Published in final edited form as:

J Autoimmun. 2021 January ; 116: 102559. doi:10.1016/j.jaut.2020.102559.

Natural Killer Cell Transcript 4 promotes the development of Sjögren's Syndrome via activation of Rap1 on B cells

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Abstract

Autoimmune disorders are the third most common diseases in the United States, and affect the daily lives of millions of people. In this study, we analyzed patient samples, utilized a transgenic mouse model and human B cells to reveal Natural Killer Cell Transcript 4 (NK4) as a novel regulator that promotes the development of autoimmune disorders. NK4 was significantly elevated in samples from patients with Sjögren's Syndrome (SS). SS patients show elevated NK4 levels. There is a strong and positive correlation between the increased levels of NK4 and the duration of SS. Interestingly, transgenic expression of NK4 in a mouse model led to the development of autoantibodies and lymphocytic infiltration in salivary glands similar to those in SS patients. Those phenotypes were associated with increased B1a cells in the peritoneum, plasma cells in the spleen, and increased IgM, IgA, and IgG2a in serum of the NK4 transgenic mice. The autoimmune phenotypes became more severe in older mice. Moreover, after NK4 transfection, human naïve B cells were activated and memory B cells differentiation into IgG and IgA-plasmablasts, resulting in an increased production of autoantibodies.NK4 regulated the differentiation and activation of B cells through activating Rap1 activity. NK4 also promoted B cell migration in a paracrine fashion through an induction of CXCL13 in endothelial cells. Collectively, these findings identify NK4 as a promoter of the development of autoimmune disorders through its roles on B cells. Therefore, NK4 may be a novel therapeutic target for the treatment of autoimmune diseases.

Keywords

NK4; autoimmune; Sjögren's Syndrome; B cell; Rap1

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Author statement

PC Lin* conceived and designed the work; PC Lin and H. Young coordinated technical support and funding; PC Lin and P. Qu wrote the manuscript; P. Qu, T. Wuest and Y.F. Min. performed the experiments and collected the samples; P. Qu and T. Wuest acquired, analyzed, and interpreted the data; I. Alevizos and H. Young participated in data collection and analysis. All authors read and approved the final manuscript.

The authors declare no potential conflicts of interest.

Introduction

Autoimmune diseases arise from abnormal immune responses against substances and tissues normally present in the body. Depending on the type, an autoimmune disease can affect one or many different organs or tissues, damaging organ functions. According to the NIH Autoimmune Diseases Coordinating Committee Report 2002, autoimmune diseases are the third most common diseases in the United States after cancer and cardiovascular disease; they affect approximately 5% to 8% of the population. Thus, identification of molecular mediators and investigating underlying functional mechanisms involved in the development of autoimmunity are timely and necessary. Sjögren's Syndrome (SS) is a chronic autoimmune disease affecting exocrine glands, primarily salivary and lachrymal glands. Primary SS (pSS) is a common systemic autoimmune disorder that affects approximately 0.1 to 0.4% of the general population. The pathogenetic mechanisms of this autoimmune exocrinopathy have not been fully elucidated (Voulgarelis and Tzioufas, 2010).

NK4 is a pro-inflammatory cytokine that activates p38 MAPK and NF-κB, thereby inducing pro-inflammatory cytokines. The gene was originally cloned as natural killer cell transcript(Kim et al., 2005) from activated human NK cells and T cells. It was renamed NK4 due to its ability to induce production of cytokines such as TNFα, IL-1, IL-6 and IL-8(Dinarello, 2010; Kim et al., 2005). Currently the human NK4 receptor has not been identified, and no rodent orthologs of NK4 have been reported. NK4 is highly expressed in immune cells including monocytes and activated B cells (Dinarello and Kim, 2006; Kim et al., 2005), as well as endothelial cells (Dinarello and Kim, 2006; Kobayashi and Lin, 2009; Nold-Petry et al., 2009) . NK4 has been shown to play a role in infection, including the defense against intracellular bacteria and viruses (Kobayashi et al., 2010; Schenk et al., 2012). The levels of NK4 were significantly increased in RA patients (Heinhuis et al., 2011; Joosten et al., 2006). Overexpression of NK4 in synovial fibroblasts resulted in a greater induction of pro-inflammatory cytokines, and intra-articular introduction of NK4 in mice resulted in joint inflammation (Heinhuis et al., 2011). These reported findings suggest a potential role of NK4 in the development of autoimmune conditions.

In analysis of patient samples, as well as utilizing a transgenic mouse model and human B cells, we have discovered a causative role for NK4 in the promotion of development of autoimmune phenotypes. SS patients show elevated NK4 levels in serum and tissues. There was a strong and positive correlation between the serum levels of NK4 and the duration of SS. Consistent with the human data, transgenic expression of NK4 in mice led to spontaneous development of autoimmune phenotypes with elevated autoantibodies in the circulation, immunoglobulin deposition and lymphocyte infiltration into the salivary glands, similar to pSS patients.

Mechanistically, NK4 promoted human B cell migration through the induction of CXCL13 in endothelial cells and monocytes, and efficiently stimulates naïve B cells activation and mediates memory B cells differentiation into IgG and IgA-plasmablasts (PBs) through upgrading the activity of Ras-proximity-1 (Rap1). These results reveal a novel role for

NK4 in promoting B cell maturation and activation, which leads to the production of autoantibodies and contributes to the development of autoimmune phenotypes.

Materials and Methods

Human samples and mouse models

The human specimens were obtained through NIH Institutional Review Board approved protocols. Patients with primary Sjögren's Syndrome (pSS) and healthy controls were recruited to the NIDCR Sjögren's Syndrome Clinic. Minor salivary glands were obtained from the lower lips, and serum was isolated according to the clinic's standard operating procedures. All primary Sjögren's patients met the American-European Consensus Group criteria for diagnosis and there was no evidence of presence of other autoimmune diseases, such as Systemic Lupus Erythematosus (SLE). The pSS population consisted of 22 females and 7 males with a mean age of 54.3 years and a standard deviation of 15.7 years (Suppl. Table 1). Twenty of the patients were Caucasians, seven were African American one was Asian and one was Hispanic. Healthy controls included 15 females with a mean age of 46.3 years and a standard deviation of 9.1 years. Eight of the females were Caucasians and seven were African American. All healthy controls were carefully examined for Sjögren's syndrome and other autoimmune diseases.

PECAM-NK4 transgenic mice in B6 background and wild type littermates were maintained in a pathogen-free facility at the National Cancer Institute (Frederick, MD) in accordance with Animal Care and Use Committee regulations. Sex and age matched mice were used in all the studies.

Primary cells and cell lines

Human peripheral mononuclear cells (PBMCs) were isolated from pooled healthy donors (19-65 years age) at the NIH Blood Bank by centrifugation over a discontinuous Lymphoprep gradient. Human B cells were purified using CD19-mAb conjugated magnetic beads (Miltenyi Biotec, Auburn, CA), respectively. More than 97% purity of the cells was attained for use in subsequent studies. Primary human umbilical vein endothelial cells (HUVECs) were from ATCC and Lonza, respectively. HUVECs were incubated in serum free EBM-2 (CC-3156, Lonza, Allendale, NJ) overnight, followed by the treatment of NK4 transfection or shRAP1.

Western blotting

Human B cells isolated from Human PBMCs were transfected with Vector, NK4 or Rap1 shRNA, NK4 or Rap1 protein expression was analyzed in cell lysates by and probed with a goat anti-NK4 antibody (Ybdy biotech, South Korea) or anti-Rap1A+Rap1b antibody (Abcam, Cambridge, MA). NK4 plasmid was pCMV-EGFP-CMV-SV40 plasmid. The band intensities of Western Blot were analyzed using imageJ software. The intensity ratios were quantified.

