Partial NK cell tolerance induced by radioresistant host cells in rats transplanted with MHC-mismatched bone marrow

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Abstract

We have studied the effect of radioresistant host cells in inducing tolerance and adaptation of the MHC recognition repertoire of donor-derived NK cells in stem cell allotransplanted (allo-SCT) rats. Sub-lethally irradiated PVG.1AV1 rats (RT1av1) were transplanted with bone marrow from fully MHC-mismatched allotype-marked PVG.7B (RT1c) rats; MHC-identical PVG (RT1c) controls were transplanted in parallel. In the PVG.7B → PVG.1AV1 allogeneic chimeras, NK cells were donor derived and showed partial tolerance toward host cells. Allogeneic chimeras failed to efficiently reject PVG.1AV1 cells by an NK-mediated mechanism in vivo (allogeneic lymphocyte cytotoxicity), and IL-2-cultured NK cells derived from these chimeras showed diminished cytolytic activity against PVG.1AV1 cells in vitro. There were corresponding changes in the phenotype and function of the highly alloreactive Ly49i2⁺ NK cells, which are specifically inhibited by a donor MHC class I ligand, RT1-A1c. The ligand-negative host MHC haplotype apparently induced expression of a second uncharacterized inhibitory MHC receptor responsible for the partial tolerance toward host-derived cells, along with a modest increase in Ly49i2 receptor levels. The host MHC haplotype did not induce a general hyporesponsiveness in Ly49i2⁺ NK cells, which showed normal activation responses in a panel of MHC congenic strains. The data suggest that the MHC constitution of radiation-resistant host cells can have permanent, albeit not fully tolerogenic, effects on the development of a functional NK repertoire following allo-SCT.

Keywords: allogeneic stem cell transplantation, alloreactivity, MHC, NK cells, repertoire development

Introduction

NK cells express a number of receptors for MHC class I and class I-related molecules (1). While NK cells often attack MHC-incompatible target cells such as allogeneic bone marrow cells and lymphocytes, they are generally non-responsive toward MHC-identical cells (2, 3). This non-responsiveness can be maintained by expression of at least one self-specific inhibitory MHC receptor or by a generally dampened responsive capability (4, 5). Self-tolerance is presumably acquired in the bone marrow, where the developing NK cell is exposed to neighboring hematopoietic cells as well as various non-hematopoietic stromal cells. Both cell types may influence NK cell development and tolerance, but their relative importance has not yet been established in detail. In a transgenic mouse model on the H-2b background with mosaic expression of the H-2Dd class I molecule, hematopoietic cells appeared to be potent inducers of NK cell tolerance. Presence of only 20% H-2Dd⁺ leukocytes was sufficient to render the H-2Dd⁺ NK cells non-reactive toward H-2Dd⁻ targets. This effect was not permanent and was reversed upon separation of H-2Dd⁻ from H-2Dd⁺ cells in vitro (6). Other studies have pinpointed the importance of radioresistant elements in tolerance induction, in F1-hybrid resistance and MHC class I-deficient mouse models (7, 8).

In this study, we have investigated whether radioresistant host cells influence NK cell development and function in stem cell allotransplanted (allo-SCT) rats. Transplantation was performed across a full MHC barrier using congenic animals on the high NK-alloresponder PVG strain background. In this setting, successful engraftment is only obtained in NK-permissive host-versus-graft (HvG) combinations, i.e. where the host NK cells do not respond to the donor’s MHC allotype; otherwise bone marrow (BM) graft rejection occurs with reconstitution of the hematopoietic system from host stem cells (9). Thus, while PVG strain...
(RT1c MHC haplotype) NK cells show strong alloreactivity against PVG.1AV1 lymphoblast target cells (RT1\textsuperscript{av1}), PVG.1AV1 NK cells are tolerant to PVG target cells (10). In accordance with this, stem cell engraftment is only observed in the PVG→PVG.1AV1, but not the PVG.1AV1→PVG strain combination (9).

