$CD3^+$ T cells (upper panel), allo–$CD4^+$ T cells (middle panel) and allo–$CD8^+$ T cells (lower panel) in the BM and SP of poly(I:C)-pretreated and control PBS-pretreated huNOD/SCID recipients at day 3 after allo–T-cell transfer (mean ± SEM, three to four mice/group). (**C**) Representative dot plots displaying division kinetics of CFSE-labeled allo–$CD3^+$ T cells in the BM and SP of PBS-pretreated huNOD/SCID recipients and huNOD/SCID recipients pretreated with poly(I:C) 8 or 12 hours prior to allo–T cell transfer. Boxes (dotted lines) were created to calculate the proportions of CFSE-labeled allo–$CD3^+$ T cells in division number 0, 1, 2, 3, 4–8. (**D**) Proportions of CFSE-labeled allo–$CD3^+$ T cells in division number 0, 1, 2, 3, 4–8 in the BM and SP of huNOD/SCID recipients pretreated with poly(I:C) 8 hours prior to allo–T cell transfer (mean ± SEM, data collected from four poly(I:C)-pretreated huNOD/SCID recipients). *$p < 0.05$.

after transfer, because it was technically difficult to track transferred allo–T cells at earlier time points, thus our experiments cannot exclude the possibility that the allo–T cell could have been primed in other tissues prior to migration to the BM. Our data rather, argue that after allotransplantation allo–T-cell responses persist in the BM, as has been proposed for secondary lymphoid organs. In support of this proposed new role for BM as a site for allo–T-cell responses in allotransplant recipients, recent studies have shown that BM host memory cells specific for tumor antigen [4], autoreactive T cells specific for β-cell antigen [25] and also represents a primary site for immune responses to foreign antigen [5].

Furthermore, in our present study, we established that early human allo–T-cell responses after allotransplantation were regulated by their compartmentalization in the BM and secondary lymphoid tissue (e.g., SP). In the BM, allo–T cells underwent multiple divisions, including allo–T effector cell generation, while in the SP, allo–T cells were restricted to a single division, which resulted in fewer or no allo–T effector cells. Allo–T cell responses have been extensively studied using experimental murine transplantation models, however, no such compartmentalization has yet been proposed. Because human allo–T-cell responses are induced in vitro more effectively by $CD11c^+$MDC than by any other antigen-presenting cells [28], this implies that activated resident $CD11c^+$MDC are likely to be principal mediators of induction and compartmentalization of allo–T-cell responses within BM and SP of allotransplant recipients. Also, our finding that

