Expansion of an Atypical NK Cell Subset in Mouse Models of Systemic Lupus Erythematosus

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Chronic inflammatory conditions, such as in autoimmune disease, can disturb immune cell homeostasis and induce the expansion of normally rare cell populations. In our analysis of various murine models of lupus, we detect increased frequency of an uncommon subset identified as NK1.1CD11cCD122+MHC class II+. These cells share characteristics with the NK cell lineage and with cells previously described as IFN-producing killer dendritic cells: 1) they depend on IL-15 and express E4BP4; 2) they are cytotoxic and produce type I and type II IFN upon activation; and 3) they are efficient APCs both through MHC class II expression and in cross-presentation to CD8s. These atypical NK cells are responsive to TLR stimulation and thus are most abundant in mice with high copy number of the Tlr7 gene. They are highly proliferative as assessed by in vivo BrdU incorporation. In adoptive transfer experiments they persist in high numbers for months and maintain their surface marker profile, indicating that this population is developmentally stable. Gene expression analyses on both mRNA and microRNAs show a modified cell cycle program in which various miR-15/16 family members are upregulated, presumably as a consequence of the proliferative signal mediated by the increased level of growth factors, Ras and E2F activity. Alternatively, low expression of miR-150, miR-181, and miR-744 in these cells implies a reduction in their differentiation capacity. These results suggest that cells of the NK lineage that undergo TLR stimulation might turn on a proliferative program in detriment of their full differentiation into mature NK cells. The Journal of Immunology, 2015, 194: 000–000.
These cells lack some mature NK cell surface markers but they express NK1.1, CD122, and several NK-restricted transcription factors, and therefore they most likely arise from activation of an immature precursor. In this study we show that TLR activation of NK cell precursors induces considerable alterations of both microRNA (miRNA) and mRNA expression patterns that could explain the expansion of immature populations under conditions of chronic activation conditions.

Materials and Methods

Mice and experimental protocols

The generation of TLR7g and B6.FcyRIBB" mice has been described earlier (8, 10). All experiments in this study used the transgenic line 7.1 that harbors 8–16 copies of the TLR7 gene. All other C57BL/6 (B6), IL-15/-, and B6.pptm mice were obtained from the National Institute of Allergy and Infectious Diseases mouse colony at Taconic Farms. B6.FcyRIIB" mice were crossed to B6yaa mice to obtain B6.FcyRIIB"yaa mice. Housing at the National Institutes of Health facility met the Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Mice were used at 8–12 wk of age and were age and sex matched within experiments. Twelve-week-old B6 mice received an i.p. injection every other day for 2 wk with 50, 100, and 200 μg imiquimod or resimiquimod (R848; InvivoGen, San Diego, CA).

In vivo assays for BrdU incorporation by proliferating cells used the BrdU staining kit from eBioscience (San Diego, CA), used according to the manufacturer’s protocol. In brief, mice were fed with water containing 0.8 mg/ml BrdU for 14 d. Incorporated BrdU was detected by flow cytometric analysis after permeabilization and staining with anti-BrdU FITC Ab.

Flow cytometry

Spleen, liver, and lymph nodes prepared to single-cell suspensions were stained with various fluorochrome-conjugated Abs purchased from eBioscience (San Diego, CA) and BD Biosciences (San Jose, CA). Cd block (CD16/32) was added to prevent unspecific binding. FACS analysis was done on FACScalibur and LSR II machine (BD Biosciences), and data were analyzed using FlowJo software.

Bone marrow chimeras

Bone marrow (BM) cells were isolated from femurs and tibias of TLR7.1tg or B6.pptm mice aged 6–7 wk and then mixed in equal proportions for the transfer. Eight to 10-wk-old B6 recipient mice were irradiated with 940 krad 24 h prior to the transfer. BM cells (1 × 10^7) were injected retroorbitally into each recipient. All mice, donors and recipients, were female.

Bone marrow chimeras were analyzed using FlowJo software.

