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An Innate Response to Allogeneic Nonself Mediated by Monocytes

Daniel Zecher,* Nico van Rooijen,† David M. Rothstein,** Warren D. Shlomchik,‡ and Fadi G. Lakkis²*

The mammalian innate immune system has evolved diverse strategies to distinguish self from microbial nonself. How the innate immune system distinguishes self-tissues from those of other members of the same species (allogeneic nonself) is less clear. To address this question, we studied the cutaneous hypersensitivity response of lymphocyte-deficient RAG⁻/⁻ mice to spleen cells transplanted from either allogeneic or syngeneic RAG⁻/⁻ donors. We found that RAG⁻/⁻ mice mount a specific response to allogeneic cells characterized by swelling and infiltration of the skin with host monocytes/macrophages and neutrophils. The response required prior priming with allogeneic splenocytes or skin grafts and exhibited features of memory as it could be elicited at least 4 wk after immunization. Neither depletion of host NK cells nor rechallenging immunized mice with F₁, hybrid splenocytes inhibited the response, indicating that the response is not mediated by NK cells. Depletion of host monocytes/macrophages or neutrophils at the time of rechallenge significantly diminished the response and, importantly, the adoptive transfer of monocytes from allogeneized RAG⁻/⁻ mice conferred alloimmunity to naive RAG⁻/⁻ hosts. Unlike NK- and T cell-dependent alloresponses, monocyte-mediated alloimmunity could be elicited only when donor and responder mice differed at non-MHC loci. These observations indicate that monocytes mount a response to allogeneic nonself, a function not previously attributed to them, and suggest the existence of mammalian innate allore cognition strategies distinct from detection of missing self-MHC molecules by NK cells. The Journal of Immunology, 2009, 183: 7810–7816.

Immunity in most studied vertebrates is dependent on two integrated immune systems, the innate and adaptive, whose principal function is to protect the host against infection (1). Cells of the innate immune system express germline-encoded pattern recognition receptors that detect conserved molecular patterns on microbes (1, 2). Activation of these cells by microbes leads to an acute inflammatory response that limits the infection but does not necessarily eliminate it. Cells of the adaptive immune system, in contrast, express a highly diverse repertoire of Ag receptors, generated by RAG-mediated gene rearrangements, that specifically recognize microbial peptides presented to them in the context of MHC molecules on activated innate cells. Once triggered by the innate immune system, the adaptive system eliminates the microbe and provides long-term protection of the host against re-infection (immunological memory).

Vertebrate immunity, however, is not restricted to antimicrobial responses. All vertebrates studied so far are capable of allore cognition, the ability to discriminate between self tissues and those of other members of the same species (3, 4). Allorecognition in mammals is best exemplified by the rejection of transplanted cells, tissues, or organs (allografts) and by graft vs host disease after bone marrow transplantation (5). It is also observed under natural conditions such as the maternal response to paternal alloantigens on fetal cells and the rejection of transmissible allogeneic tumors (6–9). In the adaptive immune system, T lymphocytes are the principal cell type responsible for allorecognition due to their ability to detect molecular variations in MHC molecules and/or the endogenous peptides bound to them (10).

How the innate immune system recognizes allogeneic nonself is less clear. Cells of the innate system are activated within minutes to hours after transplanting allogeneic tissues to humans or experimental animals and are present in large numbers at the feto-maternal interface in the gravid uterus (11, 12). The prevailing view is that innate immune cells, with the exception of NK cells, do not engage in specific allorecognition but instead respond to inflammatory ligands released by stressed or dying cells at the time of transplantation (13, 14). These ligands are not unique to allografts but are common to self and nonself tissues subjected to hypoxic or other forms of injury (15, 16). NK cells, in contrast, engage in “missing self recognition” in that they are inhibited by self-MHC molecules but become more amenable to activation by stimulatory ligands when they encounter MHC-deficient or MHC-mismatched targets (17–19). Although NK cells contribute to innate allorecognition of MHC-mismatched grafts (20, 21), they are not expected to recognize allogeneic tissues transplanted between MHC-matched individuals. Therefore, a major gap in our understanding is whether innate cells other than NK cells do or do not distinguish between self and allogeneic nonself and whether such allorecognition would be dependent on or independent of MHC polymorphisms.