Real-time PCR

CXCL13 mRNA expression was determined by qPCR with the primer sets for murine:
5’-GGGTGCCCAAAAAGAGAAATC-3’

5’-GATGGGAGGGTTCAAGCATACA-3’

human:

5’-GAGTTCAAGGTCTCCTAAGAC-3’

5’-CTTCCAGACATTCGGAGACC-3’

Human Iγ-Cμ:

Forward: 5'-GCCATGGGGTGATGCCAGGATGGGCAT-3'

Reverse: 5'-AGACGAGGGGGAAAAGGGTT-3'

Human Iα-Cμ:

Forward: 5'-CAGCAGCCCTCTTGGCAGGCAGCCAG-3'

Reverse: 5'-AGACGAGGGGGAAAAGGGTT-3'

Autoantibody detection

Anti-dsDNA IgG in serum was measured by ELISA following the manufacturers’ protocol (Alpha Diagnostic International, San Antonio, TX). Antinuclear antibody (ANA) was detected by an immunofluorescence assay on HEp-2 cells (BMD) with 1/100 diluted serum. ANA positive nuclei were counted in randomly selected fields under light microscopy. Mouse Ig Isotyping Array was used to determine immunoglobulin subclasses (IgG1, IgG2a, IgG2b, IgG3, IgA, IgD, IgE, and IgM) (Raybio® Rapid, Inc. Norcross, GA). Sixteen sera from WT and PECAM-NK4 mice were mixed with a Cy3 equivalent dye labeled antibody, and applied to the array. The slides were visualized and quantitated using a Genepix 4000A laser Scanner (Molecular Devices Inc. San Jose, CA). The SSA and SSB autoantibody ELISA is performed for the detection of IgG antibody to SSA or SSB in mouse serum following the manufacturers’ protocol (SE120117, SE120118, Sigma, St Louis, MO).

Rap1 activity measurement

Briefly, Goat Anti-Mouse IgG Magnetic Beads (S1431S, New England Biolabs, Ipswich, MA) were washed with lysis buffer and incubated with milk for 15 min. The anti-active Rap1a (26912, NewEast Biosciences, King of Prussia, PA) were then added and incubated for 30 min. The active Rap1 antibody suspension was washed and resuspended in difference lysis buffer separately. 100ul active Rap1 antibody suspension above were added to the tubes containing 900uL of human B cells lysate and incubated with rotation for 30 min at 4°C. The samples with beads above were pelleted using magnet, and washed twice. The remaining buffer were removed carefully.

The samples with beads above were resuspended in 1X Laemlii with reducing agent TCEP (tris(2-Carboxyethyl) phosphine), and were boiled to remove beads at 95°C for 5min. The samples were harvested for western blot. For the positive control use GTPyS containing lysis buffer, negative control use GDP containing lysis buffer, and experimental samples above need to be washed with buffer containing GTP and GDP.

Histological analysis

Mouse kidneys and parotid glands were harvested, fixed in 10% formalin, sectioned and stained with H&E for histological evaluation. For immunohistochemistry, the tissues were fixed in 4% paraformaldehyde, sectioned and stained with anti-mouse CXCL13, B220, IgM, IgG antibodies, respectively. For Immuno-staining of human pSS patient's salivary gland, anti-human CD68, B220 and NK4 antibodies were used.

Flow cytometry

Cells were stained with panels of antibodies including anti-mouse CD19-pacific Blue, CD138-APC, CD21-PEcy7, CD5-FITC, CD43-PE, IgD-APCcy7, CD38-PE-Cy7, GL-7-Percp (BioLegend, San Diego, CA), and IgM-APCcy7 (Miltenyi Biotec, Auburn, CA). For the human B cell phenotypes, the following antibodies were used: anti-CD19-pacific blue, anti-CD38-PECy7, anti- CD20-PE, anti-CD27-APCcy7 (BioLegend), and anti– human IgG-PE, anti– human IgA-APC (Miltenyi Biotec). BD Cytofix/ Cytoperm and BD Perm/ Wash (BD) were used for IgA and IgG intracellular staining per manufacturers' instructions. Data were analyzed on a BD Biosciences LSRII machine and Flowjo software 29.

Protein Array and ELISA

Sera were collected from age and sex matched mice, and subjected to mouse Igs array and cytokine array (Proteome Profiler Array Mouse Cytokine, Raybio® Rapid, Inc. Norcross, GA).

NK4 from the supernatant of human was detected by ELISA, as previously described as the protocol (reference in BioLegend). Briefly, anti-NK4 mouse monoclonal antibody (2 μg/ml, #513601; BioLegend, San Diego, CA) was coated onto a 96-well ELISA plate (BD Falcon, Franklin Lakes, NJ, USA). After blocking each well with 10% FBS in PBS supplemented with 0.05% Tween 20 (PBST) RT one hour, human serum or a cultured medium supernatant was applied into each well, followed by Biotin anti-human NK4αβγδ Antibody (2ug/ml, #513503; Biolegend) with enzyme SAV-HRP (BD bioscience, 1:250) for 2h RT. Color development was measured by an ELISA reader. Recombinant IL-32 protein was used as standard protein (ab256059, Abcam, Cambridge, MA)

The ELISA for IgG2a, IgG3, IgA and IgM were performed in 96-well plates coated with anti-murine IgG2a, IgG3, IgA and IgM (Sigma-Aldrich), respectively. For IgM ELISA, sera were diluted 1/2,000 and for I IgG2a, IgG3 or IgA ELISA, sera were diluted 1/25,000 in PBS. Sera from NK4 -transgenic and WT mice were incubated in coated wells, washed, and then alkaline phosphatase-conjugated anti-murine IgG2a, IgG3 or anti-murine IgM was added, respectively. Plates were analyzed with a spectrophotometer after incubation with substrate.

Sandwich ELISAs were used to measure human IgM, IgG and IgA. Capture and detection antibodies for Ig ELISAs were purchased from Southern Biotech. Human reference serum (Bethyl Laboratories, Inc. Montgomery, TX) was used to generate the standard curve for Ig ELISAs. Plates were read with a SpectraMax M2 (Molecular Devices Inc., Downingtown, PA) and analyzed with SoftMaxPro V5 software (Molecular Devices Inc. San Jose, CA).

B cell migration

Purified human CD19+ B cells were seeded in the upper chamber in 3-μm Trans-well plates (Corning Costar, Cambridge, MA); HUVECs pre-transfected with Empty vector or NK4 were seeded in the bottom chamber.

The Transwell was maintained at 37°C for 24hrs with 5% CO2. The number of B cells that had migrated into the bottom chamber was determined by flow cytometry for CD19+ cells. For CXCL13 neutralization, increasing concentration of neutralizing CXCL13 antibody or an isotype control antibody (10 μg/ml) (R&D Systems) was used. Recombinant human CXCL13 (R&D Systems, Minneapolis, MN) at 500 ng/ml were applied as positive control in the bottom chamber.

Statistical analysis

Statistical analyses were performed using ANOVA by comparison of data between SS patient samples and healthy normal subjects. We performed Cox proportional hazard regression to examine the relationship between NK4 serum levels and sustained times of Sjogren's syndrome. Paired Student t test was used for mouse studies and in vitro assays. The differences were considered statistically significant when P-value<0.05.

Results

NK4 levels are elevated in patients with pSS; and transgenic expression of NK4 in mice leads to the development of autoimmune phenotypes that resemble pSS.

NK4 regulates the production of pro-inflammatory cytokines and its expression is elevated in chronic inflammatory and rheumatoid arthritis(Joosten et al., 2006). To test the hypothesis that NK4 may have a causative or triggering role in the development of autoimmune disease, we first examined the expression of NK4 in patients with pSS, a chronic autoimmune disease. Patients with primary Sjögren's Syndrome (pSS) and healthy controls were recruited to the NIDCR Sjögren's Syndrome Clinic. The patients were examined in detail when patients first started feeling the oral and ocular dryness. All pSS patients met the American-European Consensus Group criteria for diagnosis and there was no evidence of presence of other autoimmune diseases. All healthy controls were carefully examined for Sjögren's syndrome and other autoimmune diseases (Suppl. Table 1).