Ag presentation assays

The APCs, that is, splenocytes or NK1.1" cells purified from spleens of wild-type (WT)/TLR7tg mice, were irradiated with 4000 rad to prevent their proliferation, and 2 × 10^6 were injected for 72 h with OVA-specific CD8 T cells (CD4" selection of OT-II splenocytes by beads from StemCell Technologies) in the presence of different concentrations of OVA257-264 peptide (AnaSpec, Fremont, CA). CD4" cells were labeled with CFSE (Molecular Probes/Invitrogen, V12883) before incubation so that T cell proliferation could be measured by CFSE dilution in flow cytometry.

The procedure for cross-presentation of cell-associated Ag was as described earlier (26). Briefly, OVA-specific CD8 T cells were isolated from spleens of OT-I mice using a RoboSep CD8" selection kit (StemCell Technologies) and were labeled with CFSE (Molecular Probes/Invitrogen, V12883). They were then cultured with 2 × 10^5 OVA-albumin-coated irradiated H-2bm1 splenocytes or NK1.1" cells purified from either WT or TLR7tg spleens. Proliferation was quantified 60–65 h later by calculating the number of CD8 cells with reduced green fluorescence by flow cytometry.

Cytotoxic responses

YAC-1 cells (susceptible to NK cytotoxic activity) and reference cell line EL4 were labeled with 1 or 5 CFSE μM, respectively. NK1.1" isolated from WT or TLR7tg spleens were incubated for 20 h with these two cell lines at different ratios between effector and target cells. The change in ratio between CFSE* and CFSE* cells was determined by flow cytometry and interpreted as cytotoxic activity relative to background apoptosis of cells. Additionally, cytotoxic activity was measured by caspase activity in live cells by using CytoToxLum Plus (OncoImmunin) according to the manufacturer’s instructions.

Adoptive transfer

Transferred NK1.1"CD11c" cells were sorted by FACS (BD Biosciences) or RoboSep (StemCell Technologies). Transferred NK1.1" cells were purified by a combination of CD4"NK1.1 and CD11c" bead selection (RoboSep, StemCell Technologies) from cell suspension depleted of CD4 cells by a CD4" selection kit (StemCell Technologies). Cells (3–5 × 10^6) were injected i.v. per mouse. Recipients were untouched WT mice.

Genotyping and real-time PCR

For genotyping, IL-15/- mice we used following primers: aczo, 5'-GAA TGG CAC CTG GAG CCC TTC CTC G-3'; downstream, 5'-TCA TAT CCT CTG CAC CCT TAC TG-3'; upstream, 5'-GAG GCC TAA ACG TCA TGA TGC GTG TG-3'; exon 3, 5'-GAG CTG CATG ATG GCC GCG GGC-3'. Quantitative PCR on genomic DNA and cDNA were used to measure levels of TLR7, as described previously (6); mRNA levels of samples were normalized to actin. For isolation of mRNA, purified cells were resuspended in TRIzol (Invitrogen, Carlsbad CA) and incubated for 5 min. RNA was extracted using chloroform and precipitation with isopropanol. RNA pellets were washed and resuspended in nuclease-free water, and integrity was tested by using an Experion RNA StdSens electrophoresis machine.

cDNA was synthesized using an iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

Transmission electron microscopy

Spleens were obtained from WT and TLR7tg mice, frozen in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) on dry ice, and stored at −80°C until sectioning. Organs were sectioned at 5–10 μm using a Leica 1850 cryostat (Leica, Wetzlar, Germany) and stored at −20°C until used. Before staining, sections were allowed to equilibrate to room temperature, fixed in cold acetone, and rehydrated in PBS. To reduce nonspecific staining, 10% horse serum was applied for 20 min to all sections before addition of Abs. Slides were stained with primary Abs for 30 min and examined using a Zeiss LSM 710 microscope, and data were acquired using Zen 2009 software.

Immunohistochemistry

Spleens were obtained from WT and TLR7tg mice, frozen in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) on dry ice, and stored at −80°C until sectioning. Organs were sectioned at 5–10 μm using a Leica 1850 cryostat (Leica, Wetzlar, Germany) and stored at −20°C until used. Before staining, sections were allowed to equilibrate to room temperature, fixed in cold acetone, and rehydrated in PBS. To reduce nonspecific staining, 10% horse serum was applied for 20 min to all sections before addition of Abs. Slides were stained with primary Abs for 30 min and examined using a Zeiss LSM 710 microscope, and data were acquired using Zen 2009 software.