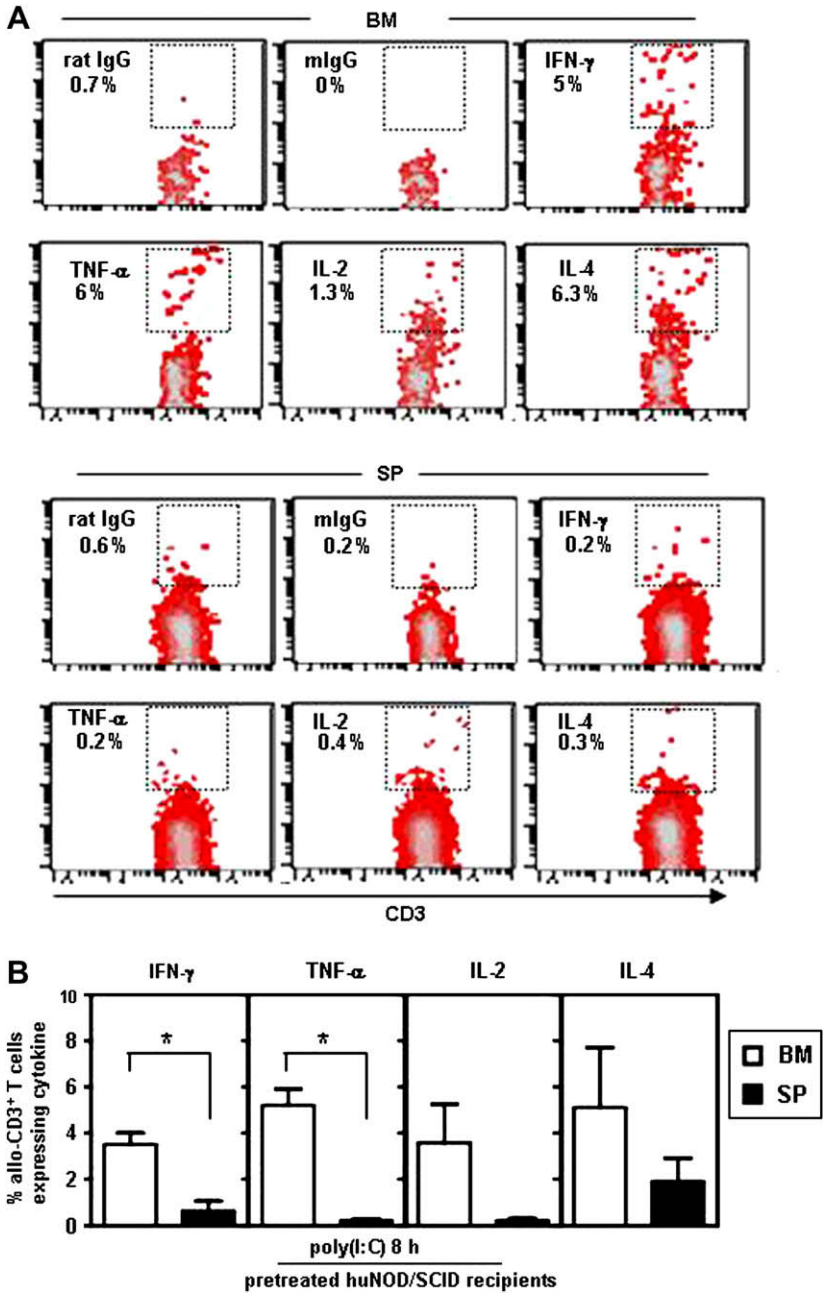

Figure 5. Effector allo–CD3$^+$T cells in the BM and SP of huNOD/SCID recipients. (**A**) Representative dot plots showing the proportions of activated allo–CD3$^+$ T cells producing huIFN-γ, hu TNF-α, huIL-2 or huIL-4 in the BM and SP of huNOD/SCID recipients pretreated with poly(I:C) 8 hours prior to allo–T cell transfer. Events that correspond to allo–T cells producing cytokines are indicated by dotted boxes and numbers. (**B**) The proportions of allo–T cell producing huIFN-γ, huTNF-α, huIL-2, or huIL-4 in the BM and SP of huNOD/SCID recipients pretreated with poly(I:C) 8 hours prior to allo–T cell transfer (mean ± SEM, three to six mice/group). *$p < 0.05$.

allo–T-cell responses occurred exclusively in the BM and SP containing activated human resident MDC argues for the role of activated resident MDC in the induction of allo–T-cell responses, but further studies are required to affirm this.

We found that splenic CD11c$^+$MDC, when compared with BM MDC from poly(I:C)-pretreated allo–T-cell recipients, had a more activated phenotype and were endowed with CD86 and IL-12 signals, both essential for allo–T-cell response generation. Therefore, it is reasonable to expect that allo–T-cell responses would be generated more effectively in the SP with its "professional" CD11c$^+$MDC than in the BM of poly(I:C)-pretreated recipients. Unexpectedly, allo–T-cell responses were accelerated in the BM compared to SP 3 days after allo–T-cell transfer. This cannot be attributed to a delay in allo–T-cell responses in the SP compared to BM, because even 5 days after allo–T-cell transfer, allo–T-cell responses were still clearly accelerated in the

BM as compared with the SP of poly(I:C)-pretreated huNOD/SCID recipients.

