Natural Killer Cells from Patients with Recombinase-Activating Gene and Non-Homologous End Joining Gene Defects Comprise a Higher Frequency of CD56bright NKG2A+++ Cells, and Yet Display Increased Degranulation and Higher Perforin Content

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Mutations of the recombinase-activating genes 1 and 2 (RAG1 and RAG2) in humans are associated with a broad range of phenotypes. For patients with severe clinical presentation, hematopoietic stem cell transplantation (HSCT) represents the only curative treatment; however, high rates of graft failure and incomplete immune reconstitution have been observed, especially after unconditioned haploidentical transplantation. Studies in mice have shown that Rag<sup>−/−</sup> natural killer (NK) cells have a mature phenotype, reduced fitness, and increased cytotoxicity. We aimed to analyze NK cell phenotype and function in patients with mutations in RAG and in non-homologous end joining (NHEJ) genes. Here, we provide evidence that NK cells from these patients have an immature phenotype, with significant expansion of CD56<sup>bright</sup> CD16<sup>−/low</sup> CD57<sup>−</sup> cells, whereas a further subset expressing the CD56<sup>bright</sup> CD16<sup>−/low</sup> KIR<sup>+</sup> phenotype are thought to represent exhausted NK cells (6, 11). We have previously shown that the CD56<sup>bright</sup> NK cells, expressing CD57 represent terminally differentiated NK cells, whereas a further subset expressing the CD56<sup>−</sup> CD16<sup>+</sup> CD57<sup>+</sup> KIR<sup>+</sup> phenotype are thought to represent exhausted NK cells (12).

RAG1 and RAG2 mutations in humans are associated with a broad spectrum of clinical and immunological phenotypes, including T<sup>+</sup> B<sup>+</sup> severe combined immune deficiency (SCID) (13), Omenn syndrome (OS) (14), atypical SCID (AS) (15–17), and combined immune deficiency with granuloma and/or autoimmunity (CID-G/A) (18–21). We have previously shown that the severity of the clinical and immunological phenotype in patients with RAG mutations correlates with the residual recombination activity of the mutant recombinase-activating gene (RAG) protein.
(22), which may differently affect diversity and composition of T and B cell receptor repertoires (23), whereas NK cell differentiation proceeds unaffected. For patients with severe RAG mutations presenting with SCID, OS, or AS, HSCT represents the only option of definitive cure; however, an increased rate of allograft rejection has been observed as compared to patients with other forms of SCID (24, 25). An important role of NK lymphocytes in mediating rejection of bone marrow allografts has been known for decades (26), but why patients with RAG deficiency would have a higher risk of graft rejection than other forms of NK+ SCID (such as IL7R or CD3 deficiency) remains unknown.

Although RAG genes are not required for NK cell development, data in mice indicate that RAG deficiency affects NK cell phenotype and function. It has been shown that expression of the RAG genes begins in common lymphoid progenitor cells that give rise to T, B, and NK cells (27–29). Studies in mice harboring transgenic reporters for Rag expression or recombinase activity have demonstrated the existence of two populations of mature NK cells: those that have been exposed to transient Rag expression during lymphoid differentiation (here termed as Ragonne) and NK cells that were not previously exposed to Rag (Ragaive NK cells) (30). These two populations differ for their proliferative capacity and interleukin-2 (IL-2)-mediated Stat5 phosphorylation, and a progressive decrease in the proportion of Raggone cells has been observed during NK cell differentiation (29). Furthermore, Ragaive NK cells display an activated phenotype, increased cytotoxicity, and enhanced apoptosis, thereby resulting in poor survival and impaired DNA damage response as compared to their Ragonne counterpart (30). It has been postulated that Rag expression in lymphoid progenitors would favor selection of cells with optimal levels of expression of proteins involved in DNA break repair, including ARTEMIS and DNA ligase 4 (LIG4), thereby marking functionally distinct subsets of NK cells, and providing Ragaive NK cells with improved survival and “fitness.” If confirmed also in humans, the hypothesis that RAG deficiency results in the presence of NK cells with a distinctive phenotype and enhanced cytotoxic potential, would provide novel mechanistic insights to account for the high rate of primary graft failure, incomplete T cell reconstitution, and lack of B cell engraftment that are frequently observed following haploidentical HSCT for RAG deficiency, unless chemotherapy is used (25).

To test this hypothesis, we have analyzed NK cell phenotype and function in a large cohort of patients with mutations in RAG and in other genes involved in non-homologous end joining (NHEJ), presenting with a variable severity of clinical and immunological manifestations, as compared to healthy donors of comparable age, and to patients with other forms of T or B cell lymphopenia.

MATERIALS AND METHODS

Patients and Controls

The patient population consisted of 66 subjects with molecularly confirmed biallelic mutations in gene involved in V(D)J recombination, in particular 35 patients with RAG1, 11 with RAG2, 15 with DCLRE1C (ARTEMIS), 3 with LIG4, and 2 with NHEJ1 (Cernunnos/XLF) mutations. Based on the clinical and immunological phenotype (Table 1), these patients were assigned to the following subgroups: T- B+ NK+ SCID (n = 19), OS (n = 11), AS (n = 13), and delayed onset combined immune deficiency (CID, n = 23). For the purposes of analysis, patients with OS and with AS, were combined into a single group (OS/AS, n = 24).

In addition, 22 patients with various other conditions characterized by numerical and/or functional defects of T lymphocytes served as a control for T cell deficiency. This group of other T cell defects (TCDs) included four patients with CD3G mutations, two patients with Di George syndrome, two patients with IL7R deficiency, and one patient each with mutations in MSN, JAK3, IL2RG, CD3D, CD3E, RMRP, genes, or with MHC class II deficiency. In seven cases with T cell deficiency, the underlying diagnosis was unknown, including two infants with severe T cell lymphopenia diagnosed at birth with low T cell receptor excision circle (TRECs). Based on established criteria (31), 7 of these patients met definition of SCID (with <300 T cells/µL), and the remaining 15 had either less severe numerical TCDs or had functional T cell abnormalities. None of these patients with TCD carried mutations in RAG1/2, DCLRE1C, PRKDC, LIG4, or NHEJ1 genes.

As a control for B cell lymphopenia, nine patients with molecularly confirmed mutation in the BTK gene, causing X-linked agammaglobulinemia (XLA), were also studied.

Finally, 19 healthy infants (age ≤2 years) and 29 healthy subjects of >2 years of age served as normal controls for patients with SCID and OS/AS, or for patients with CID, respectively.

The study was approved by the Institutional Review Boards of Boston Children's Hospital, the National Institute of Allergy and Infectious Diseases (protocols 93-I-0119 and 16-I-N139), and of all other referring centers. Blood samples were obtained upon written informed consent of the subject or, in the case of minors, of their parents or legal guardians. Because of limitations in blood volume and/or in the number of cells, not all analyses could be performed in each individual included in the study.

Analysis of NK Cell Phenotype

Peripheral blood was collected in EDTA- or heparin-containing vacutainers. Red blood cells were lysed from 200 µl of EDTA-blood using RBC lysis buffer (eBioscience). Cells were washed with FACS buffer (PBS containing 2% FBS), then equally distributed in five tubes and incubated for 1 h at 4°C with primary antibodies, washed again with FACS buffer, and incubated for 30 min at 4°C with conjugated antibodies. After washing, cells were re-suspended in FACS buffer and immediately analyzed on LSR Fortessa Flow Cytometer (BD) using FACS Diva v.6.1.3 software (BD). NK cells were defined as CD56− CD3+ CD20− CD14− cells. Analysis on the expression of NK cell markers was performed using FloJo v.10.2 (TreeStar). The antibodies used to define NK cell phenotype were either commercially available or were generated in the laboratories of Alessandro Moretta and Silvia Parolini.