Microarray and miRNA analyses

For microarrays, RNA was purified from splenic NK1.1" cells isolated from WT and TLR7tg mice as described above. The Ambion Illumina TotalPrep RNA altification kit (Ambion, Austin, TX) was used to amplify the RNA and to validate the manufacturer’s instructions and as previously described (27) to prepare biotinylated cRNA from isolated total RNA. Input was normalized to 500 ng per sample. Labeled cRNA was then used for hybridization to Illumina’s mouse WG-6 per the manufacturer’s instructions. BeadChips were imaged on an Illumina HiScan-SQ scanner. Microarray target preparation and BeadChip hybridization were performed by the Genomic Technologies Section, Research Technologies Branch, National Institute of Allergy and Infectious Diseases. Microarray data were submitted to the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63829). Initially, a splenic NK sample from a single B6 WT mouse and splenic NK1.1" sample from a sample TLR7tg mouse were each probed in duplicate on a single mouse WG-6 array (no. 7011). Later, a second mouse WG-6 array (no. 4020) was probed with samples from two B6 WT mice and two TLR7tg mice. The two technical replicates from the first array were averaged together to allow the two unique biological samples from the first array to be analyzed with the four biological replicates from the second array.
For the miRNA expression analysis, total RNA was isolated from cell-sorted NK1.1+CD11c+ and conventional NK cells (NK1.1+CD11c−) using an miRNeasy Mini kit (QIAGEN). The quality of RNA was assessed by the Experion system (Bio-Rad Laboratories), and only RNAs with a RNA quality indicator >9 were used. cDNA was generated using the miScript II RT kit (QIAGEN) and reverse transcription reaction with SYBR Green (QIAGEN). miRNA expression profiles were detected using the mouse miFinder and inflammatory response and autoimmunity miScript miRNA PCR arrays (QIAGEN). Data were analyzed further with SABiosciences microarray data analysis software.

Statistical analysis
Illumina microarray data were log2-transformed and normalized for each array per the instructions from Illumina by National Institute of Allergy and Infectious Diseases Research Technologies Branch. All subsequent analyses were computed using the R project for statistical computing (http://www.R-project.org) (28). Microarray data were inspected for possible failed chips using density plots, box plots, and principal components analysis plots. Even after initial platform-specific normalization at the National Institute of Allergy and Infectious Diseases Research Technologies Branch, there were still systematic differences in the distribution of expression values between the samples from the first array (7011) with two technical replicates (average of 7011A plus 7011C B6 and average of 7011D plus 7011E Tg) and the samples from the second array (4020) with no technical replicates (4020A, 4020B, 4020D, and 4020F). Quantile normalization was applied to the six samples to eliminate these differences. Gene probe sets with log2 expression SD of ≥0.2 among all samples were removed to eliminate low information genes prior to statistical testing. Differential expression was tested using a pairwise empirical Bayes modified t test from the limma R package library (29). A probe set was considered to be differentially expressed when its false discovery rate (FDR) adjusted p value was <0.05 and its absolute fold change was ≥2. Any statistically significant differentially expressed genes were imported into Ingenuity Pathway Analysis (IPA) to identify any enriched pathway groups among the significant genes.

miRNA analyses were computed using Qiagen software and p values are reported from unpaired Student t tests. All other statistical tests were computed using GraphPad Prism, with p values reported from one-way ANOVA with Dunnett multiple comparisons against control adjustments for multiple comparisons.

Results
NK1.1+CD11c+ cells expand in autoimmune conditions
During our characterization of DC populations associated with disease in various mouse models of SLE, we found that a sizable number of CD11c+ cells in the spleen also expressed NK1.1 and CD122, a combination of surface markers normally expressed by NK cells (Fig. 1A). The mouse strains we selected represent a varied range of autoimmune pathology: 1) FcγRIIB−/− mice develop moderate lupus-like disease with 2–3 months of age (8, 30), and 3) mice of 2–3 mo of age (10). We found that within the WT CD11c+ population increases considerably to 20% in 4 mo of age (10). We found that within the WT CD11c+ population increases considerably to 20% in mice of 2–3 mo of age (Fig. 1A). As a whole, spleens from 6-mo-old FcγRIIB−/− mice or 3-mo-old FcγRIIB−/− mice contained on average 1 × 10^6 of these cells (Fig. 1B). TLR7tg mice of 2–3 mo of age had the highest number, with an average of 4 × 10^6 NK1.1+CD11c+ cells in the spleen. In fact, >90% of NK1.1+ cells originated in TLR7tg mice were also CD11c+CD122− (not shown).