Another impetus to explore lymphocyte-independent alloresponses is the intriguing observation that the rejection of allogeneic tissues is common to many invertebrate organisms that...
predate the evolution of T, B, and NK cells. The ubiquity of rejection responses, particularly among colonial marine invertebrates, has led to the proposal that all recognition evolved before the emergence of vertebrate adaptive immune systems, characterized by polymorphic MHC molecules and re-arranging lymphocyte receptors, and that it serves to protect the organism against stem cell parasitism (22–25). The recent identification of specific all recognition systems in two colonial marine organisms, Botryllus and Hydractinia (26, 27), and the characterization of their role in preventing stem cell chimerism (28, 29), have confirmed these earlier suspicions. To investigate whether the mammalian immune system mounts an allose immune response independent of lymphocytes, we studied the cutaneous reaction of RAG−/− mice to cells transplanted from allogeneic donors. We show in this study that these mice mount an allose immune response independent of T, B, and NK cells. The response requires prior priming with allogeneic nonself, is mediated by monocytes, exhibits features of memory, and is elicited by MHC-unlinked genetic disparities between the donor and responder mouse strain.

Materials and Methods

Mice

C57BL/6 (B6), BALB/c, B6.C-H2/H-2ByJ (B6.C), C3H/HeJ, B6 RAG−/−, BALB/c RAG−/−, and congenic B6 SJL RAG−/− (CD45.1+CD45.2−) mice were purchased from the Jackson Laboratory. B6 and BALB/c RAG−/− mice were purchased from Taconic Farms. (C57BL/6 × BALB/c)F1, RAG−/− mice were generated by breeding the parental strains at the University of Pittsburgh animal facility. All mice were housed in a specific pathogen-free environment and experiments were performed in accordance with the Institutional Animal Care and Use Committee of the University of Pittsburgh. Mice were generated by breeding the parental strains at the University of Pittsburgh. B6 and BALB/c RAG−/− mice were generated by breeding the parental strains at the University of Pittsburgh. BALB/c RAG−/− mice were grafted with syngeneic tail skin. One or four weeks later, baseline thickness of the ear pinnae was measured. In experiments where wild-type mice were used, wild-type (B6) and syngeneic (BALB/c) RAG−/− mice were generated by breeding the parental strains at the University of Pittsburgh. BALB/c RAG−/−, and congenic B6 SJL RAG−/− (CD45.1+CD45.2−) mice were purchased from Taconic Farms. RAG−/− mice were generated by breeding the parental strains at the University of Pittsburgh. BALB/c RAG−/− and RAG−/− mice were used interchangeably and are referred to as RAG−/− mice throughout. RAG−/− mice were tested periodically by performing serum IgM ELISA and flow cytometry on either blood or spleen.

Abs and reagents

Biotin-conjugated mAbs against CD45.1 (clone A20), CD4 (L3T4), CD8b (Ly-3), CD19 (MB19–1), F4/80 (BM8), pan-NK (DX5), CD11b (M1/70), CD11c (HL3), I-Ad (AMS-32.1), Ter119 (Ly-76), and CD16/32 (2.4G2); Allexa750-conjugated anti-CD45.2 (104); allophycocyanin-conjugated anti-F4/80 (BM8), and CD16/32 (2.4G2); Allexa750-conjugated anti-CD45.2 (104); allophycocyanin-conjugated anti-F4/80 (BM8), Phycoerythrin-conjugated anti-CD16/32 (2.4G2); and Pacific Blue-conjugated Streptavidin were obtained from eBioscience. FITC-conjugated anti-Gr-1 (RB6–8C5) and purified anti-CD16/32 (2.4G2) were from BD Pharmingen. Ethidium monoazide was from Invitrogen. Phycoerythrin-conjugated anti-Gr-1 (RB6–8C5) and purified anti-CD16/32 (2.4G2) were from BD Pharmingen. Ethidium monoazide was from Invitrogen. Phycoerythrin-conjugated anti-NK1.1 mAb and corresponding isotype control Abs were from BioXCell. Clodronate was a gift from Roche Diagnostics and was incorporated into liposomes by one of the co-authors (NvR) as described (30).