To analyze the expression of NK cell surface markers, five different tubes were prepared (Table 2), each of which contained
| Patient ID | Phenotype | Age | Gene defect and mutation | Lymphocyte subsets (cells/μL) | IgG (mg/dL) | IgA (mg/dL) | IgM (mg/dL) | IgE (kU/L) | Proliferation to phytohemagglutinin | CMV viremia | Epstein-Barr virus (EBV) status | Infections | Autoimmunity | Granulomas | Skin rash |
|------------|-----------|-----|--------------------------|-----------------------------|-------------|-------------|-------------|------------|-----------------------------------|------------|---------------------------|-----------|------------|-----------|---------|
| P1         | SCID      | 9 months | RAG1: p.R561H; p.R507G | 185 74 24 0 166 883 | <7 | <5 | <2 | Absent | neg | neg | Rotavirus, Pseudomonas, Enterococcus enteritis | No | No | No |
| P2         | SCID      | 2 months | RAG2: p.I218N; p.I218N | 25 23 0 1 155 | <7 | <5 | nd | neg | neg | PJP | No | No | No |
| P3         | SCID      | 10 months | RAG2: p.T125Rfs*10 | 230 230 4 0 362 | 929 | <7 | <5 | <1 | Absent | neg | neg | PJP, metapneumovirus, oral candidiasis | No | No | No |
| P4         | SCID      | 2 months | RAG1: p.R641C; p.F974L | 34 30 4 265 395 | 371 | 22 | 67 | 38 | Markedly reduced | neg | neg | No | No | No | No |
| P5         | SCID      | 2 weeks  | RAG1: p.R570G; p.S966T | 286 111 190 16 | 1027 | 235 | <15 | 18 | Reduced | pos | nd | CMV viremia | No | No | No |
| P6         | SCID      | 6 months | RAG1: p.E965X | 2 2 0 0 252 | <7 | <5 | nd | Absent | neg | neg | Klebsiella pneumonia | No | No | No |
| P7         | SCID      | 2 months | RAG1: p.M605I; p.R561C; p.F972S | 2 1 0 0 | 275 | 325 | <7 | <5 | nd | Absent | pos | neg | RSV bronchiolitis, adenovirus pneumonia, CMV | No | No | No |
| P8         | SCID      | 6 months | RAG2: p.C41W; p.C41W | 5 1 0 1 804 | 71 | <7 | <5 | <1 | Absent | neg | neg | Disseminated BCG | No | No | No |
| P9         | SCID      | 5 months | RAG1: p.G720D | 9 9 0 0 | 449 | 766 | <7 | <5 | <1 | Absent | neg | neg | Cutaneous S. aureus abscess | No | No | No |
| P10        | SCID      | 5 months | RAG1: p.C470D | 236 90 146 0 | 80 | 161 | <7 | <5 | nd | Absent | neg | neg | No | No | No | No |
| P11        | SCID      | 1 week   | RAG1: p.C470D | 23 13 1 | 573 | 1,449 | 754 | <7 | <5 | 7.8 | Absent | neg | neg | No | No | No | No |
| P12        | SCID      | 14 months | DOCLR1C exon1_3del | 11 0 4 10 | 1,248 | 722 | <7 | <5 | <1 | Absent | neg | neg | Disseminated BCG | No | No | No |
| P13        | SCID      | 3 months | DOCLR1C exon1_3del | 1,076 1,070 | 10 0 | 904 | 50 | <7 | <5 | nd | Absent | neg | neg | RSV pneumonia | No | No | Yes |
| P14        | SCID      | 5 months | DOCLR1C p.K157Rfs*13; p.K157Rfs*13 | 215 152 | 61 | 40 | 287 | 540 | 38 | 106 | nd | Markedly reduced | neg | neg | Pseudomonas bacteremia, recurrent otitis, skin abscesses, oral candidiasis | No | No | Poplar rash |

(Continued)
| Patient ID | Phenotype | Age | Gene defect and mutation | Lymphocyte subsets (cells/μL) | IgG (mg/dL) | IgA (mg/dL) | IgM (mg/dL) | IgE (IU/L) | Proliferation to phytohemagglutinin | Cytomegalovirus (CMV) status | Epstein-Barr virus (EBV) status | Infections | Autoimmunity | Granulomas | Skin rash |
|------------|-----------|-----|--------------------------|-----------------------------|-----------|------------|------------|------------|----------------------------------|---------------------------|------------------------|-------------|------------|-----------|----------|
| P15 SCID 1 week DCLRE1C: exon1_3del; | 0 0 10 840 nd nd nd nd | 0 0 0 10 | 498 | 1,350 | <5 | <1 | Absent | neg | neg | Sepsis | No | No | No |
| P16 SCID 23 months DCLRE1C: | 107 50 63 0 | 0 0 0 10 | 620 | nd | nd | Absent | neg | neg | PJP, RSV pneumonia, cutaneous | No | No | No |
| P17 SCID 28 months DCLRE1C: | 46 39 7 0 | 0 0 0 10 | 291 | 1,060 | <7 | <7 | <5 | Absent | neg | neg | No | No | No | neg |
| P18 SCID 4 months DCLRE1C: | 7 1 0 1 | 1 0 1 0 | 698 | 115 | <7 | <7 | <5 | Reduced | pos | neg | Adenovirus hepatitis, oral ulcers | No | No | No |
| P19 SCID 10 months DCLRE1C: | 13,997 9,851 3,221 29 | 29 274 | 830 | <7 | <7 | <7 | <7 | Reduced | pos | neg | Oral candidiasis | No | No | Generalized erythroderma |
| P20 Ommen syndrome (OS) 2 months RAG1: | 15,760 6,390 9,465 0 | 0 1,968 | 300 | <7 | <5 | <5 | <5 | Reduced | pos | neg | Candidiasis, E. coli UTI | No | No | Generalized erythroderma |
| P21 OS 1 month RAG1: | 3,424 3,368 0 0 | 5,003 | 300 | 16 | <2 | Absent | neg | neg | Rhinovirus | No | No | Generalized erythroderma |
| P22 OS 2 months RAG1: | 19,230 10,286 8,215 0 | 816 | 1,120 | 101 | >5,000 | Markedly reduced | neg | neg | Rhinovirus | No | No | Generalized erythroderma |
| P23 OS 2 months RAG1: | 4,667 3,976 696 0 | 7,231 | <7 | <7 | <5 | <5 | 233 | Absent | neg | neg | No | No | Generalized erythroderma |
| P24 OS 4 months RAG1: | 15,751 8,850 6,907 0 | 1,389 | 258 | 216 | <5 | <5 | 2,181 | Absent | neg | neg | PJP, rhinovirus | No | No | Generalized erythroderma |
| P25 OS 2 months RAG1: | 11,140 6,204 4,901 0 | 4,564 | 100 | 6 | 302 | >5,000 | Markedly reduced | pos | neg | Anti-smooth muscle ab | No | Generalized erythroderma |
| P26 OS 6 months RAG2: | 2,671 2,351 805 0 | 36 102 | 116 | 235 | <5 | Markedly reduced | neg | neg | Impetigo, otitis | No | No | Generalized erythroderma |
| P27 OS 1 month RAG1: | 5,105 5,029 228 15 | 4,458 | 634 | 17 | 8 | <2 | Markedly reduced | pos | neg | Recurrent URTI/ LRTI | No | No | Generalized erythroderma |
| P28 OS 1 month RAG1: | 170 155 15 0 | 320 | 815 | 14.4 | Markedly reduced | neg | neg | Enterococcus faecalis bacteremia | No | No | Generalized erythroderma |

