Natural-Killer-like B Cells Display the Phenotypic and Functional Characteristics of Conventional B Cells

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A recent study focusing on the expression of very commonly used lineage markers reported the existence of a population of lymphocytes harboring phenotypic traits typical of both B cells and natural killer (NK) cells together with markers not associated with either lineage (Wang et al., 2016). These newly described "NKB cells" simultaneously expressed the B cell markers immunoglobulin M (IgM), $Ig\alpha$, $Ig\beta$, CD19, CD20, and CD21 and the NK cell markers NK1.1 and NKp46, as well as several NK cell receptors of the Ly49 and CD94/NKG2 families. Despite their phenotype and specific location in the marginal zone of the spleen and mesenteric lymph nodes, they were not attributed with classical B and/or NK cell functions, such as IgM secretion or cell cytotoxicity and interferon-γ (IFN-γ) secretion. Instead, the cells were suggested to serve as a very early and necessary source of interleukin-12 (IL-12) and IL-18 in various models of viral and bacterial infections to facilitate the priming of NK cells and type 1 innate lymphoid cells (ILC1s) (Wang et al., 2016).

Given these surprising and potentially important functions of NKB cells, we sought to investigate this population further by using an array of mouse genetic models. We used a stringent gating strategy designed to preserve rare true NK1.1$^+$ events while excluding potential artifacts generated by doublets, dead cells, antibody aggregates, or auto-fluorescent cell populations and excluding irrelevant cell populations, such as NKT cells. Consistent with the findings of Wang et al., CD19$^+$NK1.1$^+$ cells were detected in various primary and secondary lymphoid organs, but the frequencies of these detected events were consistently much lower (by at least a factor of five) than previously reported (Figure S1A). These events accounted for 0.02%– 0.05% of the total CD45$^+$ hematopoietic cell population and were detected mostly in the spleen and blood rather than the bone marrow and lymph nodes, although there was no marked overall tissue specificity (Figure S1A). Notably, detailed phenotypic analysis of these events revealed that although all cells appeared to be IgM$^+$, most did not co-stain for the NK cell marker NKp46, CD63, or CD106, each of which was previously reported to be expressed by NKB cells (Figure S1B). CD19$^+$NKp46$^+$ cells were also detected in multiple organs at similar frequencies (data not shown). We excluded possible inconsistencies in antibody staining by analyzing reporter mouse models. We used Ncr1-driven Cre models, given that Ncr1 encodes the cell-surface receptor NKp46. Using both the R26R$^{eYFP/+}$ Ncr1-iCre fate map model (Narni-Mancinelli et al., 2011) and Ncr1-GFP mice (Gazit et al., 2006), we observed that most, if not all, NK1.1$^+$CD19$^+$ cells were negative for Ncr1 expression (Figure S1C). Like all ILC lineages, NKB cells were reported to express the transcription factor Id2, which is required for their development (Wang et al., 2016). However, we found that the vast majority of splenic NK1.1$^+$CD19$^+$ cells from Id2$^{GFP/GFP}$ mice lacked GFP (Figure S1C) (Delconte et al., 2016). In conclusion, although we could confirm the presence of low numbers of CD19$^+$ cells that bound monoclonal antibodies (mAbs) specific to NK1.1, NKp46 expression was rare on these cells, and the phenotype was distinct in other respects from the one described for NKB cells.

Because our findings were very different from those originally reported for NKB cells, we decided to analyze other genetic models of NK cell depletion. Mcl1$^{f/f}$ Ncr1-iCre mice are profoundly deficient in NK cells as a result of the critical role of Mcl-1 in NKp46$^+$ cell development and maintenance (Sathe et al., 2014). In R26R$^{DTA/+}$ Ncr1-iCre mice, activation of the Ncr1 promoter triggers the expression of diphtheria toxin fragment A, leading to NKp46$^+$ cell death (Deauvieau et al., 2016). We were unable to detect any modification of NK1.1$^+$CD19$^+$ frequencies in these two Ncr1-driven cell-depletion models (Figure S1D and data not shown), consistent with the infrequent expression of NKp46 by NK1.1$^+$CD19$^+$ cells, as noted above. Of note, we detected no significant effect on NKp46$^+$CD19$^+$ cells in either of these two mouse models (Figure S1D and data not shown). These data led us to conclude that NKp46 was not endogenously expressed by these cells and that the binding of anti-NKp46 (and possibly anti-NK1.1) mAbs to CD19$^+$ cells was independent of the specificities of these mAbs.