In vivo alloresponse

Five to 10-wk-old male mice were immunized by injecting 2 × 107 allogeneic splenocytes s.c. in the neck or by applying an allogeneic tail skin graft to the dorsoventral aspect of the mouse. If necessary, RAG−/− mice were used as both cell donors and recipients to avoid contamination with T and B lymphocytes. In experiments where wild-type (wt) mice served as source of splenocytes, the splenocytes were pulsed with T and B cells from wt mice. In experiments where wild-type (wt) mice served as source of splenocytes, the splenocytes were pulsed with T and B cells from wt mice. In experiments where wild-type (wt) mice served as source of splenocytes, the splenocytes were pulsed with T and B cells from wt mice. In experiments where wild-type (wt) mice served as source of splenocytes, the splenocytes were pulsed with T and B cells from wt mice. In experiments where wild-type (wt) mice served as source of splenocytes, the splenocytes were pulsed with T and B cells from wt mice.

Analysis of ear-infiltrating leukocytes

Excised ears were pooled from two to three mice per group, separated along the cartilage, digested in RPMI 1640 plus 1 mg/ml collagenase IV plus 0.01 mg/ml DNase I plus 0.25 mg/ml hyaluronidase (all from Sigma-Aldrich) for 1 h at 37°C, and purified by gradient centrifugation (Lympholyte M, Cedarlane Laboratories) before flow cytometric analysis. For tissue analysis, 6-μm acetone-fixed sections were stained with biotin-conjugated anti-CD45.1 and unconjugated anti-Ly6G followed by streptavidin-conjugated Cy3 (Jackson ImmunoResearch Laboratories) and anti-rat Alexa488 (Invitrogen). Sections were counterstained with 4’,6-diamidino-2-phenylindole (Molecular Probes).

NK, macrophage, and neutrophil depletion

In vivo depletion of host NK, monocyte/macrophage, or neutrophils was accomplished by injecting 250 μg anti-NK1.1 (PK136) mAb i.p., 250 μg clodronate-loaded liposomes i.v., or 250 μg anti-Ly6G (1A8) mAb i.p., respectively. Control mice received isotype control Ab or PBS-loaded liposomes. Injections were given once, 16 h before rechallenging mice with splenocytes. Depletion of NK cells before immunization was done analogous to the above protocol except that anti-NK1.1 mAb and corresponding isotype Ab were administered 7 days before immunization. Depletion of specific leukocyte populations was confirmed by flow cytometric analysis of blood, bone marrow, and spleen (supplemental Fig. S1).

Sorting and transfer of monocytes

BALB/c RAG−/− mice were immunized with either syngeneic or allogeneic (B6) splenocytes. Seven days later, spleens were removed and F4/80−Gr-1−CD11b−CD45.1−/CD45.2− monocytes were high-speed sorted using an ARIA instrument (BD Biosciences) to >95% purity. In brief, 2.0–2.5 × 107 monocytes from either group were injected i.v. via the tail vein into naive BALB/c RAG−/− mice 24 h before measuring the alloresponse as described above.

Statistical analysis

All data are presented as mean ± SEM and were analyzed by paired or unpaired Student’s t test or one-way ANOVA. Bonferroni multiple-comparison test was applied when indicated. Significance was set at p < 0.05.

Results

Evidence for an allospecific response in RAG−/− mice

To study allospecificity in the innate immune system, we examined the response of recombination activating gene-deficient (RAG−/−) mice, which lack mature T and B lymphocytes (31), to allogeneic splenocytes. BALB/c RAG−/− mice were immunized with splenocytes from genetically disparate C57BL/6 (B6) RAG−/− mice and, 7 d later, were rechallenged by injecting allogeneic (B6) and syngeneic (BALB/c) RAG−/− splenocytes in opposite ear pinnae. This assay is analogous to the delayed type hypersensitivity (DTH) reaction widely used to study T cell immunity in vivo (32). As shown in Fig. 1a, rechallenging immunized RAG−/− mice with allogeneic cells caused significantly greater ear swelling than rechallenge with syngeneic cells. The response peaked as early as 6 h after rechallenge and declined gradually thereafter. The magnitude and kinetics of the RAG−/− response were different from that of wt mice. The latter exhibited more pronounced ear swelling that peaked at 24 h (Fig. 1b), consistent with the role of effector/memory T lymphocytes in mediating DTH to allografts in wt mice (33).

The online version of this article contains supplementary material.

1 Abbreviations used in this paper: wt, wild type; DTH, delayed type hypersensitivity.
and all subsequent experiments. As shown in Fig. 1c, naïve (unimmunized) RAG−/− mice exhibited minimal allogeneic ear swelling (primary response) compared with previously immunized mice (recall response). The response could be elicited even if the mice were rechallenged with allogeneic splenocytes 4 wk after immunization, suggesting longevity of the response.