The allogenic lymphocyte cytotoxicity (ALC) responder genes, which determine the ability of rat NK cells to respond to normal allogenic leukocytes, have previously been mapped to the Ly49 region of the NK complex on rat chromosome 4 (11). There is a clear co-association with the activating Ly49 variants (12, 13), and their direct involvement in NK alloreactivity has been demonstrated in functional studies (11, 12). The Ly49 receptors are not evenly expressed among all NK cells but focused to a major subset in some studies (14, 15). There is a clear co-association with the Ly49 region of the NK complex on rat chromosome 4 (11). There is a clear co-association with the Ly49 region of the NK complex on rat chromosome 4 (11).

In accordance with this, stem cell engraftment is only observed in the PVG→PVG.1AV1, but not the PVG.1AV1→PVG strain combination (9). The allogeneic lymphocyte cytotoxicity (ALC) responder pattern was used. Breeding pairs of the PVG strain expressing the Ly49i2 receptor, which selectively binds a classical class Ia ligand expressed in PVG strain rats, RT1-A\textsuperscript{i2} (17–20). According to the licensing model (21), recognition of RT1-A\textsuperscript{i2} would allow the Ly49i2\textsuperscript{+} NK subset to mature to full function in PVG rats, while they would remain ‘unlicensed’ and less functional in ligand-negative strains, such as PVG.1U (RT1\textsuperscript{i1}). We have speculated that this could explain why Ly49i2\textsuperscript{+} NK cells in PVG.1U rats are few in numbers and exhibit little alloreactivity. In this strain, NK alloreactivity is mainly exerted by Ly49i2\textsuperscript{−} NK cells (18).

In a second ligand-negative strain (PVG.1AV1; RT1\textsuperscript{av1}), a somewhat discrepant observation has been made. In PVG.1AV1, Ly49i2\textsuperscript{+} NK cells appear to be fully functional as they are strongly alloreactive and are also expanded in absolute numbers (17, 18). Functional studies suggested that self-tolerance was not mediated by the Ly49i2 receptor, however, but by an as yet unidentified inhibitory receptor for a non-classical class Ia MHC determinant expressed in several haplotypes including RT1\textsuperscript{av1} and RT1\textsuperscript{i1}, but not RT1\textsuperscript{i2} (18). In the present study, we have studied the effect of fully MHC-mismatched (RT1\textsuperscript{av1}) host stromal cells on the function of donor-derived NK cells following allo-SCT, with a focus on the Ly49i2\textsuperscript{+} NK subset that has previously been subject to detailed functional studies in the same panel of strains (17–19). The data suggest that radiation-resistant host cells can have specific tolerizing effects on the development of a functional MHC repertoire in rat NK cells without inducing hyporesponsiveness.

**Methods**

**Rats**

A set of MHC congenic rat strains on the PVG strain background was used. Breeding pairs of the PVG strain expressing the c rat MHC haplotype (RT1\textsuperscript{c} i.e. RT1-A\textsuperscript{a}-B\textsuperscript{d}-H\textsuperscript{e}-N\textsuperscript{M}-M\textsuperscript{F}) and from the RT7.2 allotype-marked PVG→RT7\textsuperscript{b} (PVG.7B) strain (also RT1\textsuperscript{c} MHC haplotype), the PVG→RT1\textsuperscript{a} (PVG.1U; RT1\textsuperscript{a}), PVG→RT1\textsuperscript{av1} (PVG.1AV1; RT1\textsuperscript{av1}), and the intra-MHC recombinant PVG.R23 (RT1-A\textsuperscript{a}-B\textsuperscript{d}-H\textsuperscript{e}-N\textsuperscript{M}-M\textsuperscript{F}) strains were obtained from Harlan UK Limited (Bicester, UK). These strains were reared under conventional conditions in Oslo and screened for common rat pathogens. The animals were housed in compliance with guidelines set by the Experimental Animal board under the Ministry of Agriculture of Norway and ‘The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes’. The laboratory animal facilities are subject to a routine health monitoring program and were tested for infectious organisms according to a modification of the Federation of European Laboratory Animal Science Associations recommendation.

