NFKB1 regulates human NK cell maturation and effector functions

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Article history:
Received 8 July 2016
Received in revised form 4 November 2016
Accepted with revision 24 November 2016
Available online 3 December 2016

Keywords:
NFKB1
Natural killer cells
Cytotoxicity
Interferon-γ

Abstract
NFKB1, a component of the canonical NF-κB pathway, was recently reported to be mutated in a limited number of CVID patients. CVID-associated mutations in NFKB2 (non-canonical pathway) have previously been shown to impair NK cell cytotoxic activity. Although a biological function of NFKB1 in non-human NK cells has been reported, the role of NFKB1 mutations for human NK cell biology and disease has not been investigated yet. We decided therefore to evaluate the role of monoallelic NFKB1 mutations in human NK cell maturation and functions. We show that NFKB1 mutated NK cells present impaired maturation, defective cytotoxicity and reduced IFN-γ production upon in vitro stimulation. Furthermore, human IL-2 activated NFKB1 mutated NK cells fail to upregulate the expression of the activating marker Nkp44 and show reduced proliferative capacity. These data suggest that NFKB1 plays an essential novel role for human NK cell maturation and effector functions.

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1. Introduction

The NF-κB (NF-kappaB; nuclear factor of kappa light polypeptide gene enhancer in B cells) signaling pathway plays an important role both in the innate and the adaptive immune system [1–3]. The NF-κB transcription factor family consists of five members: NF-κB1, NF-κB2, RelA, RelB and c-Rel. NFKB1 encodes the precursor p105 which is processed to the mature p50. NFKB2 encodes the precursor p100 and the mature p52. The canonical pathway, which includes NFKB1, mediates numerous immunological and inflammatory cellular responses, and may be activated upon stimulation with a broad range of stimuli, including proinflammatory cytokines, activation of innate immune receptors, T-cell receptor (TCR) and B-cell receptor (BCR) signaling, and others [1–3]. The non-canonical pathway, which involves NFKB2, has more restricted immunological functions mainly focusing on B cell homeostasis and is activated upon engagement of a limited set of members of the TNF receptor superfamily, including BAFF receptor, CD40 and the lymphotxin receptor [2–4].

Data on the NF-κB involvement in NK cell function and maturation are limited. The first indirect description of the biological role of NF-κB in NK cells was derived from in vitro pharmacological NF-κB inhibition that led to impaired NK cell cytotoxicity [5]. Studies on Interleukin-2 (IL-2) induced NK cell functional stimulation have implicated the activation of NF-κB in both, cytotoxic activity and production of Interferon-gamma (IFN-γ) [6]. However, most of these studies did not investigate the role of individual components of the NF-κB signaling pathway. Observations in Nfkb1-deficient mice suggested that p50 is a negative regulator of NK cell proliferation and IFN-γ production [7].