To investigate the antigenic specificity of the RAG−/− alloresponse and to test whether it can be elicited by priming with non-hematopoietic tissue allografts, we placed either syngeneic or allogeneic “third-party” (C3H) tail skin grafts onto BALB/c RAG−/− recipients. Seven days later, transplanted mice were rechallenged with B6 and BALB/c RAG−/− splenocytes in the ears, and allogeneic swelling was calculated. Although minimal or no allogeneic responses were observed in mice immunized with syngeneic skin allografts, allogeneic swelling was readily detectable in mice immunized with allogeneic B6 skin and, to a lesser extent, in those immunized with “third-party” C3H skin (Fig. 1d). This finding is somewhat different from T lymphocyte-mediated DTH responses in wt mice, which are elicited only if the host is rechallenged with cells from the same mouse strain it had encountered before (34). The results shown in Fig. 1, therefore, indicate that the innate immune system responds to allogeneic nonself independent of the adaptive system. This response requires prior priming with either hematopoietic or nonhematopoietic allografts and has features of memory, but does not display complete alloantigenic specificity in the combinations tested.

**Leukocyte recruitment in response to allogeneic nonself**

To begin to examine which cells participate in the innate alloresponse of RAG−/− mice, we quantitated and phenotyped host leukocytes that infiltrated the ear pinnae six and 12 h after injecting either syngeneic or allogeneic RAG−/− splenocytes. Host leukocytes infiltrating or residing in the ear were distinguished from injected cells by the congenic marker CD45.1. As shown in Fig. 2a, significantly more host leukocytes were present in ears injected with allogeneic than syngeneic splenocytes. Additional flow cytometric analysis revealed that the infiltrating leukocyte population consisted mainly of neutrophils (F4/80−Ly6C+), monocytes (F4/80intLy6C+), and macrophages (F4/80highLy6C–) (Fig. 2b). NK cells (NKP46+ were also present but in smaller proportions (flow cytometry plots not shown). Whereas NK cell numbers were comparable, macrophages, monocytes, and neutrophils accumulated to a larger extent in ears injected with allogeneic as compared with syngeneic splenocytes (Fig. 2, c and d).

**The RAG−/− alloresponse is independent of NK cells**

NK cells respond to cells or tissues with which they do not share MHC molecules (missing self reaction) and, in addition, have been recently shown to mediate a memory response to chemical haptens independent of the adaptive immune system (19, 35). We therefore investigated whether NK cells are responsible for the alloresponse of RAG−/− mice. Two independent experimental approaches failed to demonstrate a role for NK cells in this response. First, BALB/c RAG−/− mice immunized with B6 splenocytes reacted equally to (BALB/c × B6)F1, and B6 RAG−/− splenocytes despite the fact that host NK activation is inhibited by the shared H-2d MHC allele expressed on F1 cells (Fig. 3a). Second, NK depletion of the host (B6 RAG−/− mouse in this case) either throughout the experiment (data not shown)
or only before rechallenge (Fig. 3b) did not alter the allo-
response to BALB/c RAG⁻/⁻ spleen cells. We also tested the
possibility that injected (donor) NK cells mount a graft-vs-host
reaction that may contribute to the observed ear swelling.
(BALB/c × B6)F₁ mice were immunized with B6 RAG⁻/⁻
splenocytes and rechallenged 7 days later with either syngeneic
(F₁) or allogeneic (B6) RAG⁻/⁻ spleenocytes in the ear. In this
setting, host F₁ cells express the B6 MHC allotype (H-2b) and
inhibit activation of donor (B6) NK cells. As shown in Fig. 3c,
F₁ hosts mounted an allospecific response to B6 splenocytes
equivalent to that of control BALB/c hosts which do not express
H-2b. This finding strongly argues against NK-mediated graft vs
host reaction as a contributor to the ear swelling response.

Phagocytic cells and neutrophils mediate the recall phase of the
RAG⁻/⁻ alloresponse

To study the role of non-NK, innate immune cells in the allo-
response of RAG⁻/⁻ mice, we administered clodronate-loaded lipo-
somes or anti-Ly6G lytic Ab to immunized mice to deplete phago-
cytic cells or neutrophils, respectively. Depletion of either cell type
before rechallenge blunted allodependent ear swelling by >50%,
Neutrophil-depleting H9251/wk earlier with allogeneic (B6 RAG3–4/group days earlier. Twenty-four hours later, mice were challenged with syngeneic and allogeneic cells and allo-dependent swelling was determined.