To address this possibility, we examined NK1.1 staining in BALB/c mice,

which display no reaction to the NK1.1 antibody PK136 because of allelic variations in *Nkrp1b* and *Nkrp1c* (Carlyle et al., 2006), and NKp46 staining in *Ncr1*$^{GFP/GFP}$ mice, which lack NKp46 expression as a result of disruption of *Ncr1* by a GFP reporter cassette (Gazit et al., 2006). In fact, the frequencies of CD19$^+$ cells that co-stained with the NK1.1 or NKp46 mAbs were unaltered in mice that lacked NK1.1 or NKp46, respectively (Figures S1E and S1F). Thus, the binding of the NK1.1 and NKp46 mAbs to CD19$^+$ cells was independent of the antigen specificities of the antibodies.

mAbs bind to various cell types via an interaction between their Fc portion and Fc receptors, and B cells strongly express the FcγRIIB receptor. Herein, we performed all antibody staining in the presence of high concentrations of unlabeled blocking anti-CD16 and anti-CD32 antibodies (FcγRIII and FcγRIIB, respectively) to prevent Fc binding to Fc receptors. This suggested that the binding of the anti-NK1.1 and anti-NKp46 mAbs was not mediated by FcγRIIB on B cells. This conclusion was corroborated with the use of FcγRIIB-deficient mice and FcγR-deficient mice lacking FcγRI, FcγRIIB, FcγRIII, and FcγRIV (Gillis et al., 2017). The frequencies of CD19$^+$ cells that co-stained with anti-NK1.1 (CD19$^+$NK1.1$^+$ C57BL/6 mice: 0.045 ± 0.002; FcγRIIB-deficient mice: 0.06 ± 0.003; FcγR-deficient mice: 0.06 ± 0.002; mean ± SEM) or anti-NKp46 (data not shown) were similar between the mutant strains and the wild-type strain. These data formally excluded a role for Fcγ receptors in the binding of anti-NK1.1 and anti-NKp46 mAbs to B cells.

We then sought to identify more precisely the cells binding anti-NK1.1 and anti-NKp46 mAbs and the mechanisms involved. Given that NKB cells have been described as CD19$^+$IgM$^+$ and expressing the B cell identity regulator Pax5 (Wang et al., 2016), we focused on the B cell lineage. A cardinal feature of mature B cells, not shared with the reported functions of NKB cells (Wang et al., 2016), is their ability to differentiate into CD138$^+$Blimp1$^+$ antibody-secreting plasmablasts (the rate of differentiation in the presence of liposaccharide [LPS] varies between follicular B cells [slow]

and marginal zone or B1 B cells [rapid]) (Fairfax et al., 2007). Sorted NK1.1$^+$ CD19$^+$ cells stimulated for 3 days with LPS readily differentiated into CD138$^+$ Blimp-1$^+$ plasmablasts at a rate more akin to that of marginal zone B cells than follicular B cells (Figure S1G). In parallel, we evaluated the capacity of NK1.1$^+$CD19$^+$ spleen cells to proliferate and survive in the presence of factors known to support the viability and expansion of bona fide NK cells. We focused on IL-15, given that NKB cells have been reported to survive and expand in the presence of this cytokine (Wang et al., 2016). However, similar to B cells and consistent with their CD122$^-$ phenotype, splenic NK1.1$^+$CD19$^+$ cells did not survive in the presence of IL-15, and the few remaining live cells were mostly NK1.1$^-$NKp46$^-$ (Figure S1H). Together, these data strongly suggest that NK1.1$^+$CD19$^+$ cells are B cells that possess the ability to rapidly differentiate into antibody-secreting cells after LPS stimulation.