Primary immunodeficiencies provide unique opportunities for a better understanding of the human immune system. For instance, congenital mutations in NIK [8] or NEMO [9], both of which encode upstream components of the NF-κB pathways, have demonstrated their importance for NK cell function. Monoallelic mutations in NFKB2, causing a functional haploinsufficiency due to expression of unprocessable p100 precursors have been reported in CVID patients [10–11] and were shown to impair NK cell cytotoxic activity [12]. Recently, monoallelic mutations in NFKB1 leading to p50 haploinsufficiency have also been reported in three CVID families [13]. However, the impact of these NFKB1 mutations in human NK cell biology has not been investigated. In the present study, we analyzed NK cell...
Table 1
Clinical information of the 7 NFKB1 mutated CVID patients.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Nationality</th>
<th>Sex</th>
<th>Year of birth</th>
<th>Year of diagnosis</th>
<th>IgG*</th>
<th>IgA*</th>
<th>IgM*</th>
<th>Clinical features</th>
<th>Viral infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>Italian</td>
<td>M</td>
<td>1976</td>
<td>1989</td>
<td>160</td>
<td>10</td>
<td>10</td>
<td>Recurrent pulmonary infections, autoimmune thyroiditis, autoimmune enteropathy, gastric adenoma</td>
<td>No EBV, CMV, HBV, HCV (PCR neg.)</td>
</tr>
<tr>
<td>Patient 2</td>
<td>German</td>
<td>F</td>
<td>1961</td>
<td>1994 (2008)</td>
<td>670</td>
<td>30</td>
<td>30</td>
<td>Pneumonias, necrotizing tonsillitis, autoimmune cytopenia (ITP, intermittent leukopenia, anemia), splenomegaly, lymphadenopathy, interstitial lung disease, periodontitis, multiple liver hemangiomas</td>
<td>Herpes zoster (2 episodes) CMV viremia (8500 IE/ml) with CMV colitis treated with valganciclovir; intermittent low grade EBV + CMV replication</td>
</tr>
<tr>
<td>Patient 3</td>
<td>German</td>
<td>M</td>
<td>1963</td>
<td>2006</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>Recurrent sinusitis, recurrent otitis, pneumonia, salmonella enteritis, autoimmune cytopenia, vitiligo, arthritis, splenomegaly, lymphadenopathy, granulomatous lung disease, persistent CRP elevation</td>
<td>No EBV, CMV, HBV (PCR neg.); adenovirus + respiratory virus panel for respiratory infections neg. n.a.</td>
</tr>
<tr>
<td>Patient 4</td>
<td>Dutch</td>
<td>F</td>
<td>1961</td>
<td>1991</td>
<td>181</td>
<td>6</td>
<td>48</td>
<td>Recurrent sinusitis, pneumonia, otitis media, severe salmonella enteritis</td>
<td></td>
</tr>
<tr>
<td>Patient 6</td>
<td>German</td>
<td>M</td>
<td>1956</td>
<td>2003</td>
<td>270</td>
<td>&lt;6</td>
<td>21</td>
<td>Chronic sinusitis, recurrent otitis, pneumonia, skin abscesses, atopic dermatitis with fungal superinfections, autoimmune enteropathy, nodular regenerative hyperplasia, splenomegaly, lymphadenopathy, thrombocytopenia</td>
<td>JC virus encephalitis Norovirus2 infection with intermittent bloody diarrhoea EBV reactivation with 1000 copies/ml, no specific therapy, on follow-up after 1 y neg.</td>
</tr>
<tr>
<td>Patient 7</td>
<td>German</td>
<td>F</td>
<td>1957</td>
<td>1987 (2014)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>Recurrent bronchitis and sinusitis, enteropathy, splenomegaly, basal cell carcinoma, osteoporosis</td>
<td>No EBV, CMV, HBV, HCV (PCR neg.)</td>
</tr>
</tbody>
</table>

Numbers in brackets indicate follow-up examinations.

n.a. not available.

b Patients 2 and 3 are siblings.
a Expressed in mg/dl.
c Not previously described patients.
maturation and effector functions in patients with NFKB1 mutations affecting the canonical NF-κB pathway.

2. Methods

2.1. Monoclonal antibodies

The fine characterization of surface markers of resting and IL-2-activated NK cells was performed using the following monoclonal antibodies (mAbs) generated in our laboratory (Department of Molecular and Translational Medicine, University of Brescia), or in the laboratory directed by A. Moretta (Laboratory of Molecular Immunology, DIMES, University of Genoa): BAB281 (IgG1, anti-NKp46); AZ20 (IgG1, anti-NKp30); ON72 (IgG1, anti-NKGD2); C127 and SUS142 (IgG1 and IgG2b respectively, anti-CD16); C227 (IgG1, anti-CD69); 11PB6 (IgG1, anti-NKp30); ON72 (IgG1, anti-NKG2D); A6/136 (IgM anti-HLA-I).

The commercially available antibodies used in this study are: anti-CCR1 (IgG1, Santa Cruz biotechnologies, Santa Cruz, CA, USA); anti-CD62L (IgG1 R&D Systems, MN, USA); anti-CD14 (IgG1, BD-Biosciences Pharmingen, CA, USA); mixture of FITC-labeled CD3 plus PC5-labeled CD56, FITC-labeled CD14 and FITC-labeled CD20 (Beckman Coulter, Immunotech, Marseille, France); anti-human Ki-67 antigen (IgG1, Dako, Denmark A/S); Annexin V PE (BD-Biosciences, Pharmingen CA, USA); anti-human Perforin/R-PE (AnceCell) and anti-human Granzyme/R-PE (Enzo-Life Sciences).