**BM transplantation**

BM transplantation was performed as previously described (9). In brief, BM cells were obtained by flushing tibias and femora with RPMI-1640/10% FCS. The resulting cell suspension was filtered, enriched for mononuclear cells by centrifugation on NycoPrep Animal, density 1.077, washed and re-suspended in PBS. Rats were sub-lethally irradiated with 10 Gy from a Cobalt-60 source (Mobiltron 80, TEM) at a dose rate of ~0.4 Gy min\textsuperscript{−1} 1–2 h later, rats were injected intravenously with 26–38 × 10\textsuperscript{6} PVG.7B BM cells in 1 ml PBS. As judged by flow cytometry, <3% of the BM cells were CD3\textsuperscript{+} T cells.

**Flow cytometry and mAbs**

A total of 1–10 × 10\textsuperscript{5} cells were labeled with different combinations of the following conjugated mAbs: FITC-conjugated HIS41 (anti-CD45 variant RT7.2 (22), kindly provided by Dr J. Kampinga, University of Groningen, The Netherlands), G4.18 (anti-CD3) or 3.2.3 (anti-NKR-P1A (23)); PE-conjugated G4.18 or 10/78 (anti-NKR-P1A; both from BD Pharmingen); Alexa 647-conjugated STOK2 [anti-Ly49i2 (17)] and biotinylated STOK2, Fly5 [anti-Ly49s5/-i5 (15)] and Dar13 [anti-Ly49s3/-s4/-i3/-i4 (13)] followed by RPE-Cy5- or PerCP-conjugated streptavidin. The RT7.2 alloantigen is expressed by the PVG.7B, but not the PVG or PVG.1AV1 strains.

**Generation of effector cells and cytotoxicity assay**

IL-2-activated NK cells were generated from NKR-P1A\textsuperscript{+} or Ly49i2\textsuperscript{+} mononuclear splenocytes as previously described (10, 17). The generation of Con A-activated lymphoblast target cells and the 4 h \textsuperscript{51}Cr release assay was performed as previously described (24). Ten micrograms per well of purified mAbs STOK2 or the isotype control mAb 2C7 (rat IgG2a) was added to plated effector cells 20–30 min before the addition of target cells. Spontaneous release was usually between 5 and 15% of the total cpm in the cells. Results are presented as mean values from triplicates for each E:T cell ratio; error bars represent one SD (GraphPad Prism software, San Diego, CA, USA).

**In vivo ALC assay and intracellular IFN-\gamma staining**

Determination of ALC in vivo was performed as previously detailed (24, 25). In short, mesenteric and cervical lymph node (LN) cells from PVG.1AV1 rats were labeled at 10–15 × 10\textsuperscript{6} cells ml\textsuperscript{−1} with 0.4 MBq ml\textsuperscript{−1} Na\textsuperscript{35}CrO\textsubscript{4}, washed and injected (10–15 × 10\textsuperscript{6} cells per rat) intravenously into allogeneic BM chimeric rats (PVG.7B→PVG.1AV1) or their transplanted control rats (PVG.7B→PVG) or to untransplanted syngeneic PVG.1AV1 recipients. Radioactivity associated...
Results