Upon analysis of NK4 levels in serum collected from normal healthy subjects and patients with pSS using ELISA, we found a significant increase of NK4 in pSS patient samples compared to the age/sex matched normal subjects. The mean concentration of serum NK4 in pSS patients is 864.97±631.58 pg/ml and normal subjects 252.2±235 pg/ml (Figure 1A). To test whether the high level of NK4 on pSS patients is related with pSS disease development,

we detected the relationship among NK4 expression and pSS clinical characterization (such as sustain time of the diseases, IgG level and Minor Salivary Gland (MSG) focus score from those patients). There was a strong and positive correlation between the serum levels of NK4 and the duration of the disease R2=0.9137 (Figure 1B), and between the serum levels of NK4 and IgG protein level R2=0.8904 (Figure 1C). We did not find the relationship between the serum levels of NK4 and Minor Salivary Gland (MSG) focus score (Figure 1D). We also acquired minor salivary gland tissues from multiple patients and healthy control subjects. The tissue sections were subjected to immunohistochemical staining for NK4 expression. Notably, 75% of pSS patient biopsies (6 out of 8 patients) showed a positive reaction for NK4. In contrast, there was no detectable of NK4 deposition in tissues from normal healthy subjects. More NK4 positive cells were detected in pSS patients with moderate to severe inflammation compared to pSS patients with limited clinical inflammation (Figure 1E). Double Immunofluorescence staining confirm NK4 expression on CD68+ myeloid cells (Figure 1F). These clinical data demonstrated the strong correlation of NK4 expression with the patients with pSS.

To test if NK4 contributes to the development of autoimmune conditions and investigate the underlying mechanism, we used PECAM-NK4 transgenic mice, in which NK4 expression is driven by the PECAM-1 promoter to direct the transgene expression in hematopoietic cells and endothelial cells(Kobayashi et al., 2010). Autoimmune diseases are developed when the healthy tissues and cells are targeted by the body's own immune system and generate abnormal levels of autoantibodies. An analysis of serum from one and two-month-old PECAM-NK4 mice showed no detectable levels of autoantibody responses in circulation. However, starting from three-months of age, more than 50% of the female PECAM-NK4 mice (12 out of 22) about 20% of male mice (5 out of 23) developed significant amounts of circulating autoantibodies against dsDNA. These levels further increased at seven-months of age in the PECAM-NK4 transgenic mice. In contrast, no autoantibody activity was detected in serum from WT littermate controls at either age group (Figure 2A).

The presence of anti-dsDNA antibody prompted us to further characterize serum for the presence of antinuclear antibodies (ANAs). Consistently, we detected a very strong reaction of ANAs with serum from three-month old PECAM-NK4 mice, which also showed a more speckled pattern. The ANA reaction intensity further increased when testing the serum from seven-month old PECAM-NK4 mice. In contrast, there were no detectable ANAs with serum from age and gender-matched WT littermate controls. Quantification of ANA positive cells confirmed a significant reaction in three-month-old PECAM-NK4 mice compared with WT controls. The reaction intensity of serum was increased in seven-month old PECAM-NK4 mice compared with those from the younger mice (Figure 2B-C). The ANA staining patterns resemble the patterns in patients with pSS and SLE. The autoantibodies characteristics of pSS patients are Anti-SSA autoantibody (Anti-Sjögren’s-syndrome-related antigen A, also called anti-Ro) and Anti-SSB autoantibody (Anti-Sjögren’s-syndrome-related antigen B). Thus, we compared SSA and SSB expression in serum from between three-month-old PECAM-NK4 mice and age and gender-matched WT littermate controls. There is strong reaction of SSA and no reaction of SSB with seven-month-old PECAM-NK4 mice, compared with WT control (Figure 2C, Suppl. Figure 1).

The development of autoantibodies could lead to tissue damage in target organs. Therefore, we harvested salivary glands and kidneys for histological examination. The majority of female PECAM-NK4 mice (12 of 22 mice at three months of age, and 11 of 15 mice at seven months of age), but no littermate WT controls, developed multiple inflammatory cell infiltrations in salivary glands. There are not significantly difference between three- and seven-months old male mice. To determine what type of cells have infiltrated these tissues, we performed immune-histochemical staining on salivary gland from seven-month old PECAM-NK4 mice. We found the majority of the infiltrating inflammatory cells are B220+ B cells. There was more inflammatory cell infiltration in seven-month old PECAM-NK4 mice than three-month-old PECAM-NK4 mice (Figure 2E). The similar results were observed in kidney tissues from PECAM-NK4 mice and littermate WT controls (Suppl. Figure 2). These results are indicative of tissue injury, and share similarities with the phenotypes observed in patients with pSS. The data from the analysis of human samples, along with our mouse model, collectively support a causative role for NK4 in the development of autoimmune disorders.

NK4 promotes immunoglobulin production and B cell differentiation

Next, we characterized immunoglobulin subtypes developed in the PECAM-NK4 mice by using an Immunoglobulin Isotyping Array (Suppl. Figure 3A). The NK4 transgenic mice that developed autoantibodies by three-months of age showed a significant increase in Ig2a, IgG3, IgM and IgA levels compared with WT controls (Figure 3A-D). By seven months, the PECAM-NK4 mice showed a further increase in IgM levels compared to those from three-month old PECAM-NK4 mice. The seven-month-old PECAM-NK4 mice also demonstrated a significant elevation of IgG2a, which was not increased in three-month old PECAM-NK4 mice (Figure 3A-D). We did not detect any significant increase in other immunoglobulin subclasses between the two groups, such as IgG2b and IgG1 (Suppl. Figure 3B). Moreover, immune-histochemical analysis revealed a strong deposition of IgG and IgM in salivary glands harvested from PECAM-NK4 mice, but not from littermate controls at either three or seven-month of age (Figure 3E). This is in complete agreement with B cell infiltration observed in salivary glands of the PEACM-NK4 mice (Figure 2D). Similar deposition of IgG and IgM proteins was also detected in kidney tissues from the PECAM-NK4 mice and not in WT littermate controls (Suppl. Figure 3C). Collectively, these data support the idea that abnormally high levels of circulating NK4 could activate B cells to over-produce IgG and IgA, thereby contributing to the development of autoimmune phenotypes.

To further corroborate this hypothesis, we analyzed B cell development and differentiation in the mouse bone marrow. There was no significant difference in the total number of bone marrow cells between the PECAM-NK4 mice and age/sex matched WT littermate controls (Suppl. Figure 4A). Nor did we detect any significant difference in the number of pre B cells (B220low IgM-) and immature B cells (B220low IgM+) between the two groups. However, there was a significant increase in the number of mature circulating B cells (B220high IgM+) in the PECAM-NK4 mice compared to WT mice (Suppl. Figure 4B and 4C). This result implicates a specific role for NK4 in the regulation of B cell maturation and activation.

Moreover, we examined subpopulations of B cells in the spleen and peritoneal cavities of mice at seven-months of age. A flow cytometry analysis of B1a cells (CD19+CD5+) detected a significant increase of these cells in the peritoneal cavity of the NK4 transgenic mice compared to WT littermate controls (Figure 3F). Similarly, an analysis of spleen B cells using anti-CD19, CD21, CD38, CD138, GL-7, IgM and IgD antibodies showed a significant increase of CD19+/CD21^{hi}/IgM+ marginal zone B cells, CD19+/B220+/GL-7+/IgDlo/-germinal center B cells and CD19−/CD138+ plasma cells in the NK4 mice compared to WT controls. The percentage of total CD19+ B cells in the spleen did not change between the two groups (Table 1).

Collectively, these findings reveal a novel role for NK4 in promoting B cell maturation and activation, which leads to the production of autoantibodies and contributes to the development of autoimmune phenotypes.