Given that NK1.1+CD11c− cells were found to be most abundant in mice with increased expression of TLR7, we considered that this cell population might be especially sensitive to TLR7 stimulation. To determine whether the NK1.1+CD11c− cells were intrinsically responding to increased TLR7 activity, we generated BM chimeras using an equal mixture of WT (CD45.1+/CD45.2−) and TLR7tg (CD45.2+) BM to reconstitute an irradiated WT host. Two months after reconstitution, NK1.1+CD11c+ cells were 4-fold more likely to be derived from TLR7tg BM than from WT cells (81.7% CD45.1+ versus 19.3% CD45.1−). In contrast, conventional DCs (CD11c−NK1.1+) or NK cells (NK1.1+CD11c−) were equally likely to originate from TLR7tg or from WT BM (Fig. 1C). Thus, the CD11c+NK1.1+ population expands as a consequence of the higher expression of TLR7 within these cells, which presumably leads to continued activation of this innate pathway. To test whether chronic activation of TLR7 was enough for the expansion of NK1.1+CD11c+ cells in the spleen, we repeatedly injected WT mice with a TLR7 ligand: we used imiquimod at doses ranging from 50 to 200 μg or R848 at 100 μg. We observed that twice weekly i.p. injections of a TLR7 ligand for 3 wk significantly increased the number of NK1.1+CD11c+ cells up to 1.5 million per spleen in WT mice (Fig. 1D).

Next we studied the in situ distribution of NK1.1+CD11c+ cells in the spleen by confocal microscopy. We used fluorescent Abs against the NK1.1 cell surface marker, in addition to B220 Abs to detect splenic B cells, and peanut agglutinin for germinal center identification. As shown in Fig. 1E, NK1.1 staining is greatly amplified in TLR7tg spleens compared with WT spleens. Conventional NK cells in WT spleens are scarce and located in the red pulp; NK1.1 staining on TLR7tg spleen shows cells primarily in the red pulp but also in T cell areas surrounding germinal centers. Because most NK1.1+ cells in TLR7tg are NK1.1+CD11c+CD122+, we infer that this particular cell population resides primarily in the red pulp but also spreads into the T cell areas. Moreover, CD11c+NK1.1+ cells were found in organs beyond the spleen: they represented 2–4% of splenocytes, 1% of lymph node cells, 2–3% of peripheral blood, and 5–6% of liver-associated immune cells in TLR7tg mice of 2–3 mo of age (Fig. 1F, 1G).

NK1.1+CD11c+ cells from TLR7tg mice share features of the NK lineage
A flow cytometric analysis showed that, compared with conventional NK cells, TLR7tg-derived NK1.1+CD11c+ cells expressed no DX5, very low B220, low NKGD2, and high MHC class II (MHC-II) surface expression, but comparable levels of CD122 (Fig. 2A, top). Compared to WT pDCs, TLR7tg-derived NK1.1+CD11c+ cells expressed similar levels of MHC-II and CD11c, very low B220, and lacked Siglec-H and PDCA-1, two hallmark markers for pDCs (Fig. 2A, bottom). The lack of DX5 in the CD11c+NK1.1+ cells rules out a mature NK cell population; however, expression of NK1.1 and CD122 is usually restricted to the NK lineage (31, 32). We then determined whether these cells required IL-15 for development, as this cytokine is essential for cells of the NK lineage but dispensable for pDCs (33). We found that TLR7tg mice that lacked IL-15 had negligible levels of CD11c+NK1.1+ cells by flow cytometry (Fig. 2B). We further confirmed the NK cell lineage of CD11c+NK1.1+ cells by detecting the NK cell–specific transcription factor E4BP4, as well as high levels of T-bet and intermediate levels of Eomes and granzyme B (Fig. 2C). Overall our analysis shows that these cells belong to the NK cell lineage and that their surface expression pattern is reminiscent of the one reported for IKDCs (21), although these cells seem to express higher levels of MHC-II and lower levels of B220 compared with IKDCs.