(Continued)
### TABLE 1 | Continued

| Patient ID | Phenotype  | Age     | Gene defect and mutation | Lymphocyte subsets (cells/μL) | lgG (mg/dL) | lgA (mg/dL) | lgM (mg/dL) | lgE (ng/mL) | Proliferation to phytohemagglutinin | Cytomegalovirus (CMV) status | Epstein-Barr virus (EBV) status | Infections | Autoimmunity | Granulomas | Skin rash |
|------------|------------|---------|--------------------------|-------------------------------|-------------|-------------|-------------|-------------|----------------------------------|----------------------------|--------------------------------|------------|-------------|-----------|----------|
|            |            |         |                          | CD3 CD4 CD8 CD19 CD16/56      |             |             |             |             |                                  |                            |                                |            |             |           |          |
| P29        | OS         | 4 months| RA61: p.R032X; p.L1025fs*39 | 34,115 22,416 8,364 0 1,338 | <7         | <7         | <5         | 600         | Reduced                                    | neg                       | neg                           | Enterovirus pneumonia, Moraxella bacteremia | No         | No          | Generalized erythroderma |
| P30        | OS         | 4 months| DCLRE1C: exon1_3del; splicing defect intron6 | 26,295 5,085 19,382 0 1,498 | 120 602 26 192 nd | neg         | neg         | PJP, rhinovirus                           | No                        | No                           | Generalized erythroderma |
| P31        | Atypical SCID | Not known | RA62: p.R123C; p.R123C | 1,056   96 780 23 67 206 11 14 1 nd | neg         | pos         | Skin abscess, recurrent pneumonia, candidiasis | Lymphadenopathy            | No                        | No          |            |           |          |
| P32        | AS         | 4 months| RA61: p.H375D; p.H375D | 870 610 210 350 390 370 nd 89 nd | Absent     | nd          | Candidiasis                                      | No                        | No          | Candida rash |
| P33        | AS         | 17 months| RA61: p.R966C; p.M435V | 360 180 150 31 370 1,036 <7 145 77 | Markedly reduced | neg         | neg         | Vaccine strain varicella zoster virus (VZV), chronic diarrhea | AHA, vasculitis            | No                        | Vasculitis |
| P34        | AS         | 3 years | RA61: p.H612R; p.A857V | 2,063 225 878 612 1,068 2,080 <7 172 1.3 | Reduced     | pos         | Adenovirus pneumonia, Kibesella pneumonia, candidiasis | AHA, TPO Ab               | No                        | No          |            |           |          |
| P35        | AS         | 2 years | RA61: p.P939C; p.P939C | 729 110 206 41 733 1,263 55 215 78 | normal      | nd          | BCG infection                                      | No                        | No                        | No          |            |           |          |
| P36        | AS         | 2 months| RA61: p.T173fs*27; p.T173fs*27 | 399 93 209 3 1,205 392 10 <5 113 | absent      | pos         | Pneumonia                                         | AHA                       | No                        | No          |            |           |          |
| P37        | AS         | 16 months| RA62: p.G315A; p.G315A | 716 277 102 105 209 229 75 327 nd | Reduced     | neg         | Recurrent pneumonia (Pseudomonas, Kibesella, H. influenzae), nail candidiasis | AHA, psoriasis            | ND                        | Psoriasis |
| P38        | AS         | 25 months| RA61: p.R841W; p.R841W | 950 220 20 121 666 538 >146 54 2 | Normal      | neg         | Adenovirus, rhinovirus                           | Tubulointerstitial nephritis with lymphohytic infiltrate | No                        | No          |            |           |          |
| P39        | AS         | 13 months| RA61: p.K866V*33; p.K866V*33 | 1,010 80 840 620 460 2,420 194 328 5.6 | Markedly reduced | pos         | pos         | Pseudomonas sepsis, CMV, BCGitis             | Miller-Fisher             | No                        | No          |            |           |          |
| P40        | AS         | 4 years | DCLRE1C: p.T656; p.T656 | 558 207 243 36 108 560 19 54 5 nd | neg         | nd          | nd          | HPV                                      | No                        | No          | No          | Warts      |

(Continued)
| Patient ID | Phenotype | Age | Gene defect and mutation | Lymphocyte subsets (cells/μL) | lgD (mg/dl) | lgE (mg/dl) | lgG (mg/dl) | lgA (MGU/L) | Proliferation to phytohemagglutinin | Colchicine-induced (CAM) status | Epstein-Barr virus (EBV) status | Infections | Autoimmunity | Granulomas | Skin rash |
|------------|-----------|-----|--------------------------|------------------------------|-------------|------------|-------------|-------------|--------------------------------|-------------------------------|--------------------------------|------------|-------------|-----------|----------|
| P41        | AS        | 10 years | DCCLRE1C: p.T65I; p.T65I | CD3 | CD4 | CD8 | CD19 | CD16/56 | <7 | 87 | 5 | nd | nd | HPV, recurrent URTI/LRTI | No | No | Warts |
| P42        | AS        | 2 years | DCCLRE1C: p.T65I; p.T65I | 547 | 217 | 268 | 22 | 226 | 240 | <7 | 35 | 5 | Absent | nd | nd | HPV, BCG | No | No | Warts |
| P43        | AS        | 9 months | NHEJ1: p.R57X; p.R57X | 426 | 299 | 98 | 259 | 265 | 600 | <7 | 849 | nd | Reduced | neg | neg | Adenovirus | No | No | No |
| P44        | CID       | 6 years | RA62: p.T215I; p.T215I | 840 | 560 | 180 | 70 | 190 | 2,480 | <7 | 170 | nd | Reduced | nd | pos | Recurrent URTI, intermittent diarrhea | Neutropenia | No | No |
| P45        | CID       | 19 years | RA62: p.G335V; p.M322T | 457 | 236 | 184 | 165 | 5,781 | <100 | <7 | <5 | nd | Reduced | neg | pos | Single episode of RTI requiring admission at age 13 years | No | Skin granulomatosis | Skin granulomas |
| P46        | CID       | 11 years | RA61; p.R404Q; p.R404Q | 4,520 | 3,300 | 1,220 | 61 | 1,004 | <200 | <7 | 30 | nd | Absent | pos | pos | CMV retinitis, esophaegal candidiasis, EBV | Colitis | Colitis granulomatosis | No |
| P47        | CID       | 8 years | RA61: p.H612R; p.H612R | 526 | 361 | 144 | 373 | 106 | 859 | <7 | <5 | 1 | Markedly reduced | na | na | Recurrent pneumonia, MAC, cellulitis | AHI | No | No |
| P48        | CID       | 17 years | RA61: p.K86Vfs*33; p.H612R | 581 | 530 | 80 | 460 | 450 | 390 | <7 | 38 | <1 | Reduced | pos | pos | Herpes zoster, H. influenzae, Corynebacterium pyrocinum, Pseudomonas | ITP, ulcers | Lung granulomatosis | No |
| P49        | CID       | 11 years | RA61: p.L514R; p.L514R | 2,831 | 1,658 | 837 | 0 | 309 | <140 | <7 | 130 | <18 | normal | neg | pos | Recurrent URTI/ LRTI, warts, molluscum, EBV-LPD | Autoimmune cytopanias | Granulomas in liver and lungs | Warts, molluscum |
| P50        | CID       | 31 years | RA62: p.F62L; p.F62L | 380 | 225 | 162 | 78 | 215 | 1,000 | <7 | 25 | nd | nd | na | na | Disseminated VZV, cryptococcosis menigitis, URTI, Pseudomonas pneumonia | No | Lung granulomatosis |
| P51        | CID       | 12 years | RA61: p.R624H; p.Y728H | 665 | 406 | 219 | 23 | 177 | 8 | <7 | 10 | <2 | Normal | neg | pos | Moderately severe VZV, zoster, recurrent URTI/LRTI | No | No | No |
| P52        | CID       | 9 years | RA62: p.V8I; p.D409H | 2,253 | 1,275 | 774 | 293 | 205 | 910 | 257 | 33 | 29 | Normal | neg | neg | S. pneumoniae sepsis, severe VZV infection, recurrent URTI/ LRTI, penial abscess | No | No | Severe atopic dermatitis |
| P53        | CID       | 16 years | RA61: p.H612R; p.H612R | 629 | 390 | 141 | 69 | 60 | 637 | 37 | 45 | nd | Normal | neg | neg | Recurrent otitis, recurrent pneumonias, S. aureus skin infection | Alopeia areata, AHI, neutropenia | No | No |