We further explored the FcγR-independent mechanisms by which anti-NK1.1 and anti-NKp46 mAbs bound to B cells by considering the possibility that this binding might be due to direct recognition of the mAbs by surface Igs expressed by a subset of B cells. We therefore investigated whether restricting the B cell receptor (BCR) repertoire would alter the binding of the anti-NK1.1 and anti-NKp46 mAbs to B cells. We used MD4 transgenic mice, in which most, if not all, B cells express a single anti-HEL BCR (Goodnow et al., 1988). NK1.1$^+$CD19$^+$ and NKp46$^+$CD19$^+$ events were extremely rare in analyses of peripheral-blood cells from these mice. Restriction of the BCR repertoire, therefore, strongly limited anti-NK1.1 and anti-NKp46 mAbs binding to B cells (Figure S1I). These data support our hypothesis that staining with anti-NK1.1 and anti-NKp46 mAbs results from binding of these mAbs to a subset of BCRs present in the normal polyclonal B cell repertoire.

In summary, our findings demonstrate that NK1.1$^+$CD19$^+$ and NKp46$^+$CD19$^+$ cells do not express NK1.1 or NKp46 and are not a distinct population of NKB cells; instead, they display the phenotypic and functional characteristics of conven-

tional B cells. The expression of CD21 and their anatomical localization (Wang et al., 2016), combined with the rapid differentiation into plasmablasts upon LPS exposure, suggest that many of the CD19$^+$ cells binding NK1.1 or NKp46 mAbs in the spleen are marginal zone B cells, but our identification of these cells in other organs that lack marginal-zone structures suggests that other mature B cell populations also fall into the NKB cell gate. Overall, these data highlight the need for extreme vigilance in the analysis of antibody-based cell-identification experiments, particularly as they apply to IgM$^+$ B cells.

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Supplemental Information

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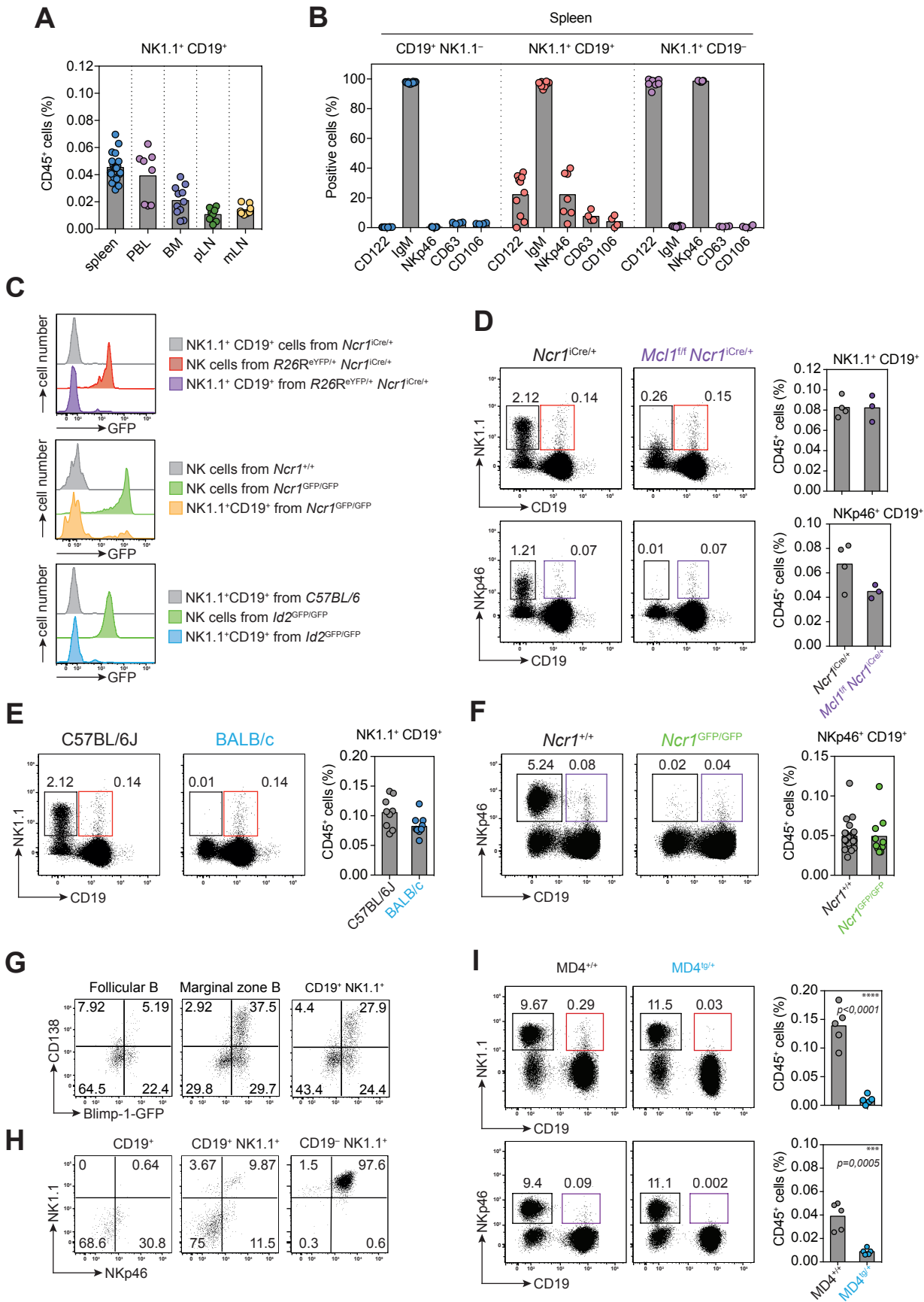
A