The overall morphology of CD11c+NK1.1+ cells, determined by electron microscopy, was distinct from conventional pDCs or conventional NK cells: they presented small size, high nuclear/cytoplasmic ratio, round shape, smooth plasma membrane with
short pseudopodia, and abundant and large electron-dense granules in the cytoplasm (Fig. 2D). Overall, these cells had a generally lymphoid morphology and a vesicular-rich cytoplasm.

**Multiple functionalities of lupus-associated atypical NKs**

Given that we found atypical NK cells in high numbers associated to lupus models, we then investigated whether their functionalities might make these cells potentially pathogenic in autoimmune conditions. We first evaluated the ability of a highly purified NK1.1+CD11c+ population to produce cytokines upon stimulation. Single-cell–sorted (FACSAria) NK1.1+CD11c+ cells from TLR7tg spleens produced TNF-α, IL-10, and IL-6 when stimulated with the TLR7 agonist imiquimod (Fig. 3A). Additionally, purified TLR7tg NK1.1+ cells produced IFN-γ in response to IL-2 (Fig. 3B) as well as IFN-α/β and IFN-γ upon TLR7 stimulation (Fig. 3C).

Because we observed that these atypical NK cells from TLR7tg spleens expressed high levels of MHC-II, we tested their ability to process and present Ag to CD4 cells. TLR7tg NK1.1+ cells were as efficient as whole splenocytes at presenting OVA peptide at two different concentrations (Fig. 3D) to OT-II CD4+ cells. We also tested these cells’ ability to cross-present Ag to CD8+ cells, as this has been shown to be an important aspect described for IKDCs (33, 34). We confirmed this function in TLR7tg-derived NK1.1+ cells using cell-associated Ag on class I–mismatched APCs (Fig. 3E). NK1.1+ cells purified from WT or TLR7tg spleens were incubated with CFSC-labeled OT-I CD8+ cells as well as with 2 × 10^6 OVA-albumin–coated irradiated H-2^bm1^ splenocytes. H-2^bm1^ APCs cannot present OVA to OT-I cells owing to the bm1 mutation. Thus, any proliferation of CD8 cells in this experiment comes as a result of capture of Ag by the APC harboring the unmutated H-2^b^ and its rerouting through the MHC class I pathway. As shown in Fig. 3E, NK1.1+ cells from TLR7tg very efficiently cross-present Ag to CD8 cells.

Finally, we determined that TLR7tg-derived NK1.1+ CD11c+ cells pretreated with IL-2 have quite efficient cytotoxic activity compared with conventional NK cells. This test was performed in two separate systems: using the YAC-1 lysis assay (Fig. 3F) and by the caspase-6 assay (Fig. 3G).

**NK1.1+CD11c+ cells from TLR7tg mice are highly replicative and developmentally stable**

As NK1.1+CD11c+ cells appear in great numbers in TLR7tg spleens, we sought to determine whether they are expanding and actively...
dividing. We fed BrdU to TLR7tg mice for 14 d and tested BrdU incorporation in various splenic populations. As shown in Fig. 4A, most CD11c+ NK1.1+ cells, also DX52, were positive for BrdU compared with less than a fourth of the BrdU+ cells within the DX5+NK1.1+ conventional NK cells, independent of their transgenic or WT origin. To determine their survival rate after adoptive transfer, we sorted CD11c+NK1.1+ cells from TLR7tg spleens and injected 3–5 million i.p. into WT mice. Sorted cells were of 98% purity and were almost exclusively DX52 and MHC-II+ (Fig. 4B). After the transfer, donor cells could be identified as CD45.1+ whereas cells from recipient mice were CD45.12. Four weeks later, donor CD11c+NK1.1+ cells represented on average 2% of the spleen, equivalent to 1 million cells, in reconstituted mice (Fig. 4C). This is a remarkable 30% recovery rate of the total number of cells injected. The surface marker expression in donor cells was maintained as NK1.1+DX52 MHC-II+, although a few of the donor cells had lost their CD11c expression (Fig. 4D). Overall, the CD11c+NK1.1+ cells that developed in TLR7tg demonstrated an elevated in vivo proliferation rate and an exceptional capacity for survival.