2.2. Patients, cell preparations and flow cytometry analysis

Seven (7) CVID patients harboring monoallelic NFKB1 mutations were included in this study (Table 1). Since Patients 2 and 3 are siblings and carry the same NFKB1 mutation (Table 1), and due to limitation of biological material available, only one of the two patients was included in each set of experiments. Peripheral blood mononuclear cells (PBMCs) derived from patients and healthy donors, were obtained from heparinized or EDTA blood by density gradient centrifugation over Ficoll (Sigma, St. Louis, MO). PBMCs were resuspended in RPMI 1640 medium, supplemented with 2 mM glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin and 10% heat-inactivated FCS (Fetal Calf Serum, Sigma, St. Louis, MO).

To obtain polyclonal NK cell lines, NK lymphocytes were isolated from PBMCs using negative selection (NK cell isolation Kit, Miltenyi), then cultured on allogenic irradiated feeder cells in the presence of phytohemagglutinin (GIBCO Ltd) [14]. To obtain polyclonal NK cell lines, NK lymphocytes were isolated from PBMCs using negative selection (NK cell isolation Kit, Miltenyi), then cultured on allogenic irradiated feeder cells in the presence of phytohemagglutinin (GIBCO Ltd) [14].

To perform flow cytometry analysis, PBMCs and IL-2-activated NK cells derived from peripheral blood were first stained with appropriate primary monoclonal mouse antibodies, followed by PE-conjugated isotype-specific goat anti-mouse secondary reagent (Southern Biotechnology, Birmingham, AL); cells were subsequently stained with a mixture of FITC-labeled CD3 and PC5-labeled CD56, and FITC-labeled CD14 and FITC-labeled CD20. NK cells analysis by flow cytometry was performed by gating on CD56+CD14+ and CD20+ cells. Cell acquisition was performed on a FACSCanto flow cytometer, and data were analyzed using the Diva software (Becton Dickinson, Mountain View, CA).

For Annexin V staining, IL-2 activated NK lymphocytes were resuspended in 1× Binding Buffer (BD Biosciences) at 1 × 10^6/ml and stained with 5 μl of fluorochrome-conjugated Annexin V PE to 100μl of cell suspension. Analysis of Annexin V was performed by gating on CD56+CD3+ cells. Cells were analyzed on a FACSCanto (BD Biosciences).

2.3. Analysis of NK cell degranulation and IFN-γ production

For degranulation assay against human erythroleukemia cell line K562, PBMCs derived from patients and from healthy donors were incubated with or without 600 IU/ml rh-IL-2 (Proleukin; Chiron) at 37 °C overnight. Then, samples were co-incubated with target cells at a ratio of 1:3 (K562), in a final volume of 200 μl in round-bottomed 96-well plates at 37 °C and 5% CO2 for 3 h in culture medium supplemented with anti-CD107a-PE mAb. After 1 h of co-incubation, GolgiStop (BD Biosciences Pharmingen, San Diego, CA, USA) was added at a 1:100 dilution. Surface staining was done by incubating the cells with anti-CD3, anti-CD14, anti-CD20 and anti-CD56 mAbs for 30 min at 4 °C. Cells were washed and analyzed by flow cytometry (FACS Canto, Becton Dickinson). Analysis of NK cells was made on CD56+CD3+CD14+ CD20+ gated cells.

To detect intracellular production of IFN-γ, PBMCs derived from patients and from healthy donors were incubated overnight at 37 °C with IL12 (20 ng/ml, Peprotech) plus IL18 (100 ng/ml, Peprotech). Cells were then washed, fixed and permeabilized with Cytotix/Cytoperm kit (BD Biosciences Pharmingen). IFN-γ production was detected by subsequent intracellular staining with anti-IFN-γ PE antibody (BD Biosciences Pharmingen) upon gating on CD56+CD3+CD14+ CD20+ cells. For both degranulation and IFN-γ expression experiments, the percentage of positive cells was calculated subtracting the baseline CD107a or IFN-γ expression in controls cultures in the absence of stimuli (target cells or cytokines).