(Continued)
| Patient ID | Phenotype | Age | Gene defect and mutation | Lymphocyte subsets (cells/μL) | lgG (mg/dL) | lgM (mg/dL) | lgA (mg/dL) | lgG (kU/l) | Proliferation to phytohemagglutinin | EBV status | Lymphoma | Autoimmunity | Granulomas | Skin rash |
|------------|-----------|-----|---------------------------|-----------------------------|-----------|------------|------------|-----------|-----------------------------------|------------|---------|-------------|-----------|---------|
| P54 CID 9 | RA51: p.R474C; p.L506F | 9 | CD3 900 500 400 300 130 560 20 60 5 | Normal | neg | pos | VZV pneumonia, bronchiectasis | No | No | No |
| P55 CID 39 | RA51: p.R108K | 14 | 16 6 1,346 420 320 nd | Reduced | neg | neg | Pneumonia, warts, oral candidiasis, MRS A skin infection | Alopeia | Autoimmune | Autoimmune hypogammaglobulinemia | No | No |
| P56 CID 16 | RA51: p.H375D; p.Y562C | 0 10 0 | 0 300 300 | Reduced | neg | neg | Pneumonia | ITP, neutropenia | Granulomatosis of skin, liver, spleen, lungs | No | No |
| P57 CID 10 RAG1: p.R108X; p.W522C | 5 | 300 130 | 560 10 | Reduced | neg | neg | Recurrent LRTI | Alopeia, vitiligo | No | No |
| P58 CID 40 | RA51: p.R108K | 0 10 0 | 0 300 300 | Reduced | neg | neg | Recurrent LRTI | Alopeia, vitiligo | No | No |
| P59 CID 36 | RA52: p.N173S; p.E437K | 106 976 100 | 0 300 300 | Reduced | neg | neg | Severe arthritis, recurrent pneumonia, warts | Alopeia | Mucocutaneous | No | Psoriatic rash |
| P60 CID 30 | DCLRE1C: p.T65I | 10 500 0 | 420 520 | Reduced | neg | pos | Echovirus, adenovirus | AHA | Recurrent LRTI | No | Perianal rash |
| P61 CID 2 | DCLRE1C: p.T65I | 10 500 0 | 420 520 | Reduced | neg | pos | Echovirus, adenovirus | AHA | Recurrent LRTI | No | Perianal rash |
| P62 CID 12 | DCLRE1C: p.S147fs*6; p.S147fs*6 | 1,635 173 | 11 2 | Reduced | neg | neg | JC virus-associated PML | Vascularitis | Cutaneous granulomatous vasculitis | Vascularitis |
| P63 CID 7 | NHEJ1: p.P67Q | 60 60 | 10 104 | Reduced | neg | neg | Recurrent LRTI | No | No | No |
| P64 CID 3 | Ligase 4 (LIG4): p.R278H | 1,400 176 | 177 177 | Reduced | neg | neg | Chronic calicivirus | No | No | No |
| P65 CID 8 | LIG4: p.K424Rfs*20; p.R278H | 622 622 | <1 15 2 | Reduced | pos | pos | Pneumonia, otitis | No | No | No |
| P66 CID 17 | LIG4: p.K424Rfs*20 | 140 140 | <1 15 2 | Reduced | neg | neg | Recurrent LRTI | No | No | No |

*: on immunoglobulin replacement therapy; Ab, antibody; AIHA, autoimmune hemolytic anemia; ANA, anti-nuclear antibodies; BCG, bacillus calmette-guerin; CMV, cytomegalovirus; HPV, human papillomavirus; ITP, immune thrombocytopenia; LPD, lymphoproliferative disease; LRTI, lower respiratory tract infection; PJP, Pneumocystis jiroveci pneumonia; RSV, respiratory syncitial virus; TPO, thyroid peroxidase; URTI, upper respiratory tract infection; UTI, urinary tract infection; VZV, Varicella zoster virus.
TABLE 2 | Combination of antibodies used to characterize natural killer cell phenotype.

| Tube # | Antibody | Source |
|--------|----------|--------|
| 1      | IgG1 isotype control | Biolegend |
|        | IgG2a isotype control | Biolegend |
|        | IgG2b isotype control | Biolegend |
|        | PE-conjugated goat anti-mouse IgG1 | Southern Biotech |
|        | PE-conjugated goat anti-mouse IgG2a | Southern Biotech |
|        | PE/Cy7-conjugated goat anti-mouse IgG3b | Southern Biotech |
|        | FITC-conjugated anti-CD3 | Beckman Coulter |
|        | PCS5-conjugated anti-CD56 | Beckman Coulter |
|        | FITC-conjugated anti-CD14 | Beckman Coulter |
|        | FITC-conjugated anti-CD20 | Biolegend |
|        | APO/Cy7-conjugated IgG1 | Biolegend |
|        | BV510-conjugated IgG1 | Biolegend |
|        | BV421-conjugated mouse IgM | Becton-Dickinson |
| 2      | Anti-NKG2A (Z199) [IgG2b] | A. Moretta, S. Parolini |
|        | Anti-KIR2 DL1/S1 (AZ115) [IgG1] | A. Moretta, S. Parolini |
|        | Anti-KIR2 DL2/S2 (GL183) [IgG1] | A. Moretta, S. Parolini |
|        | Anti-KIFSD (AZ158) [IgG2a] | Southern Biotech |
|        | PE-conjugated goat anti-mouse IgG1 | Southern Biotech |
|        | PE-conjugated goat anti-mouse IgG2a | Southern Biotech |
|        | PE/Cy7-conjugated goat anti-mouse IgG2b | Southern Biotech |
|        | FITC-conjugated anti-CD3 | Beckman Coulter |
|        | PCS5-conjugated anti-CD56 | Beckman Coulter |
|        | FITC-conjugated anti-CD14 | Beckman Coulter |
|        | FITC-conjugated anti-CD20 | Biolegend |
|        | APO/Cy7-conjugated anti-CD16 | Biolegend |
|        | BV510-conjugated anti-CD69 | Biolegend |
|        | BV421-conjugated anti-CD57 | Becton-Dickinson |
| 3      | Anti-SIGLEC7 (Z176) [IgG2b] | A. Moretta, S. Parolini |
|        | Anti-LR1 (F278) [IgG1] | Southern Biotech |
|        | PE-conjugated goat anti-mouse IgG1 | Southern Biotech |
|        | PE/Cy7-conjugated goat anti-mouse IgG2b | Southern Biotech |
|        | FITC-conjugated anti-CD3 | Beckman Coulter |
|        | PCS5-conjugated anti-CD56 | Beckman Coulter |
|        | FITC-conjugated anti-CD14 | Beckman Coulter |
|        | FITC-conjugated anti-CD20 | Biolegend |
|        | APO/Cy7-conjugated anti-CD16 | Biolegend |
|        | BV510-conjugated anti-CD69 | Biolegend |
|        | BV421-conjugated anti-CD57 | Becton-Dickinson |
| 4      | Anti-NKG2C [IgG2b] | R&D Systems |
|        | Anti-KIR2 DL1/S1 (1PB6) [IgG1] | A. Moretta, S. Parolini |
|        | Anti-KIR2 DL2/S2 (GL183) [IgG1] | A. Moretta, S. Parolini |
|        | Anti-KIFSD (AZ158) [IgG2a] | Southern Biotech |
|        | PE-conjugated goat anti-mouse IgG1 | Southern Biotech |
|        | PE-conjugated goat anti-mouse IgG2a | Southern Biotech |
|        | PE/Cy7-conjugated goat anti-mouse IgG3b | Southern Biotech |
|        | FITC-conjugated anti-CD3 | Beckman Coulter |
|        | PCS5-conjugated anti-CD56 | Beckman Coulter |
|        | FITC-conjugated anti-CD14 | Beckman Coulter |
|        | FITC-conjugated anti-CD16 | Beckman Coulter |
|        | APO/Cy7-conjugated anti-CD16 | Biolegend |
|        | BV510-conjugated anti-CD69 | Biolegend |
|        | BV421-conjugated anti-CD57 | Becton-Dickinson |
| 5      | Anti-CXCR1 [IgG1] | Santa Cruz |
|        | PE-conjugated goat anti-mouse IgG1 | Southern Biotech |
|        | FITC-conjugated anti-CD3 | Beckman Coulter |
|        | PCS5-conjugated anti-CD56 | Beckman Coulter |
|        | FITC-conjugated anti-CD14 | Beckman Coulter |
|        | FITC-conjugated anti-CD20 | Biolegend |
|        | APO/Cy7-conjugated anti-CD16 | Biolegend |
|        | Pacific Blue-conjugated anti-CCR7 | Biolegend |