Monocytes are responsible for the long-lived alloresponsiveness they exit from the bone marrow (36), we then tested whether shown. Because neutrophils have a short lifespan (37), we inferred alloresponsiveness to naive RAG/H11002 containing spleen cell population enriched for neutrophils and monocytes, but host was tested in the ear swelling assay. In initial experiments, a harvest spleen cells from BALB/c RAG/H11002 mice. One day later, alloresponsiveness of the adoptive host was tested in the ear swelling assay. In initial experiments, a spleen cell population enriched for neutrophils and monocytes, but containing <2% NK cells, dendritic cells, or macrophages, conferred alloresponsiveness to naive RAG/H11002 hosts (data not shown). Because neutrophils have a short lifespan (<2 days) after they exit from the bone marrow (36), we then tested whether monocytes are responsible for the long-lived alloresponsiveness observed in our model. We found that F4/80/H11002 splenic monocytes, high-speed sorted from the spleens of alloimmunized RAG/H11002 mice (Fig. 4c), conferred full alloreactivity to naive RAG/H11002 hosts (Fig. 4d). The monocytic lineage of the transferred population was confirmed by morphologic examination and by expression of the surface molecules CD115 (M-CSF-R) and Ly6C (Fig. 4c). Less than 1% of the transferred population expressed the NK cells marker Nkp46 (Fig. 4c). Control mice that received a similar cell population from mice immunized with syngeneic cells and allo-dependent swelling was determined. n = 3–4/group × 2 experiments (pooled data shown). Bar graphs are mean ± SEM; *, p < 0.05; **, p < 0.01.

Adoptive transfer of alloimmunity by primed monocytes

To identify which innate cell population is primed at the time of immunization and therefore confers long-lived alloimmunity to RAG/H11002 mice, an adoptive cell transfer approach was used. We harvested spleen cells from BALB/c RAG/H11002 mice immunized 1 wk earlier with allogeneic (B6 RAG/H11002) spleen cells, isolated distinct cell populations, and transferred them to naive BALB/c RAG/H11002 mice. One day later, alloresponsiveness of the adoptive host was tested in the ear swelling assay. In initial experiments, a spleen cell population enriched for neutrophils and monocytes, but containing <2% NK cells, dendritic cells, or macrophages, conferred alloresponsiveness to naive RAG/H11002 hosts (data not shown). Because neutrophils have a short lifespan (<2 days) after they exit from the bone marrow (36), we then tested whether monocytes are responsible for the long-lived alloresponsiveness observed in our model. We found that F4/80/H11002 gr-1+ monocytes, high-speed sorted from the spleens of alloimmunized RAG/H11002 mice (Fig. 4c), conferred full alloreactivity to naive RAG/H11002 hosts (Fig. 4d). The monocytic lineage of the transferred population was confirmed by morphologic examination and by expression of the surface molecules CD115 (M-CSF-R) and Ly6C (Fig. 4c). Less than 1% of the transferred population expressed the NK cells marker Nkp46 (Fig. 4c). Control mice that received a similar cell population from mice immunized with syngeneic cells and allo-dependent swelling was determined. n = 3–4/group × 2 experiments (pooled data shown). Bar graphs are mean ± SEM; *, p < 0.05; **, p < 0.01.

The RAG/H11002 alloresponse is dependent on non-MHC disparities between donor and responder strains

MHC molecules are central to the recognition of allogeneic nonself by T lymphocytes. TCR, generated by RAG-mediated gene rearrangement, directly recognize allogeneic MHC molecules on target cells or, alternatively, react to MHC or non-MHC-derived allopeptides bound to self-MHC molecules (5). Likewise, allorecognition by NK cells is dictated by MHC polymorphisms. NK cells are inhibited if they share the same MHC class I molecules with their targets but are released from inhibition if MHC class I
munized with B6 RAG<sup>−/−</sup> parities between donor and responder strains: BALB/c RAG<sup>−/−</sup> mice share the same MHC (H-2d) with BALB/c mice but differ at non-MHC loci. Allo-dependent ear swelling was calculated as in Fig. 1. Mice were rechallenged with either B6 or B6.C splenocytes. Con-}