All NK cells are donor derived in allogeneic PVG.7B→PVG.1AV1 BM chimeras

Non-lethally irradiated PVG.1AV1 strain rats (RT1av1; MHC haplotype) were transplanted with 30 × 10^6 fully MHC-mismatched BM cells from PVG.7B rats (RT1f); MHC-identical PVG control rats (RT1c) were transplanted in parallel. PVG.7B expresses a non-immunogenic variant of the CD45 marker (RT1.2) detected by mAb HIS41, but PVG.7B is otherwise used interchangeably with PVG (22). Transplanted rats were analyzed after 5–6 months. In the PVG.7B→PVG.1AV1 allogeneic chimeras, we observed a complete donor cell hematopoietic chimerism as judged by HIS41 staining. In both spleen and LNs were >99% of the mononuclear cells RT7.2⁺ (Fig. 1A and data not shown). It was difficult to ascertain whether the few remaining RT7.2⁺ cells represented residual hematopoietic host cells, non-hematopoietic cells or more immature donor-derived cells. The observation of a significant fraction (~20%) of RT7.2⁺ cells in the BM of allogeneic chimeras, being of donor origin as determined by staining with an RT7.2⁺ specific mAb (data not shown), argued for the last alternative. Occurrence of chronic graft versus host (GVH) disease in several of the allogeneic chimeras, with gross enlargement of mesenteric LNs and wasting, also suggested complete donor hematopoietic chimerism. In parallel experiments, manifestation of GVH disease correlated with conversion from mixed to complete donor cell chimerism (30). Finally, at the level of mature NK cells, there were no RT7.2⁺ cells among NKR-P1A⁺ NK cells and all the cells were RT7.2⁺ and hence of donor origin (Fig. 1B).

In the PVG.7B→PVG control chimeras, mixed chimerism was observed. In the spleen, ~95% of the mononuclear splenocytes were RT7.2⁺ and there was a significant fraction of RT7.2⁺ cells (Fig. 1A). Among NKR-P1A⁺ cells, ~96% were RT7.2⁺, the remaining 4% being RT7.2⁻ host-derived cells (Fig. 1B).

NK cells in PVG.7B→PVG.1AV1 chimeras are partially tolerant toward RT1av1 host cells both in vivo and in vitro

NK cells are responsible for the prompt rejection of MHC-mismatched allogeneic lymphocytes in non-sensitized recipients. This rejection phenomenon has been termed allogeneic lymphocyte cytotoxicity or ALC. It has been well documented that RT1av1-expressing lymphocytes such as PVG.1AV1 are rapidly eliminated in PVG strain (RT1f) recipients (25, 26, 31, 32). We tested for tolerance induction of donor-derived PVG.7B NK cells by RT1av1-expressing radio-resistant host cells. PVG.1AV1 LN cells were labeled with ^51^Cr and injected intravenously. After 20 h, radioactivity was measured in cervical LNs of chimeras and compared with untransplanted syngeneic recipients and the results were expressed as an LN index. A low value is a reliable measure of acute rejection and reflects the failure of injected lymphocytes to recirculate back to LNs. In the PVG.7B→PVG control chimeras, we observed a low LN index (<0.15; Table 1). This is in line with previous observations for this donor-recipient strain combination. By contrast, in the PVG.7B→PVG.1AV1 allogeneic chimeras, the LN index was higher (>0.5), which indicated that the majority of the injected PVG.1AV1 lymphocytes had survived and reached the LNs. The fact that the LN index was <1.0, which is the normalized value for syngeneic recipients, suggested that the donor-derived PVG.7B NK cells were not fully tolerant to RT1av1 cells but were weakly host reactive.

Splenic NK cells from individual chimeras were isolated and expanded by IL-2 culture. Flow cytometric analysis confirmed that all IL-2-activated NK cells from allogeneic chimeras were RT7.2⁺ and donor derived (data not shown). Their reactivity against host Con A lymphoblast targets was tested.

Fig. 1. Mononuclear splenocytes are donor derived in allogeneic PVG.7B→PVG.1AV1 BM chimeras. (A) Mononuclear splenocytes from allogeneic (PVG.7B→PVG.1AV1) or control (PVG.7B→PVG) chimeras were stained with mAb HIS41 reacting with the donor CD45 allotype marker RT7.2. More than 99% of the splenocytes were HIS41⁺ and of donor origin in the allogeneic chimeras, while there was a distinct population of HIS41⁻ host-derived cells in the control chimeras. (B) Two-color staining with mAb HIS41 and 10/78 (anti-NKR-P1A) showed complete donor cell chimerism of NKR-P1A⁺ NK cells in allogeneic, but not control chimeras.
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in vitro. RT1<sup>av</sup> targets were lysed by NK cells from all the chimeras, but cytotoxicity was lower for the allogeneic compared with the control chimeras (Fig. 2, left panel). RT1<sup>c</sup> targets of the donor MHC type, on the other hand, were spared (Fig. 2, right panel). These data suggested that the MHC constitution of radioresistant host cells could dampen the ability of donor-derived newly developed NK cells to respond to the host and that this effect could not be reversed by IL-2 activation in vitro.