FITC-conjugated antibodies directed against CD3, CD20, and CD14, to gate out T cells, B cells, and monocytes. Furthermore, tube #1 contained isotype controls, whereas tubes #2–5 contained antibodies to NK cell markers.

Analysis of Perforin Expression

Intracellular content of perforin was analyzed in a limited number of patients with SCID/OS/AS due to RAG/NHEJ defects and in healthy infants. Briefly, PBMCs were first stained with a mixture of FITC-conjugated mAbs directed against CD3, CD20, and CD14, as well as with PC5-labeled anti-CD56 mAb, and incubated for 30 min at 4°C. After treatment with 200 µl of Cytofix/Cytoperm (BD-Bioscience, Pharmingen CA, USA) for 20 min at 4°C, cells were washed with 1 ml of saponin (0.1% solution in PBS), and then stained with 5 µl of purified RPE-labeled anti-perforin mAb (Ancyll). After washing, the proportion of CD3+ CD14+ CD20+ CD56+ cells expressing perforin and the mean fluorescent intensity (MFI) of perforin were immediately analyzed on LSR Fortessa Flow Cytometer (BD) using FACSDiva software (BD). Final analysis was done using FlowJo v.10.2 (TreeStar).

Analysis of NK Cell Degranulation

Natural killer cell degranulation activity was tested against the K562 erythroleukemia human cell line. In particular, PBMCs derived from patients and from healthy donors were obtained upon Ficoll separation of heparinized blood samples, and incubated with or without 100 U/mL recombinant human IL-2 (NIH) at 37°C overnight. Cells were then incubated with target cells at an effector:target ratio of 1:3 in a final volume of 200 µl in round-bottomed 96-well plates at 37°C and 5% CO2 for 4 h in culture medium supplemented with anti-CD107a-PE (BD Biosciences Pharmingen, San Diego, CA, USA) monoclonal antibody. Cells were then surface-stained with FITC anti-CD3, PC5 anti-CD56, FITC anti-CD14 (Beckman Coulter), FITC anti-CD20, and APC/Cy7 anti-CD16 (BD Biosciences Pharmingen, San Diego, CA, USA) Ab for 30 min at 4°C. The cells were washed, and the proportion of CD3+ CD14+ CD20+ CD56+ cells expressing CD107a was analyzed immediately on LSR Fortessa Flow Cytometer (BD) using FACSDiva v6.1.3 software (BD Biosciences, Mountain View, CA, USA). Final analysis was performed using FlowJo v.10.2 (TreeStar). The threshold to define CD107a expression in cells co-cultured with K562 target cells (in the presence or absence of IL-2) was set up on cells cultured with IL-2 alone, without K562 cells.

Analysis of Interferon-γ (IFN-γ) Production

To detect intracellular production of IFN-γ, PBMCs from patients and healthy donors were incubated overnight at 37°C with IL-12 (0.5 ng/ml), or IL-12 (0.5 ng/ml) and IL-18 (0.1 ng/ml) combined. Surface staining was done by incubating the cells with FITC anti-CD3, PC5 anti-CD56, FITC anti-CD14 (Beckman Coulter), FITC anti-CD20, and APC/Cy7 anti-CD16 (BD) mAbs for 30 min at 4°C. Cells were then washed, fixed, and permeabilized with BD Cytofix/Cytoperm kit (BD Biosciences Pharmingen). IFN-γ production was detected by subsequent intracellular staining with PE-conjugated anti-IFN-γ (BD Biosciences Pharmingen). After...
washing, the proportion of CD3−CD14−CD20−CD56+ cells expressing IFN-γ was immediately analyzed on LSR Fortessa Flow Cytometer (BD) using FACSDiva software (BD). Final analysis was done using FloJo v.10.2 (TreeStar).

Statistical Analysis
Statistical analysis of the in vitro recombination activity of mutant RAG and ARTEMIS proteins, and of the expression of NK cell markers in patients and controls was performed using Mann–Whitney test for non-parametric variables.

RESULTS

Demographic and Clinical Features
At the time of evaluation, mean age (±SEM) was significantly lower in patients with SCID (7.1 ± 1.72 months; range: 0.25–28 months) and in those with OS/AS (15.96 ± 5.41 months; range: 24–480 months) than in patients with CID (177.2 ± 28.22 months; range: 24–480 months; p < 0.0001).

A clinical history of significant infections was present in 60 patients with RAG/NHEJ gene defects (Table 1). Candida (n = 11), Pseudomonas (n = 7), human papillomavirus (HPV, n = 7), adenovirus (n = 6), varicella zoster virus (VZV, n = 6), Pneumocystis jiroveci (n = 5), Bacillus Calmette-Guerin (BCG, n = 5), and rhinovirus (n = 5) were the most common pathogens. In particular, P. jiroveci pneumonia was observed only in patients with SCID or OS/AS, whereas severe VZV infection was mostly restricted to patients with CID. Cytomegalovirus (CMV) and Epstein–Barr virus (EBV) infections were documented in 13 patients each, but were clinically significant only in 4 and 3 patients, respectively. Two SCID patients (P4 and P11) were diagnosed at birth following newborn screening, and infections were effectively prevented with isolation and prophylactic antimicrobials.

Autoimmunity and/or autoantibody production were documented in 23 patients, and were more common in patients presenting with CID (14 out of 21 patients) than in those with SCID (0/19) or with OS/AS (9/24). Cytopenias were the most frequent manifestation of autoimmunity and were documented in 11 patients (4 with OS/AS and 7 with CID). Alopecia and/or vitiligo were observed in six CID patients. Granulomatous disease was present in eight patients with CID. Finally, 12 patients (P20–P31) had typical features of OS (generalized erythoderma, lymphadenopathy, hepatosplenomegaly).