FIGURE 5. The RAG<sup>−/−</sup> alloresponse is dependent on non-MHC disparities between donor and responder strains: BALB/c RAG<sup>−/−</sup> mice immunized with B6 RAG<sup>−/−</sup> splenocytes were rechallenged 7 days later with T- and B cell depleted B6 (H-2b<sup>+</sup>) (solid bars) or congenic B6.C (H-2a<sup>+</sup>) (hatched bars) wt splenocytes. Alternatively, B6 RAG<sup>−/−</sup> mice were immunized with BALB/c RAG<sup>−/−</sup> splenocytes and rechallenged 7 d later with T and B cell-depleted B6.C (H-2d<sup>+</sup>) wt splenocytes (open bars). B6.C mice share the same MHC (H-2d<sup>+</sup>) with BALB/c mice but differ at non-MHC loci. Allo-dependent ear swelling was calculated as in Fig. 1. n = 6/group × 1–2 experiments. Bar graphs are mean ± SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

molecules are not shared (missing self hypothesis) (19). Microbial nonself recognition by nonlymphocytic innate cells, in contrast, is independent of the MHC and is determined by germline-encoded pattern-recognition receptors (1, 2). We therefore investigated whether the alldeterminants responsible for the monocytemediated alloresponse of RAG<sup>−/−</sup> mice are linked or unlinked to the MHC. To do so, we immunized BALB/c RAG<sup>−/−</sup> mice (H-2b<sup>+</sup>) with B6 RAG<sup>−/−</sup> splenocytes and rechallenged them in the ear pinnae with either B6 (H-2<sup>+</sup>) or congenic B6.C (H-2<sup>+</sup>) splenocytes. The B6.C congenic strain is identical to the B6 strain except for a 38 Mb genomic segment that encodes the H-2d MHC locus of the BALB/c strain. As shown in Fig. 5, ear swelling was comparable in mice rechallenged with either B6 or B6.C splenocytes. Conversely, B6 RAG<sup>−/−</sup> mice did not respond to B6.C splenocytes with which they differed at the MHC but shared all other genetic loci (Fig. 5). These findings indicate that the innate alloresponse of RAG<sup>−/−</sup> mice is dependent on determinants not linked to the MHC.

Discussion

We investigated in this study whether innate immune cells other than NK cells distinguish self from allogeneic nonself. We found that T and B cell-deficient RAG<sup>−/−</sup> mice mount a specific response to allogeneic nonself independent of NK cells. The re-}

specific response required prior priming with allogeneic splenocytes or skin grafts and exhibited "memory," features traditionally associated with adaptive immunity. Adoptive cell transfer and depletion studies demonstrated that innate alloimmunity in our model is conferred by monocytes but that both neutrophils and monocytes/macrophages contribute to the effector phase of the response. Finally, we demonstrated that the response is elicited by MHC-unlinked disparities between donor and responder. Our findings provide direct evidence that monocytes mediate a response to allogeneic nonself, a function not previously attributed to them, and suggest the presence of MHC-unlinked innate allorecognition systems in the mouse.

Memory has long been assumed to be a defining feature of adaptive immunity (37). This view, however, has been challenged by recent studies. In the mouse, NK cells activated by hapten, virus, or cytokines acquire classical features of memory that include extended life span, self-renewal capacity, and ability to mount a robust recall response (35, 38, 39). A memory response to pathogens has also been observed in invertebrate organisms that lack mammalian-type adaptive immune systems (40). For example, Pham et al. (41) demonstrated specific and persistent immu-

nity to bacterial pathogens in Drosophila that is mediated by phagocytes. Our finding that monocyte-mediated alloimmunity in RAG<sup>−/−</sup> mice is long-lived provides the first evidence that phagocytic cells of the vertebrate innate immune system exhibit features of memory. This observation further blurs the divisions previously thought to exist between innate and adaptive immunity.

The innate alloresponse characterized in our study is distinct from that mediated by NK cells as it is not influenced by MHC matching; instead, it is dependent on mismatching at loci unlinked to the MHC. It is not currently known what alldeterminants these loci code for. Theoretically, these could be any of many genes outside the MHC that are polymorphic among inbred mouse strains. Polymorphic, Ig domain-containing genes or gene families that are expressed in cells of the innate immune system have been identified in mice and humans (42). Recently, a role for SIRPα polymorphisms in the rejection of hematopoietic stem cell xenografts by the innate immune system has been reported (43). It is not known, however, whether SIRPα also serves as an alldeterminant. Alternatively, the innate system of RAG<sup>−/−</sup> mice may recognize differences in glycosylation patterns between mouse strains rather than amino acid polymorphisms (44). With the exception of well-characterized glycomic differences between distant species, best exemplified by the lack of α1,3-galactosyltransferase in ca-tarrhine primates (including humans) but its presence in most other mammals (45), glycomic differences between conspecifics have not yet been carefully explored.