2.4. Analysis of cellular proliferation

Cellular proliferation of IL-2 activated NK cells from patients' and healthy donors' peripheral blood was evaluated by detecting intracellular

![Fig. 1. Novel NFKB1 mutations in adult CVID patients. Electropherograms showing the novel monoallelic null NFKB1 mutations for Patient 1 (A), Patients 2 and 3 (siblings) (B), Patient 6 (C) and Patient 7 (D). Wild type control sequences for the novel NFKB1 monoallelic mutations are shown in the upper panels.](http://example.com/fig1.png)
Ki-67. Polyclonal NK-cell lines were permeabilized with saponin solution (0.1% in PBS) at 4 °C for 20 min. Then cells were incubated with Ki-67 mAb as primary antibody (IgG1, mouse anti-human, 1/40 dilution; Dako, Copenhagen, Denmark) for 1 h at 4 °C, to detect the nuclear proliferation antigen. The secondary antibody used was PE-conjugated isotype-specific goat anti-mouse (Southern Biotechnology, Birmingham, AL) and was incubated for 30 min at 4 °C, then cells were re-suspended in 500 μl of PBS solution for flow cytometry analysis.

2.5. Mutational analysis

Affected individuals were screened with targeted next generation sequencing for mutations in known and prospective genes associated with primary immunodeficiencies as previously described [13]. Sequence variants were verified by Sanger sequencing on genomic DNA according to standard procedures.
2.6. Statistical analysis

Statistical significance was analyzed with the unpaired Student’s t-test by using GraphPad Prism Version 8.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. Patients and novel NFKB1 mutations

Seven adult patients affected with CVID carrying monoallelic NFKB1 mutations were included in this study. Table 1 summarizes their clinical and immunological features. All patients presented with recurrent respiratory infections, both of the upper and the lower respiratory tract. None of the patients presented a history of mycobacterial disease. Lymphadenopathy and splenomegaly is a frequent feature of this cohort of patients (4/7 and 6/7 patients, respectively). Autoimmune manifestations appeared as prominent features of these NFKB1 mutated patients (6/7 patients) and include autoimmune cytopenias (3/7) and autoimmune enteropathy (3/7). Interestingly, 4 out of 7 patients presented a history of herpes zoster infection, a clinical complication that has not been reported in patients with NFKB1 mutations. Furthermore, Patient 2 presented high grade Cytomegalovirus (CMV) replication with CMV colitis and low grade Epstein Barr Virus (EBV) replication (Table 1). Patient 6 suffered from Poliomavirus JC (JCV) encephalitis, intestinal norovirus infection and EBV reactivation (Table 1).

Two of the affected individuals (Patients 4 and 5) were previously reported in the first description of NFKB1 mutations in CVID [13]. The remaining five patients harbor novel monoallelic NFKB1 mutations (Fig. 1A–D). In detail, Patient 1 harbors a novel frameshift mutation in exon 15 (c.1517delC) [14], which predicts expression of a truncated protein (p.Ala506Valfs*17) Patients 2 and 3 are siblings and harbor a nonsense mutation in exon 14 (c.1356delT), leading to a premature termination of translation (p.Val456*). Both, Patient 6 (c.496C>T; p.Arg157*) [15] and Patient 7 (c.295C>T; p.Gln99*) carry nonsense mutations in exon 7 and exon 6, respectively, predicting rapid decay of the severely truncated proteins and thus, p50 haploinsufficiency.

3.2. Impaired human NK cell maturation in the presence of monoallelic NFKB1 mutations

To determine whether monoallelic mutations in NFKB1 affect NK cell maturation, PBMCs from the index patients were evaluated by multicolor flow cytometry. All patients presented normal proportions of circulating CD56 NK cells (Fig. 2A). The evaluation of the expression pattern of activating and inhibitory NK cell receptors, including CD16, Natural Cytotoxicity Receptors (NCRs) (NKp46, NKp30), KIRs (killer cell immunoglobulin-like Receptors, HLA class I specific receptors), NKG2A (inhibitory receptor), NKG2C (activating receptor), CD62L (cell adhesion molecule), CD57 and chemokine receptors (CXCR1, CCR7) on human NK cells (both CD56bright and CD56dim subsets) allowed us to discriminate NK cell subsets based on their maturational and functional status [16]. Precisely, while the percentage of CD56+ NK cells expressing the NCR Nkp30 was similar between patients and controls (Supplementary Fig. 1), the percentage of CD56+ NK cells expressing the NCR Nkp46 was significantly reduced in patients carrying NFKB1 mutations when compared to healthy controls (Fig. 2B). Similar results were obtained when the expression of KIRs on patients’ NK cells was evaluated: Patients with NFKB1 mutations showed reduced percentages of CD56+ NK cells expressing KIRs when compared to healthy controls (Fig. 2C). The expression of CD57, a classical NK maturation marker [17], showed an interesting pattern (Fig. 2D). The percentages and the mean fluorescence intensity (MFI) for the CD56brightCD57low NK cell population were similar in both test groups (Fig. 2D, upper right panels). In contrast, the CD56dimCD57hi NK cell population was significantly reduced in affected individuals with NFKB1 mutations when compared to healthy controls, both in terms of percentages and MFI (Fig. 2D, lower right panels).