Activated expression pattern in NK1.1+CD11c+ cells
We characterized the gene expression profile of NK1.1+CD11c+ cells isolated from TLR7tg mice and compared them to conventional NK1.1+ cells. Data were acquired from an Illumina whole-genome microarray using three samples per genotype. The complete array featured 45,281 probe sets representing 21,563 unique genes. After transformation, normalization, and quality control assessments, we removed any probe set with log2 expression SD ≤ 0.2 among all six samples, leaving 14,982 probes representing 9,581 unique genes. We used empirical Bayes pairwise t tests to identify 1490 probe sets with statistically significant differences among the two cell populations (FDR, 0.05 and absolute fold change ≥ 2), the 97 with best scores shown in the heat map of Fig. 5A. We narrowed this list of probe sets down further to identify 964 probe sets with FDR ≤ 0.00003 and absolute fold change ≥ 2 for use in IPA software. IPA identified major molecular pathways and group patterns differentially expressed between the two experimental groups. The graph in Fig. 5B shows the number of genes in each of 14 significantly enriched pathways. The most statistically significant (p = 10−10) enriched pathway was NK cell signaling, with 92 genes differentially expressed (those with >2-fold difference shown in Fig. 5C). Genes that are normally expressed in mature NK cells but not in NK cell precursors (KLRRC1, KLRB1, KLRD1, KLRA4, HIST1H2C, LCK, ZAP70) were significantly downregulated in NK1.1+CD11c+ cells compared with conventional NK cells (Fig. 5C). In contrast, the inhibitory receptors LIR1 and CD300A were upregulated, as well as FcγRIII, perhaps as a sign of cell activation.

NK1.1+CD11c+ cells also differentially expressed a number of TLR signaling genes compared with conventional NK cells: they expressed high levels of TLR1, TLR6 plus the signaling adaptors MYD88, IL-1R–associated kinase 3, and CD14 (Fig. 5B, 5C).
TLR7 was highly upregulated, even more than expected from the expression of the transgene. Alternatively, the inhibitory receptor SIGIRR, which has TLR regulatory function, was downregulated in the same cells. Overall this pattern of expression would make NK1.1^+CD11c^+ cells highly sensitive to innate stimulation. Moreover, signaling pathways downstream of TLR or IFN were also significantly different in NK1.1^+CD11c^+ cells compared with conventional NK cells, with a pattern of activation in NF-κB, and cytokine/Jak/Stat signaling genes. IFN-inducible genes (several IFIT genes, OAS1, and STAT1) are all highly expressed in NK1.1^+CD11c^+, as expected from cells that have chronic TLR stimulation. Uniquely among cytokine signaling genes, STAT4 was downregulated in NK1.1^+CD11c^+ cells (Fig. 5C). STAT4 is an adaptor molecule important for NK cell activity, and its expression is high in mature NK cells compared with precursor NK cells (35).

A recurrent pattern in the groups selected by the IPA software in this analysis is the differential expression of cell cycle–related genes, and those expressed in oncogenic transformation. Cyclins and cell cycle regulators RB1, CDKN1A, E2F, and CCND1 were upregulated whereas CDK4 and CCND2 were downregulated in NK1.1^+CD11c^+ cells compared with conventional NK cells (Fig. 5). Finally, a number of CSFRs showed high expression in NK1.1^+CD11c^+ cells and might account for the rapid growth in vivo of these cells.

miRNA expression patterns in NK1.1^+CD11c^+ cells suggest a change in maturation potential and cell cycle programing