Immunological Phenotype
The count of circulating CD3+, CD19+, and CD3−CD14−CD20−CD56+ cells in patients belonging to the various groups (SCID, OS/AS, and CID) is shown in Figure 1. With the exception of one SCID patient (P19) who had a high count of maternal T cells (13997 cells/µl), all others had severe T cell lymphopenia (Figure 1A). By contrast, a higher count of autologous T cells was recorded in patients with CID and especially in patients with OS/AS. B cell lymphopenia was observed in the vast majority of patients, irrespective of the clinical phenotype (Figure 1B). By contrast, NK cell count was either normal or increased in most patients (Figure 1C).

Data on in vitro proliferation to phytohemagglutinin (PHA) were available in 51 patients. Response was absent (<10% of lower limit of normal, LLN) in 17, markedly reduced (10–30% of LLN) in 11, reduced (>30% of LLN, but lower than LLN) in 14, and normal in 9 patients. T cell proliferation to PHA was better preserved in patients with CID; among 18 such patients for whom information was available, 15 had either normal (n = 7) or reduced (n = 8) response.

Low serum levels of IgA and IgM were detected in the majority of SCID patients (Table 1); serum IgG in this group was less informative because of the presence of maternally derived immunoglobulins. Immunoglobulin levels were more variable in patients with OS/AS and in those with CID. In particular, among 23 CID patients, 17 had either low serum IgG (n = 9) or were under immunoglobulin replacement therapy (n = 8) and 15 had undetectable serum IgA.
Circulating NK Cells from Healthy Infants Comprise a High Proportion of CD56bright Cells

According to a widely accepted scheme, human NK cell differentiation is marked by changes in the expression of cell surface and intracytoplasmic markers, so that five distinct stages of NK cell development are recognized (33). In peripheral blood from healthy controls, both stage 4 (CD56bright CD16−) and stage 5 (CD56+ CD16hi) NK cells are detectable. In particular, in normal adults, CD56bright CD16− cells account for only 5–10% of all circulating NK cells. By contrast, a higher percentage of CD56bright cells was detected in healthy infants (Figures 3A,B). Moreover, the proportion of CD16+ cells was lower in infants than in healthy controls greater than 2 years of age (Figures 3A,C). Finally, NK cells from healthy infants included a lower proportion of cells expressing CD57 (Figures 3A,E), KIRs (Figures 3A,D), and CXCR1 (Figures 3A,F), whereas the percentage of NK cells expressing NKG2A was higher in infants than in older healthy controls (Figures 3A,G).

Markedly Increased Proportion of CD56bright NK Cells in the Peripheral Blood of Patients with RAG and NHEJ Defects Correlates with the Clinical Phenotype

A recent study in Rag−/− mice has unexpectedly disclosed abnormalities of NK phenotype and function, with increased proportion of mature, activated cells with increased cytotoxic activity and reduced survival (30). In order to assess whether similar abnormalities exist in patients with RAG or NHEJ defects, and to establish whether such abnormalities, if present, correlate with the severity of the clinical phenotype and with residual function of the mutant protein, we have analyzed the expression of CD56 and CD16 on the surface of CD3− CD14− CD20− peripheral blood NK cells from patients and controls. As shown in Figures 4A,B, patients with SCID due to RAG/NHEJ defects had a high proportion of CD56bright CD16− NK cells (mean ± SEM: 34.7 ± 4.4), that exceeded what was observed in healthy infants (13.9 ± 3.3; p = 0.0007). Similarly, the proportion of CD56bright CD16− NK cells was higher in patients with CID than in healthy controls greater than 2 years of age (10.4 ± 1.8 vs. 5.1 ± 0.7; p = 0.0431; Figures 4A,B). Among patients with RAG/NHEJ defects, the proportion of CD56bright CD16− NK cells was higher in those presenting with SCID than in those with OS/AS (p = 0.0008; Figure 4B). In order to investigate whether the increase in the proportion of each of the CD56bright NK cells was secondary to numerical and/or functional abnormalities of T and/or B cells, we also analyzed a group of patients with other forms of TCD or with XLA. The proportion of CD56bright CD16− NK cells in these groups was similar to that of healthy controls, and significantly lower than in patients with SCID due to RAGH/NHEJ defects (Figure 4B). Among patients with TCD, no correlation was observed among the proportion of CD56bright NK cells and the severity of T cell lymphopenia, indicating that the increase of CD56bright NK cells observed in patients with RAG/NHEJ defects is not simply secondary to T cell lymphopenia. Furthermore, the proportion of CD56bright CD16− NK cells, that are considered to represent an intermediate step of differentiation between CD56bright CD16− and CD56dim CD16hi cells, was significantly higher in infants with SCID due to RAG/NHEJ defects than in healthy infants, patients with TCD or with XLA (p < 0.0001; Figure 4C). A higher percentage of CD56bright CD16− NK cells was also observed in patients with CID than in healthy controls greater than 2 years of age (p < 0.05; Figure 4C). Finally, patients with SCID due to RAG/NHEJ defects had a lower proportion of CD56dim CD16hi NK cells when compared to healthy infants (p = 0.0007; Figure 4D). Similar results were obtained when comparing patients with CID versus healthy controls older than 2 years of age (p = 0.0011).

CD56bright NK Cells from Infants with RAG/NHEJ Defects Have an Immature Phenotype

The data reported above on CD56 and CD16 surface expression indicate that patients with RAG/NHEJ defects have an

**Correlation between Clinical Phenotype and Recombination Activity Sustained by the Mutant Alleles**

Molecular analysis of the patients included in this study identified a total of 41 RAG1, 16 RAG2, 11 DCLRE1C, 4 LIG4, and 2 NHEJ distinct gene mutations (Table 1). Using a flow cytometry-based assay, we have previously reported correlation between recombination activity supported by RAG and DCLRE1C mutant alleles and the clinical and immunological disease phenotype (22, 32). For the patient cohort included in this study, data on in vitro recombination activity were available for 36 RAG1, 9 RAG2, and 6 DCLRE1C mutant alleles. Upon plotting for each patient the recombination activity associated with each of the two mutant alleles, a significant genotype-phenotype correlation was observed, with higher residual recombination activity for mutant alleles associated with a CID phenotype than for those associated with OS/AS or SCID (Figure 2).
abnormal distribution of NK cell subsets, with an increased proportion of more immature cells as compared to what is observed in healthy controls. In order to further confirm this, we analyzed the expression of additional surface markers that are differentially expressed in CD56bright versus more mature CD56dim NK cells. As compared to healthy controls >2 years of age, patients with CID had a lower proportion of CD57+ NK cells ($p = 0.0022$; Figures 5A,B). Furthermore, patients with SCID
Flow cytometry analysis of CD56 and CD16 expression in natural killer cells from patients and controls. (A) Representative analysis of the expression of CD56 and CD16 in CD3−CD19−CD14−CD56+ cells from one patient each belonging to the severe combined immune deficiency (SCID), Omenn syndrome (OS), delayed-onset combined immune deficiency (CID), other forms of T cell defect (TCD), and X-linked agammaglobulinemia (XLA), respectively. (B–D) Percentage of CD56bright CD16− (B), CD56bright CD16int (C), and CD56dim CD16hi (D) cells among CD3−CD19−CD14−CD56+ cells. For each marker, positivity was defined as fluorescence intensity above that of isotype control. Bars represent mean ± SD values. *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.001.

had fewer CD57+ NK cells than patients with other forms of TCD (p = 0.0041), and a similar trend was also observed when compared to infant controls, although the difference did not reach statistical significance (Figure 5B).