CD62L belongs to the selectin family proteins and represents an additional marker for human NK cell maturation [17]. The expression of this marker on NFKB1 mutated NK cells showed an accumulation of

Fig. 3. Expression of CD62L on NFKB1 mutated human NK cells. A. Representative dot plots showing expression of CD62L on CD56+ NK cells from a healthy control and an NFKB1 mutated patient (upper left panel). Representative dot plots showing percentages of CD56brightCD62Llow (red square), CD56dimCD62Lhi (blue square) and CD56dimCD62L− NK cells from a healthy control and an NFKB1 mutated patient (lower left panel). B, C, D. Scatter plots showing percentages and MFI for CD56brightCD62Llow (B), CD56dimCD62Lhi (C) and CD56dimCD62L− (D) NK from NFKB1 mutated patients (n = 6) and healthy controls (CTRs) (n = 10). Data were obtained from single experiments performed in triplicate and statistical analysis was performed using the t-student test (** = p < 0.005; *** = p < 0.0005).
CD56brightCD62Llow NK cells, a finding that was not observed in healthy controls (Fig. 3A and B). The proportion of the CD56brightCD62Lhigh population among the NK cells with NFKB1 mutations was variable when compared to healthy controls; however, the MFI of CD62L within this subset was significantly lower in NFKB1 mutated patients when compared to healthy controls (Fig. 3A and C). Furthermore, the CD56dimCD62Lhigh subset was strongly reduced in NK cells from patients with NFKB1 mutations when compared to healthy controls, both in terms of percentages and MFI (Fig. 3A and D). These data suggest that the canonical NF-κB pathway orchestrates unique aspects of human NK cell maturation stages.

Evaluation of activating (CD16) and chemokine (CXCR1, CCR7) receptors further revealed the involvement of the canonical pathway in human peripheral NK cell maturation/homeostasis (Fig. 4). The percentage of CD56bright NK cells expressing CXCR1 were significantly reduced in NFKB1 mutated patients as compared to healthy controls (Fig. 4A). The percentage of CD56brightNK cells expressing CCR7 (Fig. 4B) was variable among the NFKB1 mutated patients, similarly as observed in the healthy controls. The percentages of CD56bright NK cells expressing CCR7 were similar between NFKB1 mutated patients and healthy controls (Fig. 4C). Interestingly, CD16 showed reduced MFI in patients with NFKB1 mutations compared to the healthy controls (Fig. 4D). The expression of the remaining NK cell receptors was normal (Supplementary Fig. 1). In summary, these data provide evidence for an essential role of NFKB1 in human NK cell maturation.

### 3.3. Impaired in vitro degranulation of NK cells derived from patients with NFKB1 mutations

Our results indicated that NK cell maturation was perturbed in the presence of monoallelic NFKB1 mutations. Based on previous published data from mice studies [7] and on our previous observations in NK cells derived from a patient with a heterozygous NFKB2 mutation [12], we next investigated the functional consequences of NFKB1 mutations in human NK cell biology. Baseline degranulation against the human erythroleukemia cell line K562, measured as CD107a expression of resting NK cells [18], was similar in both test groups (NFKB1: 19.33% mean value, n = 6; healthy controls: 24.12% mean value, n = 10) (Fig. 5A upper panel and B). Upon IL-2 stimulation in vitro (Fig. 5A lower panel and 5B), NK cell degranulation against the human erythroleukemia cell line K562 was significantly reduced in cells carrying NFKB1 mutations (44.66%; n = 6; p < 0.005) when compared to healthy controls (60.71%; n = 10). These data collectively provide evidence for a novel role for the canonical pathway in human NK cell degranulation similar to the previously described involvement of the non-canonical pathway [12].