NK1.1⁺ CD19⁺

CD45⁺ cells (%): spleen, PBL, BM, pLN, mLN

B

Spleen

CD19⁺ NK1.1⁻ | NK1.1⁺ CD19⁺ | NK1.1⁺ CD19⁻

Positive cells (%): CD122, IgM, NKp46, CD63, CD106

C

NK1.1⁺ CD19⁺ cells from *Ncr1*^iCre/+
NK cells from *R26R*^eYFP/+ *Ncr1*^iCre/+
NK1.1⁺ CD19⁺ from *R26R*^eYFP/+ *Ncr1*^iCre/+

NK cells from *Ncr1*^+/+
NK cells from *Ncr1*^GFP/GFP
NK1.1⁺CD19⁺ from *Ncr1*^GFP/GFP

NK1.1⁺CD19⁺ from *C57BL/6*
NK cells from *Id2*^GFP/GFP
NK1.1⁺CD19⁺ from *Id2*^GFP/GFP

D

Ncr1^iCre/+ | *Mcl1*^f/f *Ncr1*^iCre/+

NK1.1 vs CD19: 2.12, 0.14 | 0.26, 0.15
NKp46 vs CD19: 1.21, 0.07 | 0.01, 0.07

NK1.1⁺ CD19⁺ — CD45⁺ cells (%): *Ncr1*^iCre/+, *Mcl1*^f/f *Ncr1*^iCre/+
NKp46⁺ CD19⁺ — CD45⁺ cells (%): *Ncr1*^iCre/+, *Mcl1*^f/f *Ncr1*^iCre/+

E

C57BL/6J | BALB/c

NK1.1 vs CD19: 2.12, 0.14 | 0.01, 0.14

NK1.1⁺ CD19⁺ — CD45⁺ cells (%): C57BL/6J, BALB/c

F

Ncr1^+/+ | *Ncr1*^GFP/GFP

NKp46 vs CD19: 5.24, 0.08 | 0.02, 0.04

NKp46⁺ CD19⁺ — CD45⁺ cells (%): *Ncr1*^+/+, *Ncr1*^GFP/GFP

G

Follicular B | Marginal zone B | CD19⁺ NK1.1⁺

CD138 vs Blimp-1-GFP
Follicular B: 7.92, 5.19, 64.5, 22.4
Marginal zone B: 2.92, 37.5, 29.8, 29.7
CD19⁺ NK1.1⁺: 4.4, 27.9, 43.4, 24.4