Tolerance toward host RT1<sup>av</sup> targets is extended to the alloreactive Ly49<sup>i2</sup> NK cell subset in allogeneic chimeras

Ly49<sup>i2</sup> is an inhibitory receptor for the donor classical class Ia molecule, RT1-A<sup>c</sup> (18, 19). Notably, in donor PVG.7B/Ly49<sup>i2</sup><sup>+</sup> NK cells from allogeneic PVG.7B<sup>/</sup>PVG.1AV1 or PVG.7B<sup>/</sup>PVG chimeras but considerably enhanced compared with NK cells from untransplanted PVG.1AV1 rats (Fig. 3, upper left panels). Third-party RT1<sup>av</sup> targets from PVG.1U were efficiently lysed by NK cells from both allogeneic and control chimeras but with a slight reduction in reactivity for the allogeneic chimeras (Fig. 3, upper right panels). The somewhat reduced anti-RT1<sup>c</sup> reactivity of NK cells from RT1<sup>av</sup> rats is consistent with the MHC modulatory effects on NK alloreactivity observed in a series of MHC congenic strains (10, 33). Donor RT1<sup>c</sup> targets were spared (Fig. 3, bottom panels, open symbols).

<table>
<thead>
<tr>
<th>Rat no.</th>
<th>LN index</th>
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<tr>
<td>PVG.7B → PVG.1AV1 chimeras</td>
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</tr>
<tr>
<td>PVG.7B → PVG chimeras</td>
<td>0.62</td>
</tr>
<tr>
<td>PVG.7B → PVG.1AV1 chimeras</td>
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<tr>
<td>PVG.7B → PVG chimeras</td>
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<tr>
<td>PVG.7B → PVG chimeras</td>
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</tr>
<tr>
<td>PVG.7B → PVG.1AV1 chimeras</td>
<td>0.57</td>
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<tr>
<td>PVG.7B → PVG chimeras</td>
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Table 1. MHC allogeneic PVG.7B→PVG.1AV1 chimeras display reduced NK-mediated rejection of PVG.1AV1 lymphoblast target cells in vivo

The functional MHC repertoire of Ly49<i>i2</i> NK cells is altered in allogeneic chimeras

We next tested whether the reduced alloreactivity of Ly49<i>i2</i> NK cells from allogeneic PVG.7B→PVG.1AV1 chimeras reflected a change in their functional MHC receptor repertoire. Previous studies have suggested that a class Ib locus in PVG.7B→PVG.1AV1 chimeras selects for a second unidentified receptor that is cross-reactive with a class Ib ligand in the RT1<sup>c</sup> haplotype and able to prevent lysis of RT1<sup>c</sup> lymphoblast targets in the presence of the anti-Ly49<i>i2</i> mAb STOK2 (18). In line with previous studies, Ly49<i>i2</i> NK cells from PVG.7B→PVG control chimeras efficiently lysed RT1<sup>c</sup> targets upon addition of mAb STOK2, confirming that the Ly49<i>i2</i> receptor is important for self-tolerance in PVG strain rats. In contrast, addition of mAb STOK2 to PVG.7B→PVG.1AV1 NK cells was much less efficient in inducing lysis of PVG.7B targets (Fig. 3, bottom panels), corroborating the previous conclusion that protection of lysis of RT1<sup>c</sup> targets by Ly49<i>i2</i> NK cells from RT1<sup>av</sup> rats is not solely dependent on inhibitory effects mediated by Ly49<i>i2</i> but also by the aforementioned RT1<sup>c</sup>-reactive second receptor (18). The present data in addition suggest that selection of this receptor can be effectively mediated by radioresistant cells in the RT1<sup>av</sup> host.