### 3.4. NK cells fail to produce Interferon-γ in the presence of monoallelic NFKB1 mutations

Based on our findings on the implication of the canonical NF-κB pathway in human NK cell maturation and degranulation, as well as on studies in p50 deficient mice [7], we next investigated whether IFN-γ production, a prominent biological feature of NK cells, was also affected due to the presence of monoallelic NFKB1 mutations. We thus evaluated the production of IFN-γ in NK cells derived from patients with NFKB1 mutations and age-matched healthy control donors upon simultaneous stimulation with IL-12 plus IL-18 [19]. As expected, unstimulated NK cells (Fig. 5C upper panel and 5D) in both test group did not produce IFN-γ (NFKB1: 11%, mean value, n = 6; healthy donors: 8%, mean value, n = 10). However, upon combined stimulation with IL12 and IL-18 (Fig. 5C lower panel and D), IFN-γ production was significantly reduced in NK cells from patients carrying NFKB1 mutations (32.43%, mean value, n = 6; p < 0.001) when compared to age-matched healthy control samples (72.83%, mean value, n = 10). These results collectively suggest that NFKB1 plays a critical, previously unrecognized, role in IFN-γ production upon in vitro NK cell stimulation with IL-12 and IL-18.

### 3.5. IL-2 activated NK cells from patients with NFKB1 mutations fail to up-regulate the activation marker NKp44 and display impaired proliferative capacity

Since NK cells derived from individuals carrying NFKB1 mutations had an aberrant receptor profile expression as well as impaired
in vitro degranulation and IFN-γ production, we also tested whether IL-2 activated NK cell populations showed phenotypical or functional alterations. We therefore evaluated the expression of the NK cell activating receptor NKp44, a member of the NCR family together with NKp46 and NKp30[20]. Expression of NKp30 in both, resting (Supplementary Fig. 1) and IL-2 activated NK cells (data not shown) was normal in all analyzed samples. In contrast, the expression of the activating receptor of NKp44 on IL-2 stimulated NK cells from patients with NFKB1 mutations was strongly reduced and remained persistently lower over a 4 weeks observation period (time points of evaluation: 14, 21 and 28 days) when compared to the controls (Fig. 6A). The expression of NKp46, another member of the NCR family[20], was reduced in unstimulated NK cells from patients with NFKB1 mutations (Fig. 2B), and remained unaffected upon IL-2 stimulation after 21 days of IL-2 stimulation (Fig. 6B), suggesting that the canonical NF-kB pathway plays a distinct role on the expression of activation markers, such as NKp44, on human NK cells.

We furthermore evaluated the intracellular expression of Ki-67 as an index of the proliferative capacity of IL-2 activated NK cells derived from patients with NFKB1 mutations and healthy controls. The proliferative responses upon IL-2 activation of NK cells harboring NFKB1 mutations were impaired as compared to healthy controls (Fig. 6A). The expression of NKp46, another member of the NCR family[20], was reduced in unstimulated NK cells from patients with NFKB1 mutations (Fig. 2B), and remained unaffected upon IL-2 stimulation after 21 days of IL-2 stimulation (Fig. 6B), suggesting that the canonical NF-kB pathway plays a distinct role on the expression of activation markers, such as NKp44, on human NK cells.

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4. Discussion

The two NF-κB pathways, canonical and non-canonical, play an important role in immune cell biology. Although diverse in terms of components and type of activating stimuli, both pathways have been shown to play crucial roles in distinct biological functions of immune cells [1–3]. This diversity has been studied mainly in mouse models and mainly for B and T lymphocytes [1,4]. Data on the involvement of NF-κB in NK cell function and maturation are limited. Regarding the NFKB1-dependent canonical pathway, studies in p50-deﬁcient knock-out mice suggested that p50 is a negative regulator of NK cell proliferation and IFN-γ production [7]. However, little is known about the involvement of NFKB1 in human NK cell biology in terms of maturation and effector functions. In the present study, we analyzed NK cells derived from patients with mutations in NFKB1 as an “experimentum naturae” model and provide the first evidence for an essential role for NFKB1 in human NK cell biology.

