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Dissecting NK Cell Development Using a Novel A lymphoid Mouse Model: Investigating the Role of the c-abl Proto-Oncogene in Murine NK Cell Differentiation

Francesco Colucci,* Claire Soudais,* Eleftheria Rosmaraki,* Lesley Vanes,† Victor L. J. Tybulewicz,† and James P. Di Santo*‡

NK lymphocytes participate in both innate and adaptive immunity by their prompt secretion of cytokines including IFN-γ, which activates macrophages, and by their ability to lyse virally infected cells and tumor cells without prior sensitization. Although these characteristics of NK cells are well documented, little is known about the genetic program that orchestrates NK development or the signaling pathways that trigger NK effector functions. By crossing NK-deficient common γ-chain (γc) and recombinase activating gene (RAG)-2 mutant mice, we have generated a novel alymphoid (B−, T−, and NK−) mouse strain (RAG2/γc) suitable for NK complementation in vivo. The role of the c-abl proto-oncogene in murine NK cell differentiation has been addressed in hemopoietic chimeras generated using RAG2/γc mice reconstituted with c-abl+/− fetal liver cells. The phenotypically mature NK cells that developed in the absence of c-abl were capable of lysing tumor targets, recognizing “missing self,” and performing Ab-dependent cellular cytotoxicity. Taken together, these results exclude any essential role for c-abl in murine NK cell differentiation in vivo. The RAG2/γc model thereby provides a novel approach to establish a genetic map of NK cell development. The Journal of Immunology, 1999, 162: 2761–2765.

The c-abl proto-oncogene is the cellular homologue of the Abelson leukemia virus. c-abl encodes a highly conserved (1) intracellular protein tyrosine kinase having both DNA- (2) and actin-binding domains (3). Targets of the c-Abl kinase include the Rb gene product, RNA polymerase II, Crk, and the JNK pathway (4), and, as such, c-Abl has been proposed to control cell growth (5, 6), although it is not clear whether it acts in a positive or negative fashion (7). c-abl is expressed ubiquitously and throughout development, but is more abundantly expressed in the spleen, thymus, and testes (8). Mice deficient in c-abl develop to the perinatal period but generally fail to thrive, runt, and die around 3 wk of age (9, 10). Although the mechanism underlying the severe phenotype of c-abl mutant mice has not been elucidated, an effect of c-abl deficiency on lymphoid development has been suspected, because c-abl+/− mice develop a generalized lymphopenia and appear highly susceptible to infections (9–12). While mature T and B cells in c-abl+/− mice appear functional (9–12), a defect in NK cell differentiation could predispose c-abl+/− mice to infection. Mature, functional NK cells first appear in the mouse at 3 wk of age (13, 14), coincident with the demise of most c-abl+/− mice. As such, any potential role of c-abl in NK cell physiology and in the increased susceptibility of c-abl+/− mice to pathogens could not be addressed.

The recombinase activating gene (RAG)3-2 blastocyst complementation system introduced by Alt and colleagues has proved instrumental in defining the potential role in T and B cell development of genes that give rise to embryonic lethality (15, 16). Therefore, this approach can discriminate cell-intrinsic defects from in trans effects for any given mutation. Nevertheless, this system cannot be used to study gene effects on NK development, because RAG2-deficient mice develop NK cells (17), thereby precluding the possibility of studying the donor-derived NK cells in the absence of competing host-derived cells. While other mouse models of NK cell deficiency are available, they are not satisfactory because they either rely on temporary Ab depletion of pre-existing NK cells (18) or they are associated with major defects in lymphopoiesis, lymphoid homeostasis, or autoimmunity (19–21).

We have developed a novel alymphoid mouse strain that combines the NK-deficiency found in common γ-chain (γc); a shared component of the receptors for IL-2, -4, -7, -9, and -15) mutant mice with the T and B cell block due to mutation in the RAG2 gene (RAG2/γc double mutant mice). RAG2/γc mice offer certain advantages over RAG2 mice for studies involving lymphoid reconstitution and are well suited for the in vivo study of NK cell differentiation. In this report, we demonstrate the feasibility and utility of the RAG2/γc mouse model by examining the role of the c-abl proto-oncogene in NK cell development.

Materials and Methods

Mice

Mice with a null mutation in the γc (20) were from the fourth generation backcross to the C57BL/6 background. RAG2 mice (17) were from the tenth generation backcross to the C57BL/6 background. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used in this paper: RAG, recombinase activating gene; ADCC, Ab-dependent cellular cytotoxicity; A-LAK, adherent lymphokine-activated killer; BM, bone marrow; γc, common cytokine receptor γ-chain; FL, fetal liver; HSC, hemopoietic stem cell; NWNA, nylon wool nonadherent.