In normal subjects, circulating stage 4 CD56bright NK cells are characterized by high levels of expression of NKG2A and of the chemokine receptor CCR7, which facilitates homing to lymphoid tissues. By contrast, expression of KIRs and of the chemokine receptor CXCR1 is virtually absent in CD56bright NK cells. As compared to healthy controls, the proportion of NK cells expressing NKG2A at high density was increased in patients with SCID and CID due to RAG/NHEJ defects (Figures 5C,D), and by contrast, the percentage of CXCR1+ NK cells was reduced (Figures 5E,F). Furthermore, patients with SCID due to RAG/NHEJ defects had a lower proportion of NK cells expressing KIR than healthy infants, patients with OS and those with other forms of TCD (Figures 5G,H). While the proportion of CCR7+ cells was higher in healthy infants than in healthy controls >2 years of age (Figure 6), no significant differences were observed in the percentage of CCR7+ NK cells between patients with RAG/NHEJ defects and healthy controls.

Notably, CCR7 expression was downregulated during the transition from CD56bright CD16− to CD56bright CD16int cells (data not shown). Furthermore, the proportion of NK cells expressing Siglec-7, leukocyte Ig-like receptor (LIR)-1, or the activation marker CD69 was similar in patients and healthy controls, although interestingly a high proportion of Siglec-7+ NK cells were detected in the control group of patients with XLA (Figure 6). Overall, these data suggest the presence of CD56bright NK cells that are more abundant in patients with RAG/NHEJ defects represent immature NK cells and provide evidence for a genotype–phenotype correlation in this group of diseases.

**Impact of Viral Infections on NK Cell Phenotype**

Recombinase-activating gene and NHEJ defects lead to increased susceptibility to viral infections, including CMV, EBV, and VZV. It has been reported that viral infections (and CMV in particular) may lead to significant changes of NK cell phenotype and function, with accumulation of cytotoxic CD56dim NKG2C+ NKG2A− KIR− CD57+ cells (34). Expansion of these cells does...
Flow cytometry analysis of the expression of CD57 (a,B), NKG2A (c,D), and CXCR1 (e,F), and killer cell immunoglobulin-like receptors (KIRs) (g,h) among CD3−CD19−CD14−CD56+ cells. In panels (A,C,E,G), representative examples are shown for one patient each belonging to the severe combined immune deficiency (SCID), Omenn syndrome (OS), and delayed-onset combined immune deficiency (CID) subgroups. For each marker, positivity was defined as fluorescence intensity above that of isotype control. Bars represent mean ± SD values. *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.001.

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not require T cells, as shown in an IL7R-deficient SCID infant after CMV infection (35). A significant proportion (26/66, 39.4%) of the patients with RAG/NHEJ defects included in this study had a documented history of CMV, EBV, VZV or severe JC virus (JCV) infection (Table 1). The proportion of NK cells expressing NKG2C was not statistically different in patients with
Furthermore, when patients were divided into two groups according to their history of CMV, EBV, VZV, or severe JCV infection, no differences were observed in the proportion of NKG2C+ NK cells (Figure 7B). Even when patients with SCID/OS/AS or with CID were divided into two subgroups based on their CMV infection history, no difference was observed in the proportion of NKG2C+ (Figure 7C) and of CD56dim (Figure 7D) NK cells among NKG2C+ cells from patients and controls. For each marker, positivity was defined as fluorescence intensity above that of isotype control. Bars represent mean ± SD values. *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.001.
CD56<sup>bright</sup> Cells from Patients with RAG/NHEJ Defects Are Potent Producers of IFN-γ

Circulating CD56<sup>bright</sup> NK cells serve an immunoregulatory function through secretion of various cytokines (IFN-γ, TNF-α, IL-10) (36). In order to assess this aspect of NK cell function, we performed in vitro stimulation with IL-12 and IL-18, and analyzed intracellular expression of IFN-γ upon gating separately on CD56<sup>bright</sup> vs. CD56<sup>dim</sup> NK cells. As expected, in both patients and controls CD56<sup>bright</sup> cells were more potent producers of IFN-γ than CD56<sup>dim</sup> cells, but both subsets were capable of producing this cytokine (Figure 8). However, CD56<sup>dim</sup> cells from patients with SCID and with OS/AS due to RAG/NHEJ defects showed impaired IFN-γ production when compared to equivalent cells from healthy infants or patients with TCD. A similar trend was observed in patients with CID versus healthy controls greater than 2 years of age (Figure 8).

NK Cells from Patients with SCID due to RAG/NHEJ Defects Have Increased Degranulation Capacity in the Absence of IL-2-Mediated Stimulation, and Express a Higher Amount of Intracellular Perforin than NK Cells from Healthy Infant Controls

Previous data had shown that NK cells from rag<sup>−/−</sup> mice have increased cytotoxic activity (30). To investigate the cytolytic machinery of NK cells, we have analyzed NK cell degranulation in response to K562 target cells, in the absence or presence of IL-2. It is well known that human CD56<sup>bright</sup> NK cells have reduced cytotoxic activity when compared to CD56<sup>dim</sup> cells (8). In spite of the markedly increased proportion of CD56<sup>bright</sup> NK cells, we observed that in the absence of stimulation with IL-2, NK cells from patients with SCID due to RAG/NHEJ defects have increased degranulation capacity when compared to NK cells from healthy infants (Figure 9A, left panel). In particular, CD56<sup>dim</sup> NK cells from patients with SCID showed increased degranulation when compared to the equivalent subset of NK cells from healthy infants (Figure 9A, middle panel). A similar trend was observed also for CD56<sup>bright</sup> NK cells, but the difference between SCID patients and controls did not reach statistical significance (Figure 9A, right panel). Stimulation with IL-2 significantly increased NK cell degranulation capacity to similar levels in patients and controls (Figure 9A, right panel). Although the amount of intracellular perforin could be measured only in a small number of patients with SCID, a significantly higher mean fluorescent intensity (MFI) was observed in unstimulated NK cells from patients than from healthy infants (Figure 9B).

**DISCUSSION**

In this manuscript, we have demonstrated that NK cells from healthy infants comprise a higher proportion of CD56<sup>bright</sup> CD16<sup>++/+</sup> CD57<sup>−</sup> cells than what is observed in older healthy subjects, confirming previous observations (37–39). Several lines of evidence indicate that CD56<sup>bright</sup> cells represent precursors to CD56<sup>dim</sup> cells. In particular, it has been demonstrated that appearance of CD56<sup>bright</sup> cells precedes that of CD56<sup>dim</sup> cells after HSCT (40) and that it is possible to induce differentiation of CD56<sup>bright</sup> into CD56<sup>dim</sup> NK cells in vitro in response to signaling via fibroblast growth factor receptor 1 (41). On the other hand, the model according to which CD56<sup>bright</sup> cells precede CD56<sup>dim</sup> cells during NK cell development has been challenged by NK cell lineage tracking experiments with genetic bar coding in macaques, which have suggested that these subsets may have distinct ontogeny (42).

In any case, the demonstration that composition of the peripheral NK cell compartment varies with age indicates that appropriate age-matched controls should be used when analyzing NK cell phenotype in pathological conditions.