Modest increase in cell surface level of Ly49<i>i2</i> in allogeneic chimeras

To directly study Ly49<i>i2</i> receptor expression in the chimeras, we isolated splenic NK cells ex vivo and investigated the percentage of Ly49<i>i2</i> NK cells and expression levels of this receptor. The Ly49<i>i2</i> NK subset had expanded somewhat in the PVG.7B→PVG.1AV1 chimeras compared with the PVG.7B→PVG control chimeras (data not shown), consistent with the positive effect of the RT1<sup>av</sup> haplotype in selecting for these cells (18). Receptor expression on individual NK cells was also increased (Fig. 4). This could be related to the absence of the RT1-A<sup>c</sup> ligand in the radioresistant host, which specifically down-regulates Ly49<i>i2</i> surface levels (18). The data are in
agreement with earlier studies showing that non-hematopoietic elements play a role in regulation of Ly49 receptors (34). MHC molecules on hematopoietic cells are apparently also important, as Ly49i2 levels were still markedly lower in the allogeneic chimeras than in PVG.1AV1 control rats (40%; Fig. 4) (35).

The Ly49i2+ NK cell subset is not hyporesponsive in RT1-A1c-negative strains

Finally, we wanted to ascertain that the altered MHC repertoire of Ly49i2+ NK cells in the allogeneic chimeras was not due to a general state of hyporesponsiveness induced by the lack of RT1-A1c in the host compartment. Ly49i2+ NK cells from RT1-A1c-positive (PVG.7B) and -negative (PVG.1U) strains showed comparable IFN-γ responses upon overnight stimulation with anti-NKP46 mAb or IL-12 in combination with IL-2, suggesting that they were fully functional (Fig. 5A and data not shown). This contrasted with the reduced IFN-γ-producing capacity of a minor subset of Ly49s3+ NKR-P1B+ NK cells (data not shown) (28). From the results below, it can be deduced that the hyporesponsive cells were Ly49i2- as all Ly49i2+ cells co-expressed Ly49s3.

We have previously shown that Ly49 expression is focused to a subset of rat NK cells expressing the Ly49s3 receptor (as judged by two different mAbs, Dar13 reacting with Ly49s3, -s4, -i3 and -i4 and STOK6 specific for Ly49s3). The alloreactive responsiveness is also confined to the Ly49s3 NK subset (13, 16). As shown in Fig. 5(B), co-expression of Ly49s3 did not vary between the three strains tested; virtually all Ly49i2+ NK cells expressed this receptor.

The Ly49s5/i5 receptors are reactive with a class Ib ligand in the RT1u haplotype and are detected by mAb Fly5 (15). Co-expression of Ly49s5/i5 by Ly49i2+ NK cells was comparable in PVG.7B and PVG.23 rats, the latter strain expressing RT1av1 in the MHC class Ib (RT1-CE) and RT1u in the class Ia (RT1-A) region. The percentage of Ly49s5/i5+ cells was more than three times higher than among all NKR-P1A+ NK cells both in PVG.7B (54 versus 16%) and PVG.23 (64 versus 20%) (Fig. 5C and data not shown). The increased frequency of Ly49s5 expression explained the enrichment of anti-RT1u alloreactivity within the Ly49i2+ subset (18). By contrast, in PVG.1U rats, co-expression of Ly49s5/i5 was only observed in ~10% of the Ly49i2+ cells (as compared with 5.3% among all NK cells). It could be

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Fig. 3. Host reactivity of the highly alloreactive Ly49i2 NK subset is dampened in allogeneic chimeras, in which its inhibitory ligand (RT1-A1c) is lacking on host stromal cells. Individual cultures of Ly49i2+ NK cells, constituting a minor highly alloreactive donor NK subset, were generated from six allogeneic (PVG.7B -> PVG.1AV1) and four control (PVG.7B -> PVG) chimeras. Cytolytic activity against RT1av1 (PVG.1AV1), RT1u (PVG.1U) and RT1c (PVG.7B) Con A blasts was tested in two separate experiments (Exp.1 and Exp. 2). IL-2-activated Ly49i2+ NK cells from untransplanted PVG.1AV1 rats were included as a negative control in the two upper left panels. In the two bottom panels, we added blocking quantities of mAb STOK2 (filled symbols) or an isotype control mAb (2C7; open symbols) to the cytotoxicity assays.