NK4 promotes Naïve B cell activation

To validate the findings from mouse models, we examined the effects of NK4 on human cells. Purified naive B cells (CD19+IgD+CD27−) were transfected with empty vector or NK4. NK4 over-expression on Naïve B cells were detected through western blotting (Figure 4A). The flow cytometry results demonstrated that NK4 cannot activate naïve B cells directly yet. (Figure 4B). However, when purified naive B cells (CD19+IgD+CD27−) were transfected with empty vector or NK4 in the presence of IL-2 and CpG, NK4 enhanced CPG function on the activation of human naïve B cells (Figure 4C-D). IL-2 can support human B cell proliferation and Ig productions (Arpin et al., 1995). CpG activates human naive B cells (Huggins et al., 2007), resulting in enhanced proliferation and survival (Joo et al., 2012). The results suggest that NK4 activate naïve B cells in the present of CPG and IL-2.

NK4 promotes memory B cell differentiation into IgG and IgA-plasmablasts

We then tested whether NK4 could promote the differentiation of both CD19+IgD+CD27+ and CD19+IgD−CD27+ memory B cells into plasmablast. NK4 effectively induced IgD+CD27+ B cell differentiation into plasmablast (Figure 5A-B), as measured by up-regulation of CD38 and down-regulation of CD20 expression. NK4 also induce those B cells to produce switched isotypes, especially IgG and IgA (Figure 5C). ELISA results confirmed that NK4 promote IgD+CD27+ B cell secrete IgG and IgA (Figure 5D-E). Similar to IgD+CD27+ B cells, after IgD−CD27+ memory B cells were transfected with NK4, CD20−CD38+ plasmablast were induced for differentiation with secreted increased levels of IgG and IgA (Figure 6A-C). Real-time PCR analysis of switch circle transcripts revealed that memory B cells transfected with NK4 had significantly increased levels of Iγ-Cμ and Iα-Cμ (Figure 6D-E), which regulate IgM switch to IgG and IgA (Joo et al., 2012). Collectively, NK4 displays a potent capability to enhance memory B cell differentiation into IgG- and IgA-plasmablasts.

NK4 regulates B cell migration via induction of CXCL13

To further investigate the mechanism that regulates B cell migration and infiltration into target organs, we performed a protein array and compared cytokine expression in serum from both PECAM-NK4 mice and age/sex matched WT littermate controls. Notably, there

was a strong signal for CXCL13 in serum samples from the PECAM-NK4 mice compared to littermate controls (supple Fig 5A, Figure 2A). Since CXCL13 is also called B cell chemo attractant, we reasoned that this chemokine might be responsible for B cell infiltration in salivary glands observed in the NK4 mice. Thus, we performed tissue staining and detected a significant amount of CXCL13 reactivity in the inflammatory cells of salivary gland tissues from the PECAM-NK4 mice compared to controls (Figure 7B). Similar results were also obtained upon an analysis of kidney tissue samples from seven-months-old PECAM-NK4 mice (Suppl. Figure 5B). In PECAM-NK4 mouse model, CXCL13 was expressed in inflammatory cells and vessels, suggesting that NK4 induces the production of CXCL13 in host cells such as endothelial cells, and CXCL13 is responsible for the recruitment of B cells to target organs. In parallel studies, we found that CXCL13 was also expressed in vessels in salivary gland from pSS patients (Suppl. Figure 5C). To test NK4 roles on endothelial cells, we performed Transwell migration assays by seeding human naïve B cells in the upper chamber and HUVEC transfected with empty vector or NK4 in the bottom chamber. Flow cytometry was performed to quantify the number of B cells that migrated into the bottom chamber. The results demonstrated that B cell migration was significantly increased (Figure 7C), due to more CXCL13 induction of NK4-treated HUVECs (Figure 7D). Interestingly, addition of CXCL13 neutralizing antibody significantly blocked NK4/endothelial cell-induced B cell migration in a dose-dependent manner (Figure 7E). These data suggest that NK4 promotes B cell migration in a paracrine fashion through an induction of CXCL13 in surrounding cells such as endothelium in target organs. Tissue deposited CXCL13 provides the chemotactic signal for B cell recruitment. Notably, CXCL13 was abundantly expressed in endothelial-like structures throughout the inflamed salivary tissues from patients with Sjögren's Syndrome.

NK4 regulates B cell differentiation via the induction of Rap1 activity

After Naïve B cells were transfected with Empty vector or NK4 for 5-6 days, the increased activity of Rap1 were shown in NK4-transfected group (intensity ratio is 20.36, compared Vec group), but total Rap1 protein level is slightly evaluated (intensity ratio is 1.57, compared Vec group, Figure 8A), suggesting that NK4 upgrades the Rap1 activity. To confirm the concept further, we use Rap1shRNA to inhibit Rap1, after Human B cell were transfected with NK4 plasmid. The data demonstrated that Rap1 shRNA block the activation of NK4-transfected naïve B cells (Figure 8B-D). Thus, data indicated that NK4, as GEF protein, upregulate activity of Rap1 to control B cells activation and differentiation.

Discussion

Autoimmune diseases are serious medical conditions affecting approximately 5% to 8% of the US population. Therefore, identifying molecular mediators and studying the underlying mechanisms for autoimmunity are critically important. In present study, we used a combination approach of patient sample analysis and a genetic mouse model as well as human Naïve and memory B cells to demonstrate a novel role for NK4 in promoting the development of autoimmune disorders through upgrading the activity of Rap1. NK4 is significantly increased in the circulation and tissue deposition in patients with pSS, a chronic autoimmune disease affecting the body's exocrine glands. The levels

of circulating NK4 strongly and positively correlate with clinical characterization of pSS. In line with the patient data, transgenic expression of NK4 in a mouse model led to spontaneous development of autoimmune phenotypes that resembles to those observed in pSS patients, and the autoimmune properties worsen with age. Mechanistic studies show that NK4 regulates Naïve B cell activation, promote memory B cells differentiation into IgG and IgA-plasmablasts through up-regulating the activity of Rap1, and promotes B cell migration in a paracrine fashion through induction of CXCL13 in endothelial cells, thereby contributing to the development of autoimmune phenotypes (Figure 8E).

Although the pathogenesis of autoimmune diseases remains unclear, T cells, B cells, macrophages and endothelial cells are known to play crucial roles in the development of the diseases. NK4 is a pleiotropic factor that affects various types of cells including lymphocytes, myeloid cells and endothelial cells (Dinarello, 2010; Dinarello and Kim, 2006).

NK4, as GEF protein (unpublished data), regulates the activity of Rap1, is a member of Ras family of small G protein ubiquitously expressed in most tissues and mediates broad cellular activities. The Rap1 mediate cell integrins and other adhesion molecules to control cell-cell and cell-matrix adhesion (Bos, 2005). Rap1GTP is inactivated by Rap1 GTPase-activating protein, including Rap GAPs and SPA-1. The mice deficient for SPA-1 had shown the increased numbers of B1a cells producing anti-dsDNA antibody and SLE through regulating the B cell receptor (BCR) repertoire (Ishida et al., 2006). Those results suggest that Rap1 signal may regulate the autoreactivity of BCR repertoire in BM immature B cells and tissue localization of potentially autoreactive mature B cells to lead to autoimmune disease (Ishida et al., 2006; Tanigaki et al., 2002). Our results also confirmed it. PECAM-NK4 Tg mice exhibited the increased number of peritoneal B1 cells and elevated serum IgM, IgG and IgA, and had increased surface IgG+ B cells infiltration into tissues (Such as salivary gland and kidney), suggesting that NK4 could promote Ig isotype switching *in vivo*, and induce tissues damage through regulating Rap1 signaling. In addition, our *in vitro* human data also demonstrated that NK4 can enhance Naïve B cell activation following treatment with TLR9 agonist CPG and IL-2, indicating that, after human Naïve B cells were treated with NK4, Rap1 activity in B cells were upregulated to modify BCR repertoire to upregulate TLR9 expression in naïve B cell (Bernasconi et al., 2003; Ishida et al., 2006). Even though TLR9 agonist--- CpG can activate naive CD27− B cells regardless of BCR cross-linking, their subsequent plasma-cell differentiation is a function of the in vitro culture conditions rather than an intrinsic biologic limitation (Capolunghi et al., 2008; Huggins et al., 2007). Therefore, NK4 lower response threshold for membrane-associated antigens to promote CPG-stimulated activation of B cells and cannot induce Naïve B cell differentiate into Ig switching plasmablast.