It has been proposed that the mammalian innate immune re-

response to allografts is largely a response to endogenous inflammatory ligands released by stressed or dying cells at the time of transplantation (13, 14). These nonpolymorphic ligands, which include uric acid and the nuclear protein high-mobility group box 1, among others, are common to both self and nonself tissues subjected to hypoxia and signal through innate pattern recognition receptors on innate immune cells (15, 16, 46). The pattern recognition receptors include Toll-like receptors and components of the inflammasome, all of which participate in the recognition of microbial products as well. Our study does not exclude that these ubiquitous recognition pathways contribute to the initiation of the adaptive alloimmune response, but provides direct evidence that innate immune cells other than NK cells respond either directly or indirectly to non-MHC determinants. Although our data provide strong evidence that innate alloresponsiveness in RAG<sup>−/−</sup> mice is mediated by monocytes, they do not determine whether monocytes directly recognize allogeneic nonself. It is quite possible that other components of the innate immune system recognize allogeneic nonself and then stimulate monocytes.

Specific allorecognition systems that predate the emergence of lymphocytes and the MHC have been identified in colonial marine invertebrates (26, 47). These systems prevent fusion between genetically disparate members of the same species and thus, serve as safeguards against stem cell and germline parasitism (25, 28). It is therefore tempting to speculate that the vertebrate innate allorecognition phenomenon described in this study is an echo of ancient but essential allorecognition systems. It is conceivable that monocytes provide innate immune surveillance that alerts the host to the presence of allogeneic stem cells encountered under either natural or artificial conditions. In mice, blood monocytes patrol healthy tis-
been shown to rapidly deploy to sites of acute inflammation, for example the infarcted myocardium, where they play a role in tissue repair (49). In addition, recruitment of monocytes to the peritoneal cavity of SCID mice has been observed in response to xenogeneic pig cells and to a significantly lesser extent to allogeneic cells (50). Examples of natural allogeneic encounters in vertebrates include entry of fetal stem cells into the maternal circulation during pregnancy, described in humans (6), and the transmission of allogeneic tumors between members of the same species, documented in dogs and Tasmanian devils (8, 9). Artificial allogeneic encounters, in contrast, are represented by solid organ and hematopoietic stem cell (bone marrow) transplantation in humans and experimental animals. We hypothesize that in these situations monocytes serve as sentinels that continually alert the adaptive immune system to the presence of allogeneic nonself and could inflict damage on the target tissue or organ.

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Disclosures

The authors have no financial conflict of interest.

References

Supplementary Information

Supplementary Figure 1. *In vivo* monocyte/macrophage and neutrophil depletion. (A) Depletion of CD115⁺ F4/80<sup>int</sup> blood monocytes 28 hrs after i.v. administration of clodronate-loaded liposomes (CLL) compared to mice treated with PBS-loaded control liposomes, analyzed by flow cytometry. (B) Kinetics and compartment-specific depletion of monocytes/macrophages after CLL administration determined by flow cytometry. Line graphs depict % depletion of CD115⁺ F4/80<sup>int</sup> blood monocytes (Blood), splenic F4/80⁺ Gr-1<sup>int</sup> monocytes/macrophages (Spleen), and CD115⁺ F4/80⁺ bone marrow macrophages (BM) compared to baseline numbers in untreated mice. Data points are means of 3 - 5 mice per time point. (C) Depletion of Gr-1⁺ blood neutrophils 28 hrs after i.p. administration of α-Ly6G mAb compared to isotype treated mice, analyzed by flow cytometry. (D) α-Ly6G mAb does not deplete monocytes/macrophages. Absolute number of F4/80<sup>int</sup> blood monocytes was determined by flow cytometry after administration of α-Ly6G mAb. Control mice received isotype control Ab. Data points are means of 3 – 5 mice/group.
Fig. S1

A) PBS and 28h post CLL

B) Mφ depletion (%)

C) Isotype and 28h post α-Ly6G

D) F4/80 X 10^6 / ml