H

CD19⁺ | CD19⁺ NK1.1⁺ | CD19⁻ NK1.1⁺

NK1.1 vs NKp46
CD19⁺: 0, 0.64, 68.6, 30.8
CD19⁺ NK1.1⁺: 3.67, 9.87, 75, 11.5
CD19⁻ NK1.1⁺: 1.5, 97.6, 0.3, 0.6

I

MD4⁺/⁺ | MD4^tg/+

NK1.1 vs CD19: 9.67, 0.29 | 11.5, 0.03
NKp46 vs CD19: 9.4, 0.09 | 11.1, 0.002

CD45⁺ cells (%): MD4⁺/⁺, MD4^tg/+
p<0,0001 ****
p=0,0005 ***

Figure S1. Tissue distribution and phenotypic analysis of 'NKB-like' cells

(A) Cumulative frequencies of NK1.1$^+$CD19$^+$ events among CD45$^+$ cells from peripheral lymphoid tissues. Pooled data from 8 independent experiments, including 8 to 20 mice in total.

(B) Phenotypic analysis of B cells, NK cells and NK1.1$^+$CD19$^+$ events in the spleen of C57BL/6 mice. Pooled data from 4 to 10 mice per group, analyzed in 2 independent experiments.

(C) Representative comparison of Ncr1 'fate map' YFP ($R26R^{eYFP/+}$ $Ncr1^{iCre/+}$), Ncr1-driven GFP ($Ncr1^{GFP/GFP}$) and $Id2$ driven GFP expression in NK1.1$^+$CD19$^+$ cells and NK1.1$^+$CD19$^-$ cells (NK cells) from naïve spleen of mice of the indicated genotype. (n= 10 $Ncr1^{+/+}$ and 10 $Ncr1^{GFP/GFP}$ total analyzed in 4 independent experiments) (n=8 $R26R^{eYFP/+}$ $Ncr1$-iCre and 5 $Ncr1$-iCre total analyzed in 3 independent experiments) (n=4 $Id2^{GFP/GFP}$ and 4 C57BL/6 total analyzed in 3 independent experiments)

(D, E, F) Representative flow cytometry analysis and cumulative frequencies of CD11b and autofluorescence$^-$CD3ε$^-$CD45$^+$ live spleen NK1.1$^+$CD19$^+$ cells and NK1.1$^+$CD19$^-$ cells (NK cells) from mice of the indicated genotypes. For each panel, one dot represents one mouse, bar graphs show the mean.

(G) Follicular B cells (CD19$^+$CD23$^+$CD21$^-$), marginal zone B cells (NK1.1$^-$CD23$^-$CD21$^+$) and NK1.1$^+$CD19$^+$ cells were sorted from the $Prdm1^{GFP/+}$ reporter strain and cultured in the presence of LPS (5 µg/ml) for three days. Dot plots show representative expression from one of three independent experiments (n=3 pooled mice/experiment).

(H) Sorted B cells (CD19$^+$), NK1.1$^+$CD19$^+$ cells, and NK cells (NK1.1$^+$CD19$^-$CD3ε$^-$) were cultured in the presence of IL-15 (10 ng/ml) for three days. Cells were assessed by flow cytometry for cell viability (top panel) and the expression of NKp46 and NK1.1 (bottom panel).

(I) Peripheral blood mononuclear cells from wild-type and anti-HEL MD4 BCR-transgenic mice were analyzed for NK1.1 and CD19 antibody binding to CD11b and autofluorescence$^-$CD3ε$^-$CD45$^+$ live cells. For each panel, one dot represents one mouse, bar graphs show the mean. P values compare the genotypes using a Mann-Whitney test.