Naïve human B cells produce both IgM and IgD, which are the first two heavy chain segments in the immunoglobulin locus. After activation by antigen, these B cells proliferate and became activated or memory B cells. When those B cells encounter specific signaling molecules via BCR or cytokine receptors, they undergo antibody class switching to produce IgG, IgA or IgE antibodies. Our results demonstrated that NK4 can promote human memory B cells differentiation into IgG-and IgA-plasmablast in the presence of CPG and IL-2.

Human memory B cells can proliferate and differentiate into immunoglobulin (Ig)–secreting plasmablast in response to CpG (Bernasconi et al., 2003). However, Immunoglobulin class switching seldom occur. Toll-like receptor ligands (such as CPG) also sensitize BCR signaling that require actin severing by cofilin, which is regulated by activation of the Rap GTPase (Freeman et al., 2015). Therefore, memory B cells have lower activation thresholds and these cells can be rapidly activated in a non-antigen-specific manner by the combination of Toll-like receptor (TLR) agonists and a proliferation-inducing ligand (APRIL) (TNFSF13A) or BAFF (TNFSF13B) as well as by the combination of cytokines, such as IL-21 and BAFF (Ettinger et al., 2008). Therefore, NK4 has potential capacity to induce Ig switching on memory B cells. The transgenic expression of NK4 also leads to elevated production and tissue deposition of IgM autoantibodies, B cell infiltration in salivary glands, and mild immune-complex mediated nephritis; all of which resemble the characteristics of patients with pSS and SLE, which are known to involve excessive activation of B cells and the production of autoantibodies(Kotzin, 1996). Notably, lymphocytic infiltration into salivary glands occurred months earlier than in the kidneys in PECAM-NK4 mice. This is also a feature common to pSS patients whose salivary glands are the first affected organs. Besides, the NK4 mice display increased numbers of B1a cells in their peritoneal cavities and splenic marginal zone B cells, as well as enhanced responses to vaccinations with antigens. These results provide cellular mechanisms for elevated serum levels of IgM in the NK4 transgenic mice since the majority of IgM is derived from B1a cells and marginal zone B cells (Guinamard et al., 2000; Martin et al., 2001). Constitutive activation of Rap1 can also antagonize Ras-dependent mitogen-activated protein kinase (MAPK) activation, likely via indirect effects of Rap1 on T cell ROS production

In this study, we found that NK4 had no direct effect on B cell migration, but it promoted B cell migration in a paracrine fashion through induction of CXCL13 in endothelial cells. CXCL13 is a B cell chemokine and its levels are increased in several autoimmune diseases(Barone et al., 2005; Moreth et al., 2010; Reif et al., 2002). In the current study, we observed CXCL13 deposition in the salivary gland and kidney from the autoimmune NK4 transgenic mice. Based on these data, it is conceivable to suggest that elevated NK4 upregulates CXCL13 expression in inflamed endothelium and inflammatory cells in salivary glands, which provide a chemotactic signal for B cell recruitment to the target tissues. In addition, CXCL13-induced B-cell migration and adhesion are strongly dependent on activation of the Rap1 GTPase (Durand et al., 2006). This interpretation is supported by the histological analysis of tissue sections from the NK4 mice that showed preferential accumulation of B cell infiltrates around blood vessels in target organs. Endothelial cells are known to produce CXCL13 (Kanemitsu et al., 2005), and this chemokine is abundantly expressed on endothelial-like structures throughout the inflamed salivary tissues from patients with pSS (Amft et al., 2001; Rangel-Moreno et al., 2006).

In summary, the identification of NK4 as a critical component in the process of B cell maturation and activation resulting in the production of autoantibodies provides a new mechanism for the pathogenesis of autoimmune diseases. NK4 is elevated in patients with autoimmune disorders. NK4 mediates B cell migration, promotes B cell autoimmunity, and enhances autoantibody responses to vaccinations. Thus, NK4 might be both a target for treating autoimmune diseases and a biomarker for the diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Intramural Research Program at NCI, NIH. We thank Drs. Ira Darr at NCI for proof reading the manuscript, and Christopher Campbell for advice and technical assistance. We thank CCR-Frederick Flow Cytometry Core for cell sorting support.

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Highlights

- Natural Killer Cell Transcript 4 (NK4) is significantly elevated in samples from patients with Sjögren's Syndrome (SS), as a novel regulator that promotes the development of autoimmune disorders.
- Old mice with transgenic expression of NK4 develop autoantibodies, sialadenitis, and immune complex-mediated nephritis similar to those in Sjogren's Syndrome patients.
- NK4 efficiently activated human naïve B cells and induced memory B cell differentiation into IgG and IgA-plasma blasts to promote the development of autoimmune phenotypes as a GEF for Rap1.
- NK4 promotes B cell migration in a paracrine fashion through an induction of CXCL13 in surrounding inflammatory cells and endothelial cells.

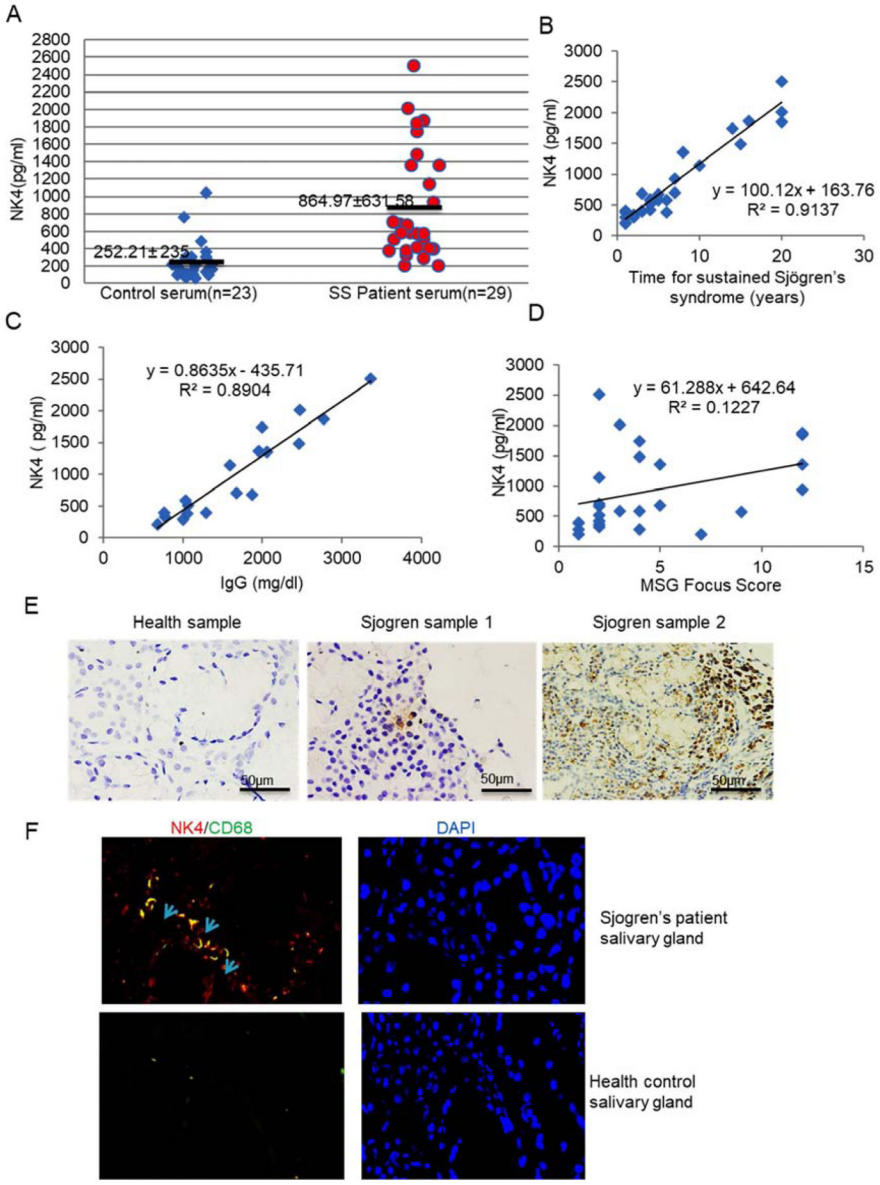

Figure 1.