We characterized miRNA expression differences in NK1.1^+CD11c^+ cells compared with conventional NK cells so that we could identify gene regulatory patterns in the newly characterized cell population. We tested our samples in two separate arrays: one miRNA PCR array that contained 84 miRNAs abundantly expressed in immune cells (mouse miFinder miRNA PCR array, MIMM-1001Z, Qiagen) and another array that contained 84...
miRNAs linked to autoimmunity and inflammation (mouse inflammatory response and autoimmunity miRNA PCR array, MIMM-105Z, Qiagen). We used these two arrays to compare miRNAs prepared from NK1.1+CD11c+ TLR7tg cells with conventional NK cells sorted as NK1.1+CD11c2 cells. Fig. 6A shows the list of miRNAs that were expressed at detectable levels in all samples. Among the miRNAs with significantly lower expression in NK1.1+CD11c+ cells compared with conventional NK cells, we found two that have been reported to be important for NK cell maturation in mice (miR-150) and in humans (miR-181) (36, 37). We validated the differences on miR-150 expression by real-time PCR (Fig. 6B). A well-characterized target of miR-150 is the Myb gene, which is highly expressed in lymphoid progenitors and downregulated upon maturation (38). We confirmed using RT-RCP that the c-Myb transcript was upregulated in TLR7tg NK1.1+CD11c+ cells compared with WT NK cells (Fig. 6C). Importantly, miR-150 levels are directly regulated by TLR7, because the addition of imiquimod to NK1.1+CD11c+ cells resulted in further downregulation of miR-150 expression (Fig. 6D).

The IPA software provided information on the regulatory network between miRNAs and their targets from the combined analysis of the Illumina array data and the miRNA PCR assay. Fig. 7 shows a number of identified connections among differentially regulated genes and miRNAs stemming from the comparison between NK1.1+CD11c+ cells and conventional NK cells. NK1.1+CD11c+ cells had very low expression of miR-744 compared with conventional NK cells (Fig. 6A). miR-744 has been linked to tumor growth through regulation of cyclin B1 expression (39). In the context of the immune response, miR-744 also regulates expression of TGF-α and its receptor (40). High levels of TGF-βR can compensate low expression of the signaling molecule SMAD3 and directly impacts miR-181 expression and cell differentiation through increase expression of phosphatases PTPN1 and PTPN6 (Fig. 7) (41).

Among the miRNAs that showed higher expression in NK1.1+CD11c+ cells relative to conventional NK cells, we found the miR-15/16 family and the related miR-27/195/222. All of them are induced by high expression of the transcription factors E2F1 and E2F2 and as a consequence of RAS and NF-κB activation in the cells (42). Overall, NK1.1+CD11c+ cells activated by TLRs or by CSFRs turn on the transcription of genes involved in proliferation (e.g., cyclin D1, HDAC9, and E2F). Ultimately this program is regulated in feedback fashion by the expression of miR-15 and miR-16, both of which target the cell cycle regulators CDK4, cyclin D2, and the apoptotic factor Bcl2. Activation of NF-κB also leads to upregulation of Bcl2-like genes such as BCL2A1D, with antiapoptotic function (Figs. 6A, 7) (43). miR-222 and miR-195a, both with high expression in NK1.1+CD11c+ cells, have been shown to affect the cell cycle by regulating p27 (44). Overall the data are consistent with an activated expression pattern in NK1.1+CD11c+ cells with concomitant induction of regulatory miRNAs (miR-15/16/27/222/195) that set a specific cell cycle transcription program.

Discussion

Activation of innate immune pathways in inflammatory and autoimmune conditions can lead to preferential expansion of cell populations that are especially sensitive to innate triggers. In this study, we report the expansion of cells of the NK cell lineage with atypical characteristics in the context of mouse models of lupus. We show that either repeated TLR activation or expression of multiple copies of the Tlr7 gene induces the expansion of this atypical NK cell population. However, these cells also appear in substantial numbers in FcγRIIB-KO, a lupus-prone mouse strain bearing WT levels of TLR7.
Given their multiple functional abilities, ascribing mouse NK1.1+CD11c+ cells to a developmental lineage has been controversial. Our full characterization of these atypical cells, defined as NK1.1+CD11c+CD122+ cells, suggests that they arise from immature NK cells because they require IL-15 and express E4BP4 (NFIL3), essential for generation of the NK cell lineage (45–47).