Abbreviations used in this paper: RAG, recombinase activating gene; ADCC, Ab-dependent cellular cytotoxicity; A-LAK, adherent lymphokine-activated killer; BM, bone marrow; γc, common cytokine receptor γ-chain; FL, fetal liver; HSC, hemopoietic stem cell; NWNA, nylon wool nonadherent.
Table I. Lymphoid cellularity in reconstituted RAG2/γc mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>BM Cell No. (×10⁶)</th>
<th>Lymphoid Cells (range in %)</th>
<th>Thymus Cell No. (×10⁶)</th>
<th>Lymphoid Cells (range in %)</th>
<th>Spleen Cell No. (×10⁶)</th>
<th>Lymphoid Cells (range in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>12.5 ± 1.6 (n = 4)</td>
<td>25–30</td>
<td>144 ± 22 (n = 5)</td>
<td>81–86</td>
<td>89.2 ± 11 (n = 6)</td>
<td>67–74</td>
</tr>
<tr>
<td>RAG2/γc, c-abl+</td>
<td>11.9 ± 1.4 (n = 4)</td>
<td>13–27</td>
<td>46.3 ± 28 (n = 6)</td>
<td>81–84</td>
<td>91.0 ± 23 (n = 6)</td>
<td>64–70</td>
</tr>
<tr>
<td>RAG2/γc, c-abl−/−</td>
<td>10.4 ± 2.4 (n = 4)</td>
<td>14–30</td>
<td>58.1 ± 40 (n = 4)</td>
<td>79–85</td>
<td>84.2 ± 15 (n = 6)</td>
<td>62–71</td>
</tr>
<tr>
<td>RAG2/γc</td>
<td>7.7 ± 0.5 (n = 4)</td>
<td>10–15</td>
<td>2.3 ± 0.4 (n = 5)</td>
<td>30–50</td>
<td>4.4 ± 1.2 (n = 5)</td>
<td>25–40</td>
</tr>
<tr>
<td>RAG2/γc</td>
<td>7.2 ± 0.3 (n = 4)</td>
<td>2–10</td>
<td>0.04 ± 0.01 (n = 5)</td>
<td>5–20</td>
<td>1.2 ± 0.1 (n = 4)</td>
<td>10–20</td>
</tr>
</tbody>
</table>

* Red cell-depleted cell suspensions were enumerated from each organ of the indicated mice, and the percentages of lymphoid cells were measured by flow cytometry on the basis of forward and side scatter parameters.
mice (10,000-fold fewer thymocytes than controls). The effect of the γc mutation on the development of these early lymphoid precursors likely reflects their requirement for IL-7/IL-7Rα/γc signaling for survival (reviewed in Ref. 24). As expected, no mature IgM⁺ B cells or αβ⁺ T cells were found in mice harboring the RAG2 mutation (Fig. 1C). In addition, RAG2/γc mice completely lacked NK1.1⁺ cells in the BM (Fig. 1B) and spleen (data not shown). The presence of functional NK cells lacking the NK1.1 marker in RAG2/γc mice appeared unlikely based on the following criteria: 1) splenocytes from RAG2/γc mice show no natural cytotoxicity against YAC-1 targets in vitro at E:T ratios of 300:1; 2) RAG2/γc mice fail to augment IFN-γ blood levels following administration of murine IL-12 in vivo, and 3) RAG2/γc mice fail to reject allogeneic BM grafts or MHC class I-deficient tumors in vivo (data not shown). In contrast, RAG2 mice retain these three NK cell effector functions (Ref. 17 and data not shown). Taken together, these results demonstrate that RAG2/γc mice have no mature B, T, or NK cells and are therefore a suitable host for in vivo NK complementation.

Lymphoid reconstitution of RAG/γc mice using c-abl⁻/⁻ HSC
We hypothesized that a defect in NK cell function could contribute to the increased susceptibility of c-abl⁻/⁻ mice to infections (9, 10). Most c-abl⁻/⁻ mice succumb at 3 wk of age, which is coincident with the first appearance of functional NK cells in the mouse (13, 14). To investigate the role of c-abl in NK development, we generated hemopoietic chimeras in irradiated (0.3 Gy) RAG2/γc mice using FL cells from c-abl⁺ or c-abl⁻/⁻ embryos. Serial blood sampling demonstrated lymphoid reconstitution beginning at 4 wk posttransfer, which normalized by 8 wk posttransfer (data not shown). At this point, chimeras were sacrificed, and lymphoid development was assessed in the BM, thymus, spleen, and liver. In chimeras injected with c-abl⁻ FL cells, full lymphoid reconstitution was observed. Normal absolute numbers of thymic and splenic lymphocytes were found, representing increases of 100- to 10,000-fold compared with nonmanipulated RAG2/γc mice (Table I). The appearance of mature T and B cells in the chimeras (Fig. 2) demonstrated that donor-derived precursors could fully differentiate in this setting, and the donor origin of the resultant NK cells was confirmed by staining with anti-γc mAb (Fig. 3C). Thus, all mature lymphoid subsets (B, T, and NK) could be generated from wild-type FL cells following injection into irradiated RAG2/γc mice. These results demonstrate that the lymphoid defects in RAG2/γc mice are cell-intrinsic and that expression of the γc in BM stromal or gut epithelial cells is not required for normal lymphoid development.