NK4 levels are elevated in patients with pSS

(A) Sera from normal healthy subjects (n=23) and pSS patients (n=29) were analyzed for NK4 levels by ELISA and graphed (p=0.02). The correlation among NK4 levels in patient sera and the sustain years of pSS patients (B), serum IgG from patients(C) and Minor Salivary Gland (MSG) focus score from those patients(D) were first diagnosed with oral symptoms were analyzed and plotted. (E) Immunohistochemistry on NK4 expression was performed on salivary glands from normal healthy donors and eight pSS patients (four patients in each group with two grades). Patient 1 represents samples collected from pSS patients with limited clinical inflammation, and patient 2 represents SS patients with moderate to severe inflammation. Representative images are shown. (F) Immunofluorescence double staining for NK4 and CD68 on salivary glands of pSS patients

and health control donor, as described in Material and methods. DAPI staining is for cell nucleus. Representative images are shown.

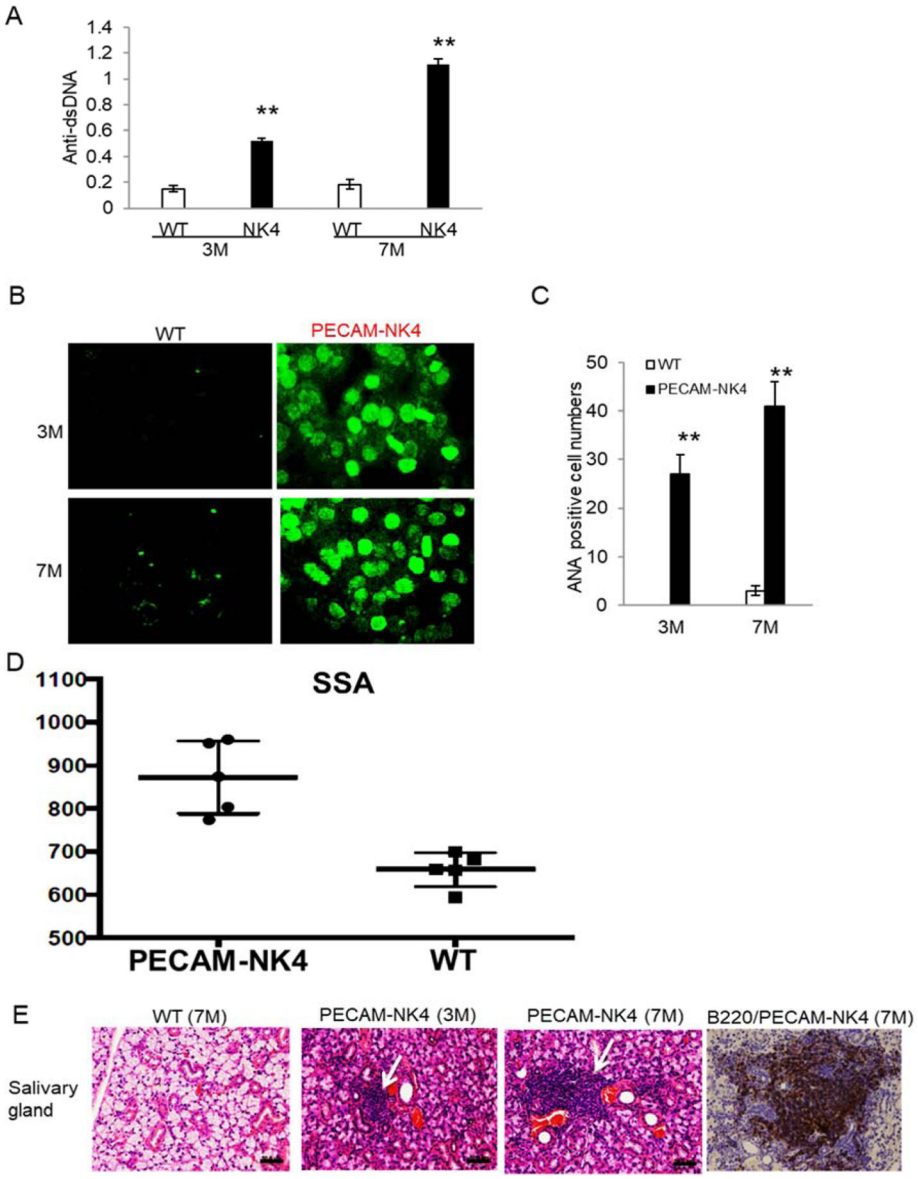

Figure 2.

Transgenic expression of NK4 in one mouse model results in spontaneous development of autoimmune phenotypes.

(A) Sera were collected from three and seven-months-old WT littermate controls and PECAM-NK4 mice. The levels of anti-dsDNA antibodies in sera were detected by ELISA and plotted (*$p<0.05$, n=23 mice at 3 months of age; n=15 mice at 7 months of age).

(B) ANA was detected on HEp-2 cells using an indirect immunofluorescence assay. Representative ANA IgG staining of Hep-2 cells incubated with serum from 3 and 7-month-old WT and PECAM-NK4 Transgenic mice. The sample from PECAM-NK4 Transgenic mice shows a specked pattern. No or few positive cells were shown after incubated with samples from 3-or 7-month-old WT mice. (C) The number of ANA positive cells was counted in 10 randomly selected high-power fields under microscopy and graphed. *$p<0.05$.

(D) Anti-SSA autoantibody (Anti-Sjögren's-syndrome-related antigen A, also called anti-Ro) was detected in sera from WT and PECAM-NK4 mice. (E) Salivary glands were harvested from 7 months old WT littermate control mice, 3 and 7 months old PECAM-NK4 mice, sectioned and stained with HE and with an anti B220 antibody. White arrows point to infiltrating inflammatory cells. Bar= 50μm.