Human NK cell maturation appeared to be impaired in the presence of monoallelic NFKB1 mutations. Whereas patients with NFKB1 mutations showed normal numbers of peripheral NK cells, mutant NK cells showed reduced expression of NKp46, KIRs and CD57. Regarding CD57 in particular, a classical maturation marker[17], while the CD56dimCD57hi NK cell subset occurred normal, NK cells with NFKB1 mutations presented a significant reduction of the CD56dimCD57hi population when compared to healthy controls. Previously published data [21–22] have shown that high CD57 expression levels, co-expressed
with NKG2C, on CD56\textsuperscript{dim} NK cells, define a terminal maturational step for CD56\textsuperscript{dim} NK cells and depict a human viral memory NK cell subset, which lacks in NK cells derived from patients with mutations in \textit{NFKB1}. Exposure to CMV has been shown to up-regulate CD57 and NKG2C on human NK cells\cite{23–24}. Within this cohort of patients, only one patient presented an infection from CMV. Thus, while the majority of the patients included in this study (6/7) resulted negative for CMV infection, it is not yet known whether the viral infections that were documented in the \textit{NFKB1} mutated patients, i.e. herpes zoster (4/7), JC virus (1/7), Norovirus (1/7) or EBV (2/7), may up-regulate the above mentioned markers in human NK cells. These observations underline a direct and specific role for the canonical pathway in this maturation step of human NK cells.

The chemokine receptors on NK cells with heterozygous \textit{NFKB1} mutations showed variable expression of CCR7, but clearly reduced expression of CXCR1 and CD62L. Regarding the latter, NK cells from patients with \textit{NFKB1} mutations showed an expansion of the CD56\textsuperscript{bright}CD62L\textsuperscript{low} subset and a severe reduction of the CD56\textsuperscript{dim}CD62L\textsuperscript{+} subset when compared to healthy controls. This particular subset of CD56\textsuperscript{dim}CD62L\textsuperscript{+} NK cells has been well studied\cite{25} and represents an intermediate stage of NK cell maturation with multiple functional capacities that can develop into terminally differentiated effectors with elevated capability of IFN-\gamma production\cite{25}. Therefore, these data provide further evidence for a specific involvement of the canonical NF-\kappaB signaling in human NK cell maturation that may also underlie defective IFN-\gamma production.
Taken together, our findings define a novel intrinsic role for NFKB1 in human NK cell maturation and effector functions. Furthermore, our data broaden the spectrum of NF-κB-related PIDs with NK cell defects (Fig. 7) and indicate that longer follow-up examinations and larger cohorts of patients are required for a better characterization of the clinical implications of human NK cell impairment in the presence of heterozygous NFKB1 mutations.

5. Conclusions

In this study, we provide evidence that monoallelic NFKB1 mutations affect human NK cell homeostasis in terms of maturation and effector functions. NFKB1 mutated NK cells showed impaired peripheral maturation with a significant reduction of the CD56dimCD57hi, CD56dimCD62L+ NK cell subsets, as well as altered expression pattern of NKP46, KIRs, CXXC1, CCCR7 and CD16. Furthermore, NFKB1 mutated NK cells showed functional impairments in terms of cytolysis, IFN-γ production, NKP44 up-regulation and proliferation. From a clinical point of view, NFKB1 haplosufficient patients show a high prevalence of viral infections. Taken together, our data suggest an important, novel role for NFKB1, thus for the canonical NF-κB pathway, in human NK cell biology and human disease.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2016.11.012.

Funding

The research leading to these results has received funding from the European Community’s Seventh Framework Programme FP7/2007–2013 under grant agreement no 201549 (EURO-PADnet HEALTH-F2-2008-201549) and from the Italian Ministerial Grant GR-2010-2315762. The research leading to these results also received funding from the “Fondazione C. Golgi”, Brescia, Italy and Associazione Immunodeficienze Primitive (A.I.P.). This study was supported by the German Federal Ministry of Education and Research (BMBF 01EO1303).

Acknowledgments

We would like to thank Fondazione Camillo Golgi, Brescia, Italy and Associazione Immunodeficienze Primitive (A.I.P.). We would also like to thank Alessandro Moretta for providing monoclonal antibodies anti-NK cells receptors, produced in the Laboratory of Molecular Immunology, DIMES, University of Genoa, Italy. We would also like to thank the patients, the patients’ families and the nurses for all their efforts. We thank Katrin Hübscher and Jessica Rojas-Restrepo for excellent technical assistance.

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Further Reading