The effect of c-abl-deficiency on B, T, and NK cell development in RAG2/γc chimeras was then assessed. The absolute numbers of lymphoid cells in the BM, thymus, and spleens of c-abl⁻/⁻ chimeras were similar to that of control chimeras (Table I). Moreover, no obvious differences in the development of phenotypically defined lymphoid subsets could be discerned between the two groups of chimeras. Similar percentages and absolute numbers of NK1.1⁺ CD3⁻ NK cells, CD4⁺ or CD8⁺ αβ⁺ T cells, γδ T cells, NK1.1⁺ αβ⁺ T cells, B220⁺ IgM⁺ B cells, and B220⁺ IgM⁻ pre-B cells were found in the lymphoid organs of c-abl⁻/⁻ and control RAG2/γc chimeras (Fig. 2, A and B, Fig. 3, A and B, and data not shown). Moreover, the absolute numbers of most lymphocyte subsets were also comparable in chimeric animals to normal C57BL/6 mice, although the development of thymic and peripheral NK1.1⁺ αβ⁻ T cells were slightly reduced in both c-abl⁻/⁻ and control chimeras (Fig. 3B).

 Phenotype and function of c-abl⁻/⁻ NK cells
A more detailed analysis of the NK cells generated in c-abl⁻/⁻ RAG2/γc chimeras was performed. Using a panel of Abs detecting Ags expressed by NK cells, a normal percentage and expression level of CD2, CD11b, CD16, CD45R, DX5, CD122, CD90, and CD117 was found on c-abl⁻/⁻ NK cells (Table I). Moreover, no obvious differences in the expression levels of the inhibitory receptors of the Ly49 family (Ly49A, Ly49C/I, and Ly49G2) and the frequencies of these different Ly49⁺ NK “subsets” in c-abl⁻/⁻ RAG2/γc chimeras was normal (data not shown).

The lytic capacity of freshly isolated splenic c-abl⁻/⁻ NK cells was tested in vitro. c-abl-deficient NWWA spleen cells demonstrated normal levels of natural cytotoxicity against YAC-1 thymoma targets (Fig. 4A). Day 8–10 A-LAK cells also mediated efficient lysis of YAC-1 targets, as well as Ab-mediated cell cytotoxicity against Ab-coated EL-4 cells (Fig. 4, B and C). Moreover, both c-abl⁺ and c-abl⁻/⁻ A-LAK cells could discriminate between class I-negative and class I-positive ConA-activated
In contrast, NK cells from both c-cells have abnormal responses to mitogenic stimulation in vitro (12). Previous studies have demonstrated that c-ABL-deficient T and B cells have abnormal responses to mitogenic stimulation in vitro (12). In contrast, NK cells from both c-ABL-/- and c-ABL+ RAG2/γc chimeras were capable of in vitro expansion in response to IL-15 in addition, the A-LAK cultures generated in this fashion were IL-12 responsive (data not shown), ruling out any essential role of c-ABL in the mitogenic responses of NK cells to IL-12 or IL-15.

To investigate in vivo NK cell function, we examined whether c-ABL RAG2/γc chimeric animals could eliminate MHC class I-negative tumor cells. Mice were injected s.c. with Tap1-deficient (RMA-S) cells (26), and tumor formation was monitored. Both c-ABL-/- and c-ABL+/+ RAG2/γc chimeras were able to control growth of MHC class I+ tumor cells (data not shown), demonstrating normal NK lytic activity in vivo.