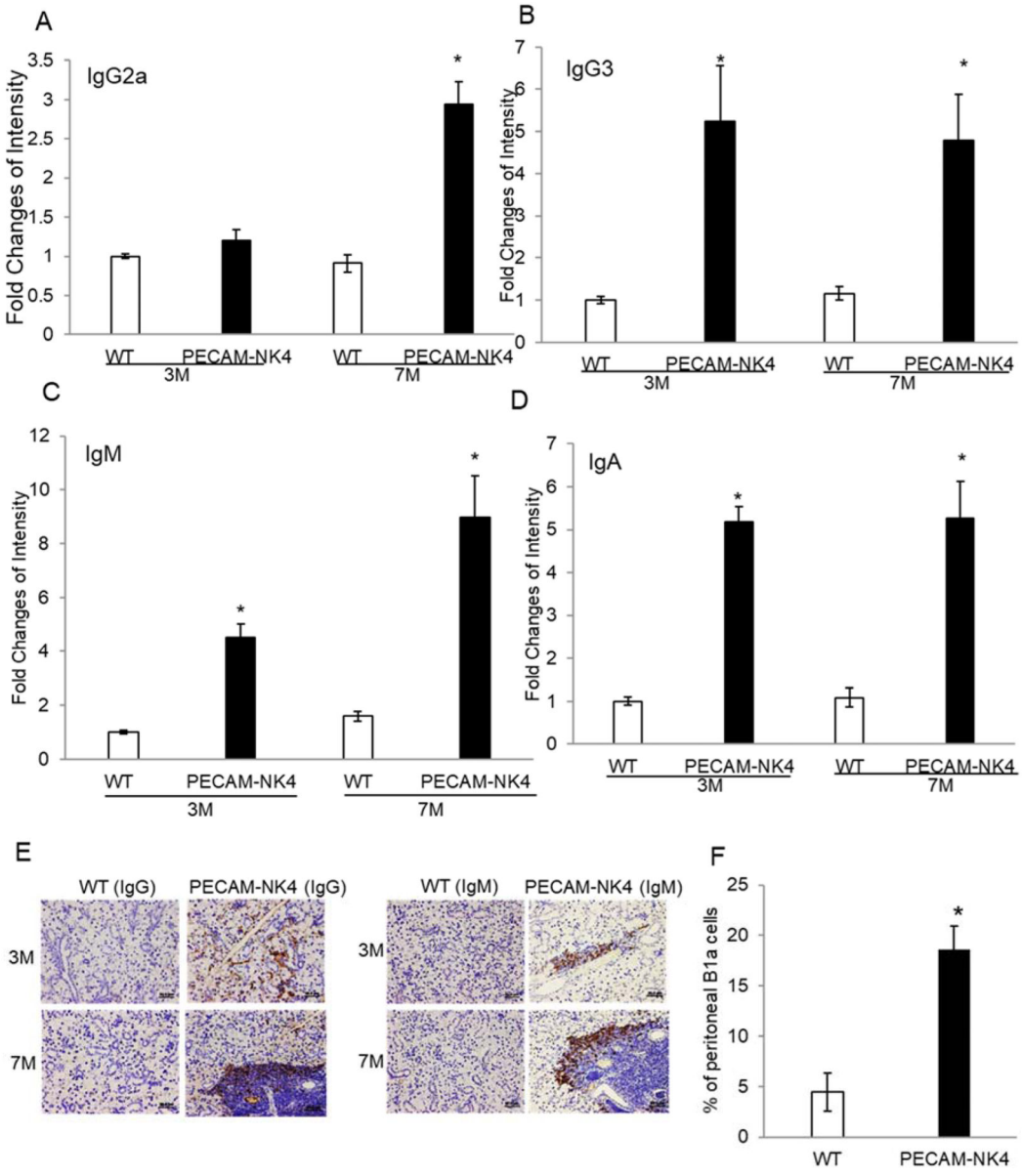

Figure 3.

NK4 promotes the production of immunoglobulins in a mouse model.

(A-D) Sera were obtained from PECAM-NK4 mice and WT littermate controls at 3 and 7 months of age. Mouse Ig Isotyping Array was performed. Data shown are the mean and SD (n=6 mice per group per time point. *p<0.05). (E) Immunohistochemistry was used to detect IgG and IgM deposition in salivary glands harvested from PECAM-NK4 mice and WT littermate controls at 3 and 7 months of age (n=6 mice per group per time point). Representative images are shown. Bar= 50μm. (F) The percentage of B1a subgroup (CD19+ CD5+) in the peritoneal of NK4 transgenic mice and WT control littermates was determined by flow cytometry (n=6 mice per group, *p<0.05). Each experiment was done in triplicate and repeated three times (*p<0.05).

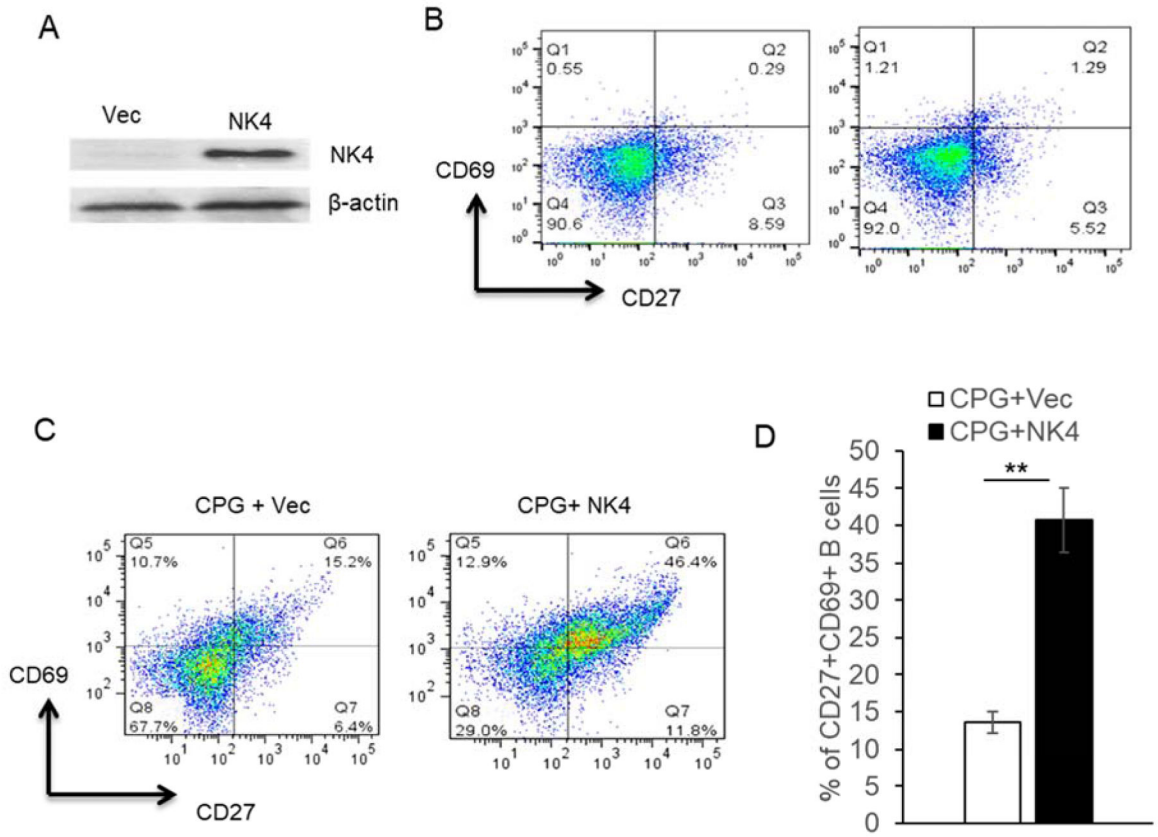

Figure 4.

NK4 activates Naïve B cells in the presence of antigen.

After naïve B cells were isolated by flow cytometry, those B cells were transfected with NK4 or empty vector. After 6 days, (A) NK4 overexpression were detected with Anti-NK4 through western blotting, (B) CD38 and CD20 expression on B cells from two groups were assessed by flow cytometry. (C) After naïve B cells were isolated by flow cytometry, those B cells were transfected with NK4 for 6 days in the presence of 50 nM CpG and 20 U/ml IL-2. Empty vector as control groups. CD69 and CD27 expression on B cells were measured on day 6th. (D) graph data are from three independent experiments with triplicate assay. *, $P<0.05$, **$p<0.01$.