**FIGURE 5.** Gene expression profile of NK1.1+CD11c+ cells from TLR7tg mice. (A) Heat map analysis of the gene expression comparison between conventional NK cells isolated from WT mice and NK1.1+ cells isolated from TLR7tg mice. Using three samples per group, the identified genes with statistically significant differences (FDR < 0.05) and absolute fold change >2 are shown. (B) Genes identified in the heat map analysis were filtered slightly further with FDR < 0.005 yielding 959 genes for pathway analysis. (C) List of genes whose expression was at least 2-fold different in NK1.1+CD11c+ samples compared with conventional NK cells.
Additionally, we demonstrate that these atypical cells are distinct from the DC lineage because they lack expression of the DC-restricted transcription factors Zbtb46 (BTBD4) and Tcf4 (E2-2). Functionally these cells are both cytotoxic and efficient APCs in a way that they resemble the previous described IKDCs (15–21). However, the cells we characterize in our lupus models seem to

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Additionally, we demonstrate that these atypical cells are distinct from the DC lineage because they lack expression of the DC-restricted transcription factors Zbtb46 (BTBD4) and Tcf4 (E2-2). Functionally these cells are both cytotoxic and efficient APCs in a way that they resemble the previous described IKDCs (15–21). However, the cells we characterize in our lupus models seem to

**FIGURE 6.** miRNA expression in atypical NK cells from TLR7tg compared with conventional NK cells. (A) WT NK cells sorted as TCRb+ NK1.1+ CD11c− and TLR7tg NK1.1+ sorted as TCRb+ NK1.1+CD11c+Siglec-H+ were used to prepare miRNAs, and differential expression was analyzed with mouse miFinder (MIMM-001Z) or inflammatory response and autoimmunity (MIMM-105Z) miRNA PCR arrays (Qiagen). Shown is the list of genes that were different between the two samples. (B) miR-150 differential expression was confirmed by real-time PCR on samples prepared as in (A). (C) c-Myb was one of the miR-150 target genes predicted using the TargetScan software; c-Myb mRNA was measured in WT NK cells or TLR7tg NK cells purified as in (A). *p < 0.05. (D) Purified NK1.1+ cells from TLR7tg were incubated with imiquimod (IQ) or left unstimulated (control) for 24 h. miR-150 expression was measured by RT-PCR relative to actin levels (control) for 24 h. miR-150 expression was measured by RT-PCR relative to U6 levels.

**FIGURE 7.** The regulatory network of miRNAs and their target genes in atypical NK cells. Representations of expression networks defined by the IPA analysis in Fig. 5 in combination with the miRNA expression analysis in Fig. 6 are shown. Genes with changes in mRNA expression in Fig. 5 are shown as colored rounded boxes whereas miRNAs differentially expressed from data in Fig. 6 are shown as rectangles marked with an miRNA symbol.
differ slightly in surface expression of some markers (MHC, B220) and, importantly, our transfer experiments show that they survive for months and in large numbers upon adoptive transfer and do not become mature immune cells, as they never acquire CD49b or DX5 expression. This difference might be due to specific activation of lupus-associated NK cells, either because of the inflammatory environment or through TLR7-dependent activation.

This type of chronic activation might lead to an oncogenic-like transformation of these cells, which would explain the highly proliferative expression profile that we uncovered in our full genome and inflammatory-associated RNA analysis. Expansion of NK1.1^CD11c^ cells correlates with the inflammatory pathology and inversely correlates with the number of mature NK cells (NKp46^DX5^) in TLR7g mice (data not shown). This is consistent with the view that activation of immature NK cells through TLR7, combined with an inflammatory environment, thwarts differentiation of these cells into mature NK cells and leads to the acquisition of markers nonspecific for NK cell lineages such as MHC-II and the integrin CD11c. The combination of unusual surface-expressed markers and novel capabilities relative to mature NK cells, combined with their block in maturation, makes this surface-expressed markers and novel capabilities relative to mature NK cells. This finding highlights the need to reevaluate immune cells. This type of chronic activation might lead to an oncogenic-like transformation of these cells, which would explain the highly proliferative expression profile that we uncovered in our full genome and inflammatory-associated RNA analysis. Expansion of NK1.1^CD11c^ cells correlates with the inflammatory pathology and inversely correlates with the number of mature NK cells (NKp46^DX5^) in TLR7g mice (data not shown).

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Disclosures

The authors have no financial conflicts of interest.