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seen as a useful adaptation of the Ly49 recognition repertoire to the RT1u environment to down-regulate Ly49s5 expression, which would otherwise render these cells potentially autoreactive (18).

**Discussion**

The MHC constitution has a profound effect on the shaping of a functional NK cell repertoire, including the ability to respond to allogeneic cells. We have studied the influence of radioresistant host cells on the function of donor-derived NK cells in a fully MHC-mismatched allo-SCT model. We focused on an informative NK cell subset (Ly49i2⁺), which is tolerized by a class Ia ligand in the donor strain and displays high alloreactivity against host strain cells. The data show that radioresistant host cells have an important and permanent tolerogenic effect on the development of a functional NK repertoire in vivo, which is not reversible by IL-2 culture.

Transplantation was performed across a full MHC mismatch (RT1c⁺/RT1av1⁻), which is associated with a strong NK cell-mediated alloresponse in the GvH, but not HvG direction (10). This MHC mismatch did not provoke acute lethal GvH in the transplanted rats. This could possibly be due to the relative paucity of T cells (<3%) in the BM graft in combination with important NK-mediated immunomodulatory effects, such as elimination of host antigen-presentation cells by donor NK cells (36).

Early studies in mice have suggested that immature NK cells require an intact BM microenvironment to acquire their full cytotoxic potential (37, 38). Other studies have shown that non-hematopoietic cells play an important role for NK cell tolerance toward class I deficient (8) and parental cells in an F₁ hybrid system (7). This notion was substantiated by the finding that Ly49 expression is dependent on contact between NK precursors and bone marrow-derived stromal cells in vitro (39, 40). Stromal cells probably provide signals required for both initiation of Ly49 expression and efficient clonal growth of NK cells. These studies also showed that stromal cell MHC class I expression play a major role in the shaping of the Ly49 receptor repertoire.

Our studies in the rat indicate that radioresistant stromal cells can have an important permanent influence on the NK cell allorepertoire in vivo. Donor-derived NK cells in the PVG.7B→PVG.1AV1 chimeras changed their functional characteristics toward NK cells of the host strain, despite the fact that the hematopoietic compartment was of donor origin. The observed effects included partial tolerance to PVG.1AV1 host cells and somewhat reduced killing of third-party PVG.1U targets. These effects were permanent as they were observed in stable long-term chimeras in vivo. They could be reproduced in vitro following cultures of NK cells with IL-2 for up to 2 weeks, in which all cells were of donor origin. It is unlikely that the whole bulk of NK cells are rendered

**Fig. 4.** Increased Ly49i2 surface expression on donor-derived PVG.7B NK cells in allogeneic chimeras, which do not express the inhibitory ligand for Ly49i2 (RT1-A¹) on host stromal cells. Ly49i2 surface expression level on NK cells was determined by three-color flow cytometry from six allogeneic (PVG.7B→PVG.1AV1) and four control (PVG.7B→PVG) chimeras, as well as from untransplanted PVG.1AV1 and PVG rats. Freshly isolated mononuclear splenocytes were co-stained with mAbs against NKR-P1A (3.2.3), CD3 (G4.18) and Ly49i2 (STOK2). The mean fluorescent channel value for STOK2 staining of NKR-P1A⁺CD3⁻ cells was taken as a measure for Ly49i2 expression level. Normalized pooled data are presented, expressed as the relative levels observed for PVG.1AV1 NK cells known to express the highest surface levels of Ly49i2.