Discussion

In this report, we describe a novel aplymphoid mouse strain harboring the RAG2 and γc mutations (RAG2/γc). Although a number of immunodeficient mouse models exist (such as beige, nude, scid, Rag, xid, and combinations thereof), RAG2/γc mice offer considerable advantages over these strains including 1) a complete absence of mature T, B, and NK cells, 2) a stable immunophenotype, 3) no increased propensity to spontaneous tumor formation, and 4) no autoimmune phenomena due to defective lymphoid homeostasis. The aplymphoid nature of RAG2/γc mice will permit the construction of mice with defined immune systems, which should prove useful in further defining the role of different lymphoid subsets (NK, NK-T, γδ T, and αβ T cells) during immune responses to infectious pathogens. The immunodeficiency in RAG2/γc mice permits stable hematopoietic engraftment (using either FL or adult BM HSCs) across classical histocompatibility barriers and without irradiation (our unpublished observations). We have recently found that RAG2/γc mice accept human PBL xenografts to a similar extent as nonobese diabetic/SCID mice (27). Thus, RAG2/γc mice should be useful for a number of applications in lymphoid development, immune responses, tumor immunology, and xenotransplantation.

Alt and colleagues revolutionized the analysis of genes involved in T and B cell development by introducing the RAG2 blastocyst complementation system (15). This powerful technique has been used extensively to study the function of "embryonic lethal" genes in the immune system (reviewed in Ref. 16). Despite this major advance, RAG2 complementation cannot be used to study NK differentiation (because RAG-deficient mice have NK cells; Ref. 17) or early lymphoid precursors (as they develop normally in the absence of RAG genes). The early precursors in RAG2 mice in principle could compete with the mutant donor cells, thereby blocking their development and giving the impression that a given gene is essential for T and/or B cell development. Because RAG2/γc mice are also severely depleted in T and B lymphocyte precursors compared with their RAG2 counterparts (Fig. 1), they should offer less competition at these early stages of lymphoid development. Consistent with this hypothesis, we have recently found that c-kit deficient (W+W) FL cells can give rise to normal numbers of T lymphocytes when grafted in RAG2/γc mutant mice (our unpublished observations), whereas the same experiment performed in RAG2-deficient mice failed to generate T lineage cells (28).

In this report, we have used RAG2/γc mice to assess the role of the c-ABL protein tyrosine kinase in NK development. Our results rule out an essential role for c-ABL in NK differentiation in vivo. In the absence of c-ABL, normal numbers of NK cells can develop in a phenotypically normal fashion and acquire a lytic capacity for a variety of targets in vitro and in vivo. The proper expression of inhibitory Ly49 receptors suggests that c-ABL is not required for the calibration of the NK cell repertoire (reviewed in Ref. 25). Moreover, c-ABL-deficient A-LAKs demonstrated natural cytotoxicity and ADCC activities similar to their c-ABL+ counterparts, and c-ABL+/+ NK cells could eliminate MHC class I+ tumor cells in vivo. Therefore, c-ABL-/- NK cells appear normal in their development and in their effector functions.

The nature of the defect in c-ABL mutant mice that causes lymphopenia and that predisposes these mice to infection remains elusive. Because c-ABL expression is ubiquitous, effects of c-ABL deficiency in trans may be difficult to dissociate from cell-intrinsic effects of the mutation. Previous studies using one strain of c-ABL mutant mice showed variable defects in BM B cell development and peripheral lymphocyte function (11). These results suggested that the c-ABL mutation could affect the function of T and B cells, although c-ABL was not required for the development of these cells (9–12). Interestingly, the observed B and T cell defects could be transferred to normal mice by adult BM, but not by FL (11), strongly suggesting an in trans effect. Our results confirm that T and B cells are not strictly dependent on c-ABL expression within fetal hematopoietic cells. Transfer of BM HSCs from adult c-ABL-/- RAG2/γc chimeras to secondary RAG2/γc recipients also generated normal B, T, and NK cell development (our unpublished observations), arguing against a cell-intrinsic difference in c-ABL-/- fetal vs adult HSCs. In addition, pre-B (B220+/IgM-) cells from c-ABL-/- RAG2/γc chimeras could generate mature B cells in vitro (our unpublished observations), in contrast to previous reports using freshly isolated pre-B cells from c-ABL-/- mice (11). A major defect in NK cell functions as responsible for the susceptibility to infection observed in c-ABL mutant mice also appears unlikely. RAG2/γc chimeras generated with c-ABL-deficient FL cells show no increased mortality when housed in conventional animal facilities up to 8 mo postgraft. Taken together, these results argue against any important cell-intrinsic defects of the c-ABL mutation for T, B, and NK cell development.
The complete absence of NK cells in RAG2/γc mice extends the RAG2 complementation system (16) to identify the genes responsible for and implicated in NK differentiation. Through the generation of somatic or hemopoietic chimeras in RAG2/γc mice, it should now be possible to establish the genetic map for the development of the NK cell lineage in vivo.

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