Because allo–T-cell responses were paradoxically accelerated in the BM as compared to SP, this suggested the possibility that BM may be a source of additional antigen-presenting cells, which can facilitate allo–T-cell responses. A previous study suggested that activated allo–T-cells, in particular these producing TNF-α, induced differentiation of $CD34^+$ cord blood cells into MDC capable of direct alloantigen presentation [29]. Consistent with this study, our experimental model suggests that activated allo–T cells producing TNF-α would be capable of recruiting new antigen-presenting cells from the engrafted human $CD34^+$ cells that were plentiful in the BM as compared to the SP, thereby creating an amplification loop for allo–T-cell responses in the BM. Furthermore, activated allo–T cells producing IFN-γ, IL-2, or IL-4 could also induce MDC differentiation from human monocytes or myeloid progenitors [30–32] that were engrafted in the BM of huNOD/SCID recipients.

Taken together, our experimental transplantation model demonstrated compartmentalization of early allo–T-cell responses specifically accelerating allo–T-cell responses in the BM as compared to the SP in the same recipient mice. The characteristics of the allo–T-cell responses generated in the BM compared to these generated in secondary lymphoid organs may have consequences for the severity of pathology associated with allotransplantation. This, for example, may help explain recent data showing severe allo–T-cell responses in recipient mice lacking all major secondary lymphoid tissues [33]. While we suggest that early allo–T-cell responses are regulated by compartmentalization in the BM and secondary lymphoid tissues, much remains to be done to provide additional clarity for this clinically relevant phenomenon.

References

1. Koni PA, Joshi SK, Temann UA, Olson D, Burkly L, Flavell RA. Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. J Exp Med. 2001;193:741–754.
2. Reis e Sousa C, Yap G, Schulz O, et al. Paralysis of dendritic cell IL-12 production by microbial products prevents infection-induced immunopathology. Immunity. 1999;11:637–647.
3. Reis e Sousa C, Hieny S, Scharton-Kersten T, et al. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. J Exp Med. 1997;186:1819–1829.
4. Khazaie K, Prifti S, Beckhove P, et al. Persistence of dormant tumor cells in the bone marrow of tumor cell-vaccinated mice correlates with long-term immunological protection. Proc Natl Acad Sci U S A. 1994;91:7430–7434.
5. Feuerer M, Beckhove P, Garbi N, et al. Bone marrow as a priming site for T-cell responses to blood-borne antigen. Nat Med. 2003;9:1151–1157.
6. Hommel M, Kyewski B. Dynamic changes during the immune response in T cell-antigen-presenting cell clusters isolated from lymph nodes. J Exp Med. 2003;197:269–280.
7. Shlomchik WD, Couzens MS, Tang CB, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. Science. 1999;285:412–415.
8. Teshima T, Ordemann R, Reddy P, et al. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. Nat Med. 2002;8:575–581.
9. Zhang Y, Shlomchik WD, Joe G, et al. APCs in the liver and spleen recruit activated allogeneic CD8+ T cells to elicit hepatic graft-versus-host disease. J Immunol. 2002;169:7111–7118.
10. Palucka AK, Gatlin J, Blanck JP, et al. Human dendritic cell subsets in NOD/SCID mice engrafted with CD34+ hematopoietic progenitors. Blood. 2003;102:3302–3310.
11. Cravens PD, Melkus MW, Padgett-Thomas A, Islas-Ohlmayer M, Del PMM, Garcia JV. Development and activation of human dendritic cells in vivo in a xenograft model of human hematopoiesis. Stem Cells. 2005;23:264–278.
12. Melkus MW, Estes JD, Padgett-Thomas A, et al. Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. Nat Med. 2006;12:1316–1322.
13. Ishikawa F, Yasukawa M, Lyons B, et al. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. Blood. 2005;106:1565–1573.
14. Traggiai E, Chicha L, Mazzucchelli L, et al. Development of a human adaptive immune system in cord blood cell-transplanted mice. Science. 2004;304:104–107.
15. Rice AM, Wood JA, Milross CG, Collins CJ, McCarthy NF, Vowels MR. Conditions that enable human hematopoietic stem cell engraftment in all NOD-SCID mice. Transplantation. 2000;69:927–935.
16. Hogan CJ, Shpall EJ, McNulty O, et al. Engraftment and development of human CD34(+)-enriched cells from umbilical cord blood in NOD/LtSz-scid/scid mice. Blood. 1997;90:85–96.
17. Kato M, McDonald KJ, Khan S, et al. Expression of human DEC-205 (CD205) multilectin receptor on leukocytes. Int Immunol. 2006;18:857–869.
18. Hock BD, Starling GC, Daniel PB, Hart DN. Characterization of CMRF-44, a novel monoclonal antibody to an activation antigen expressed by the allostimulatory cells within peripheral blood, including dendritic cells. Immunology. 1994;83:573–581.
19. Hock BD, Fearnley DB, Boyce A, et al. Human dendritic cells express a 95 kDa activation/differentiation antigen defined by CMRF-56. Tissue Antigens. 1999;53:320–334.
20. McKinlay A, Radford K, Kato M, et al. Blood monocytes, myeloid dendritic cells and the cytokines interleukin (IL)-7 and IL-15 maintain human CD4+ T memory cells with mixed helper/regulatory function. Immunology. 2007;120:392–403.
21. Osugi Y, Vuckovic S, Hart DN. Myeloid blood CD11c(+) dendritic cells and monocyte-derived dendritic cells differ in their ability to stimulate T lymphocytes. Blood. 2002;100:2858–2866.
22. Vuckovic S, Gardiner D, Field K, et al. Monitoring dendritic cells in clinical practice using a new whole blood single-platform TruCOUNT assay. J Immunol Methods. 2004;284:73–87.
23. McIlroy D, Troadec C, Grassi F, et al. Investigation of human spleen dendritic cell phenotype and distribution reveals evidence of in vivo activation in a subset of organ donors. Blood. 2001;97:3470–3477.
24. Muller M, Gounari F, Prifti S, Hacker HJ, Schirrmacher V, Khazaie K. EblacZ tumor dormancy in bone marrow and lymph nodes: active control of proliferating tumor cells by CD8+ immune T cells. Cancer Res. 1998;58:5439–5446.
25. Li R, Perez N, Karumuthil-Melethil S, Vasu C. Bone marrow is a preferential homing site for autoreactive T-cells in type 1 diabetes. Diabetes. 2007;56:2251–2259.
26. Zhang Y, Louboutin JP, Zhu J, Rivera AJ, Emerson SG. Preterminal host dendritic cells in irradiated mice prime CD8+ T cell-mediated acute graft-versus-host disease. J Clin Invest. 2002;109:1335–1344.