**Fig. 5.** IFN-γ response and expression of Ly49 alloactivation markers by Ly49i2⁺ NK cells in RT1-A¹ ligand-negative rats. (A) Ex vivo isolated splenocytes from the RT1-A¹-positive PVG.7B strain and RT1-A¹-negative PVG.R23 and PVG.1U strains were stimulated overnight with IL-2 + IL-12 or IL-2 as a control (Ctr.). Ly49i2⁺ NK cells, gated for CD3-NKR-P1A⁺ cells, were analyzed for intracellular IFN-γ expression by flow cytometry. Ly49i2⁺ NK cells were analyzed for co-expression of Ly49s3/-s4/-i3/-i4 by mAb Dar13 (B) and Ly49s5/-i5 by mAb Fly5 (C) by multicolor flow cytometry.
hyporesponsive in the allogeneic chimeras. If so, this would imply a dominant and global dampening effect mediated by radioresistant host elements, overriding the influence of the hematopoietic compartment, which was of donor origin. It is also unlikely that the mild chronic graft versus host disease symptoms observed for some of the allogeneic chimeras could explain the observed effects.

Direct evidence that the donor NK cells adapted their receptor repertoires to the non-hematopoietic host compartment was obtained by studies of the informative and highly alloreactive Ly49i2+ NK subset. In addition to partial tolerance to donor-derived targets and a slightly reduced killing of third-party cells, we observed modulatory effects on Ly49i2 surface expression and of co-expressed Ly49 receptors, as well as evidence for co-selection of a second inhibitory receptor cross-reactive with both RT1\(^{b\{v\}}\) and RT1\(^{c}\) cells. We have previously provided evidence that this receptor is selected for by a class Ib product in PVG.1AV1 rats (18). Although we have not yet identified this receptor on Ly49i2+ NK cells, we have recently characterized a pair of candidate Ly49 receptors, which react with a conserved class Ib ligand expressed in RT1\(^{b\{v\}}\) and RT1\(^{c}\) and related haplotypes (K.-Z. Dai, C. Naper and J.T. Vaage, unpublished results). We have also previously shown that the Ly49i2 receptor can provide specific protection of xenogeneic mouse Ltk cells transfected with RT1-A1\(^{c}\); i.e. in the absence of other rat class I molecules, and this protection was effectively reverted by addition of the anti-Ly49i2 mAb STOK2 (18, 19).

These data showed that the Ly49i2 receptor is fully functional in ligand-negative strains, again arguing against the hyporesponsiveness hypothesis. Further support for this was obtained in the present study, which showed a normal IFN-\(\gamma\) response in two RT1-A1\(^{c}\)-negative strains (Fig. 5A), and of our previous observation of a normal capacity of IL-2-activated Ly49i2+ NK cells to lyse a panel of tumor cells in the same strains (18).

Cell surface levels of Ly49i2 was somewhat increased in the allogeneic chimeras, even in the presence of its ligand RT1-A1\(^{c}\) on hematopoietic cells. This suggests that Ly49i2 is subject to calibration by its ligand on stromal cells and that the absence of RT1-A1\(^{c}\) on stromal cells permits a higher Ly49i2 expression level. It cannot be excluded that this effect is secondary to other changes in the Ly49 receptor repertoire alluded to above induced by allogeneic MHC. Attempts to determine the relative contribution of cis- and trans- interactions in RT1-A1\(^{c}\)-mediated down-regulation of Ly49i2 was not conclusive, however, as acid treatment of cells failed to increase receptor levels as previously observed for mouse Ly49 receptors (41), despite that MHC class I expression was lost as a result of the treatment (data not shown).

Although NK cells in the PVG.7B→PVG.1AV1 chimeras partially changed their characteristics toward the host phenotype, they still behaved more like donor NK cells. This indicates that donor-derived hematopoietic cells play a major role in shaping the NK cell repertoire. Full NK cell tolerance was not achieved in the allogeneic chimeras, in line with a previous study showing that rat NK cells are not easily tolerized (42). The ability of donor NK cells to partially adapt to the MHC environment in the radioresistant host following allo-SCT can be of importance in clinical settings, such as the tailoring of a sustained NK-mediated Graft-versus-Leukemia effect without risking detrimental GVH effects.

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Partial NK tolerance in BM allotransplanted rats


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