In particular, by performing an extensive phenotypic analysis, we have shown that NK cells from patients with RAG/NHEJ defects comprise a higher proportion of CD56<sup>bright</sup> CD16<sup>++/+</sup> NKG2A<sup>++/+</sup> cells, and a reduced percentage of CD56<sup>dim</sup> CD16<sup>−</sup> cells expressing CD57, KIRs, and CXCR1, than observed in age-matched healthy controls. Altogether, these observations suggest that NK cells from patients with RAG/NHEJ defects have a more immature phenotype when compared to age-matched healthy controls, in spite of the fact that NK cells from CID patients showed signs of progressive differentiation as compared to NK cells from patients with SCID/OS/AS. These data contrast with recent findings in Rag<sup>−/−</sup> mice, whose peripheral NK cell compartment is characterized by a predominance of cells expressing KLRG1, a marker of mature NK cells (30). Furthermore, peripheral NK cells from Rag<sup>−/−</sup> mice are largely CD62L<sup>+</sup>, indicating an in vivo activated phenotype (30). By contrast, we did not observe increased expression of CD69, an activation marker (43), in NK cells from patients with RAG/NHEJ defects.
including those with OS, a condition characterized by accumulation of in vivo-activated autologous T cells. While the reasons for these discrepancies are not clear, murine and human NK cells differ substantially, also in regard to phenotypic markers that are differentially expressed during development.

By affecting the process of V(D)J recombination, mutations in RAG and NHEJ genes profoundly affect generation of T and B lymphocytes. Consistent with this, patients with RAG/NHEJ gene defects had a markedly reduced number of circulating B and T cells, with the exception of patients with OS, some of which had a normal or even increased number of oligoclonal and activated T cells, as typically observed in this disease. Previous studies in TCRδ−/− and in μMT−/− mice had demonstrated that lack of T and B cells have opposite effects on the count of splenic NK cells. In particular, T cell deficiency is associated with a twofold to threefold increase, and B cell deficiency with a twofold to threefold decrease of NK cell count as compared to C57BL/6 wild-type mice (44). The patients with RAG and NHEJ defects included in this study had either normal or increased count of circulating NK cells. In order to rule out the possibility that their abnormalities of NK cell phenotype could be secondary to T and B cell lymphopenia, we have included in this study patients with XLA (who lack circulating B cells) and with other forms of TCD, including seven patients with SCID not due to RAG/NHEJ defects. Even when compared to these two control groups, patients with SCID due to RAG/NHEJ gene defects had a markedly increased proportion of CD56bright CD16− CD57− NKG2A+++, cells. Similar differences were also observed also when comparing NK cells from patients with OS/AS and those with TCD or XLA, with an increased percentage of CD56bright CD16− CD57− cells in the former. Altogether, these data indicate that the increased representation of immature cells within the peripheral NK cell compartment of patients with RAG/NHEJ defects is not secondary to T and B cell lymphopenia.

Because of their severe T cell immunodeficiency, patients with RAG/NHEJ defects are highly prone to viral infections, including CMV. LIR-1 is a member of the immunoglobulin superfamily which has been shown to bind the human CMV MHC class I homolog UL-18 protein (45). NKG2A and NKG2C represent inhibitory and activating forms of CD94, recognizing non-classical HLA-E molecules (46). CMV infection can drive expansion of KIR+ and/or LIR-1+, NKG2A− NKG2C+ NK cells (34). Furthermore, rapid differentiation of CD56dim NKG2C+ NKG2A− CD57− cells has been observed after CMV reactivation in recipients of HSCT (47). It has been shown that upon transfer from CMV-seropositive donors into seropositive HSCT...
recipients, NKG2C+ cells undergo expansion and produce high amounts of IFN-γ as compared to NKG2C+ cells transfused into CMV-seronegative recipients, implying that NKG2C+ cells may represent “memory” NK cells capable of a prompt response upon re-exposure to CMV (48). Finally, rapid expansion of NKG2C+ NK cells has been reported in infants acquiring perinatal CMV infection (39). Although several of the patients included in this study had a history of CMV infection, no particular expansion of NKG2C+ and/or LIR-1+ cells was observed. Monocytes are apparently required to allow expansion of adaptive/memory-like NK cells (49). Lack of expansion of NKG2C+ cells in CMV-infected, RAG-/NHEJ mutated SCID patients may reflect a requirement also for T cell help in this process and may further contribute to poor control of CMV infection in these patients.

CD56bright and CD56dim NK cells differ in their immunomodulatory and effector function. In particular, CD56bright cells are potent producers of IFN-γ, but have lower content of perforin and display reduced cytotoxic activity as compared to CD56dim cells. Analysis of IFN-γ production by CD56bright and CD56dim NK cells from patients and controls confirmed that CD56bright cells were more potent producers of IFN-γ, irrespective of the underlying diagnosis. However, the proportion of CD56dim NK cells producing IFN-γ was lower in patients with SCID/OS/AS due to RAG/NHEJ defects than in healthy infants or in patients with other forms of TCD, and a similar trend was observed for CD56dim cells from CID patients. These results suggest that RAG/NHEJ defects are associated with abnormalities of NK cells that are not restricted to their phenotype, but may also involve NK cell function. In this regard, it is particularly significant that NK cells from the patients displayed enhanced degranulation in the absence of previous stimulation with IL-2, and that CD56bright cells from patients with SCID/OS/AS had a higher content of perforin than CD56bright cells from healthy infants. Although we did not perform a chromium release assay to investigate NK cytolytic function, these data suggest that RAG/NHEJ defects are associated with enhanced effector function, similarly to what was previously reported for Rag−/− mice (30).

Expression of RAG may initiate prior to T and B cell differentiation, as indicated by the presence of incomplete rearrangements at the immunoglobulin heavy chain or T cell receptor loci in a minority of murine and human NK cells (29, 50). It has been suggested that introduction of DNA double strand breaks in lymphoid progenitor cells would facilitate selection of cells with more efficient DNA repair machinery (30). In the absence of this mechanism, NK cells from Rag-deficient animals and humans would have reduced cellular fitness. Our data indicate that indeed patients with mutations in RAG or in NHEJ genes share similar abnormalities of NK cell phenotype and function. While it is not known whether the immunoglobulin and T cell receptor loci represent the only sites in the genome that are targeted by RAG during early stages of lymphoid development, it is interesting to note that selective loss of CD56dim cells, with relative preservation of the CD56bright NK cell compartment, has been reported in patients with mutations of MCM4 and POLE2 genes, all of which control DNA replication (51–53).

In conclusion, we have identified for the first time abnormalities of NK cell phenotype and function in patients with mutations in RAG and in genes involved in NHEJ. These abnormalities are more pronounced in patients with severe mutations associated with a more severe clinical and immunological phenotype, and are not secondary to T and B cell lymphopenia. Demonstration of enhanced degranulation activity and higher amount of perforin in NK cells from patients with SCID due to RAG/NHEJ defects suggests that inclusion of serotherapy targeting NK cells in the HSCT conditioning regimen may help reduce the risk of graft rejection that has been reported in these diseases.

ETHICS STATEMENT

The study was approved by the Institutional Review Boards of Boston Children’s Hospital, the National Institute of Allergy and Infectious Diseases (protocols 93-1-0119 and 16-1-N139), and of all other referring centers. Blood samples were obtained upon written informed consent of the subject or, in the case of minors, of their parents or legal guardians.

AUTHOR CONTRIBUTIONS

JM, EM, AM, SP, and LN designed the study, interpreted the data, and wrote the manuscript; KD, GT, EC, OP, PM, SG, and DM performed experiments, acquired, and analyzed the data; WA-H, CC, MC, JB, CB, DB, SB, TC, JC, VD-C, LOdB, MtdtM, GM, AF, RG, RKG, AH, SH, C-HH, MK, AIKi, BK, AnKi, TK, BL, VL, MiMa, IM, MeMo, BN, S-YP, AP, SP, IR, JS, RS, TT, Y-JK, JW, AG, and SK contributed patient samples and clinical and immunological data; all authors have revised the work for its intellectual content, have approved its final version, and have agreed to be accountable for all aspects related to the accuracy and integrity of the work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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