27. Egner W, Hart DN. The phenotype of freshly isolated and cultured human bone marrow allostimulatory cells: possible heterogeneity in bone marrow dendritic cell populations. Immunology. 1995;85:611–620.
28. MacDonald KP, Munster DJ, Clark GJ, Dzionek A, Schmitz J, Hart DN. Characterization of human blood dendritic cell subsets. Blood. 2002;100:4512–4520.
29. Abbasian J, Mahmud D, Mahmud N, et al. Allogeneic T cells induce rapid CD34+ cell differentiation into CD11c+CD86+ cells with direct and indirect antigen-presenting function. Blood. 2006;108:203–208.
30. Sanarico N, Ciaramella A, Sacchi A, et al. Human monocyte-derived dendritic cells differentiated in the presence of IL-2 produce proinflammatory cytokines and prime Th1 immune response. J Leukoc Biol. 2006;80:555–562.
31. Delneste Y, Charbonnier P, Herbault N, et al. Interferon-gamma switches monocyte differentiation from dendritic cells to macrophages. Blood. 2003;101:143–150.
32. Vuckovic S, Clark GJ, Hart DN. Growth factors, cytokines and dendritic cell development. Curr Pharm Des. 2002;8:405–418.
33. Anderson BE, Taylor PA, McNiff JM, et al. Effects of donor T-cell trafficking and priming site on graft-versus-host disease induction by naive and memory phenotype CD4 T cells. Blood. 2008;111:5242–5251.