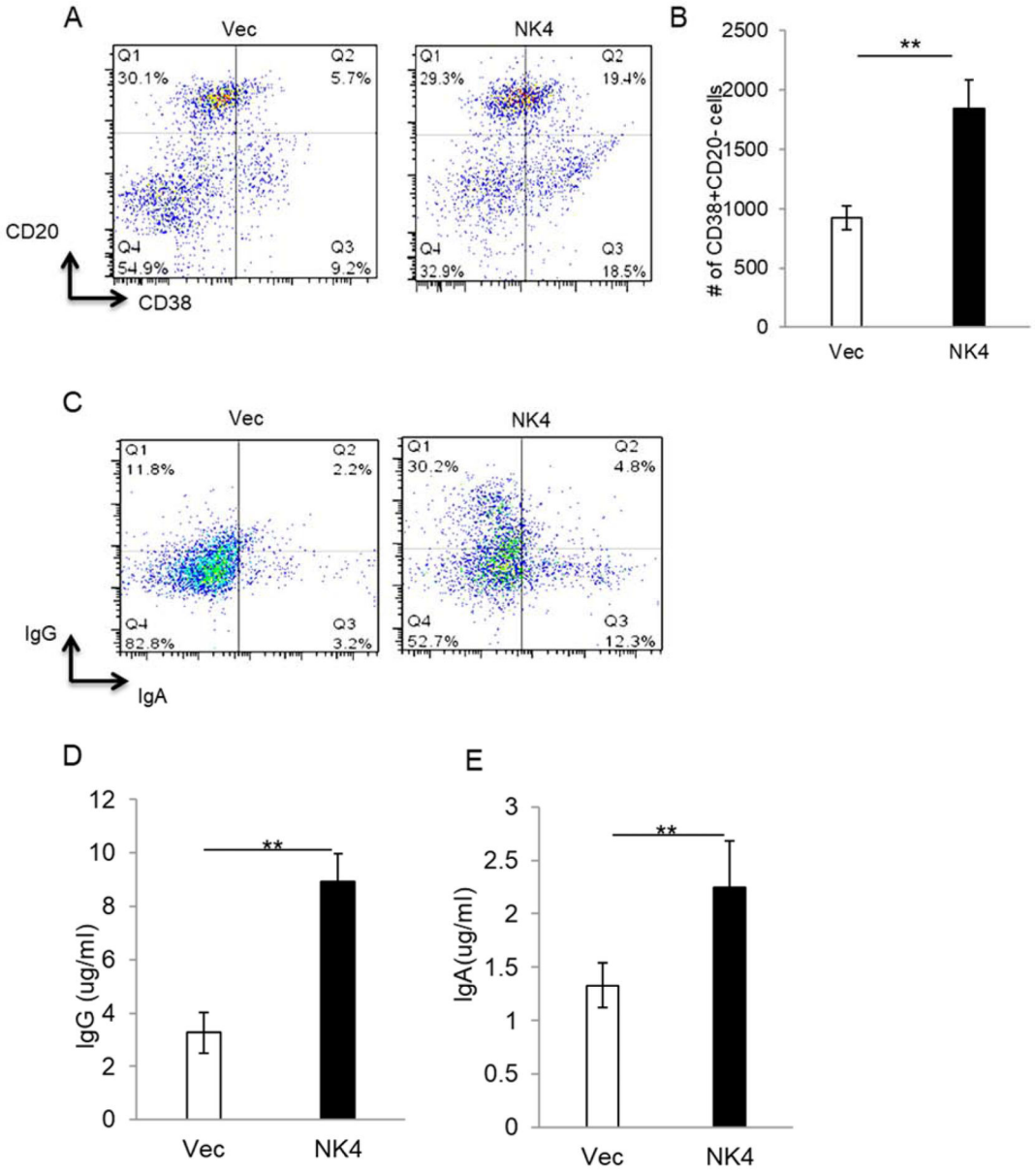

Figure 5.

NK4 induces CD19+ CD27+IgD+ B cell responses by promoting IgG- and IgA-secreting B cell responses.

CD19+ CD27+IgD+ memory B cells were isolated by flow cytometry, and were transfected with NK4 for 6 days in the presence of 50 nM CpG and 20 U/ml IL-2. Empty vector as control groups. (A) CD38 and CD20 expression on B cells from four groups were assessed after 6 days. (B) Experiments using 8 healthy human showed similar results. (C) Surface Ig staining on day 6. (D and E) Total Ig assayed by ELISA on day 6th. In B, D, E combined data (mean ± SD) from experiments using 8 healthy donors are presented. Data are from three independent experiments with triplicate assay. *, P<0.05, **p<0.01.

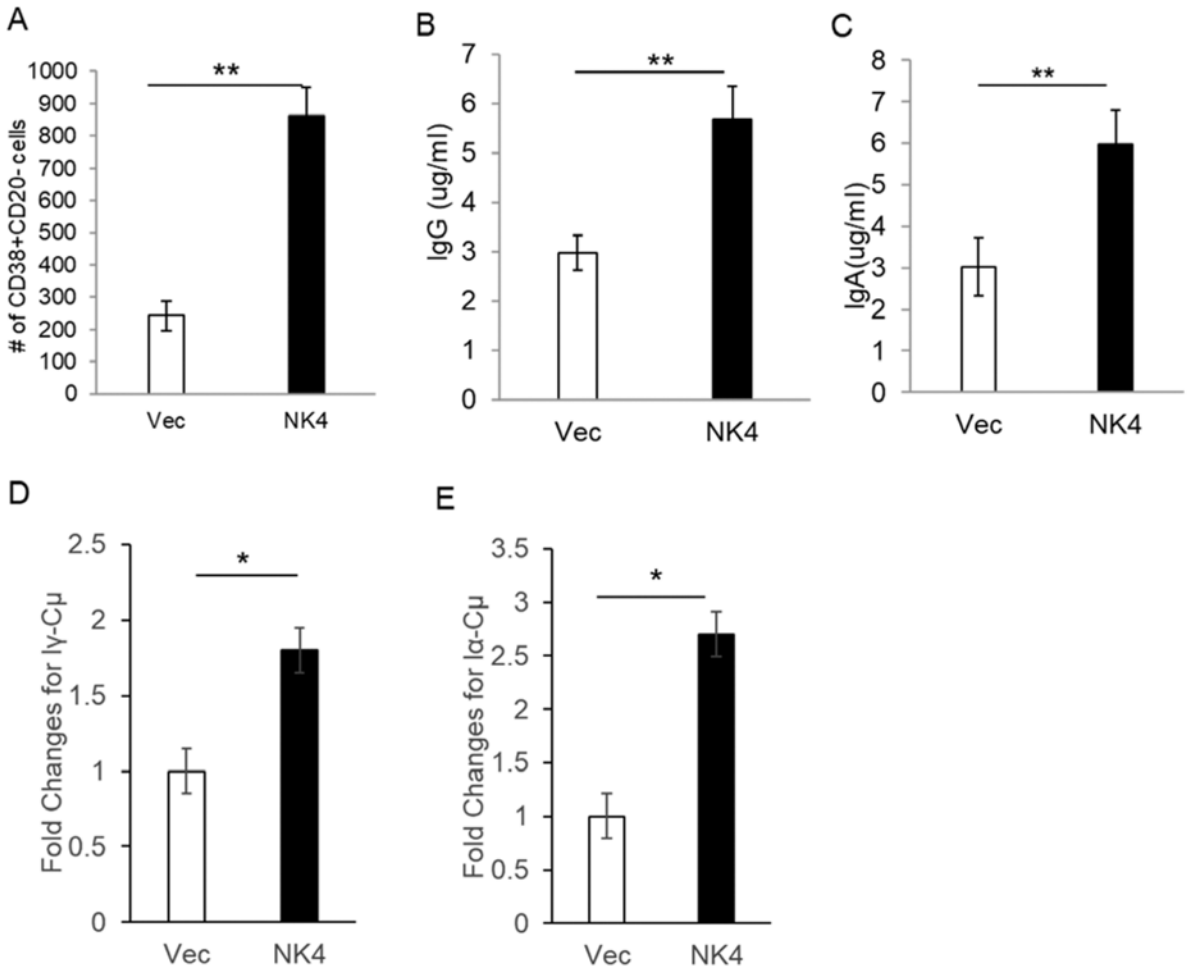

Figure 6.

NK4 induces CD19+ CD27+IgD- B cell responses by promoting IgG- and IgA-secreting B cell responses.

CD19+ CD27+IgD- memory B cells were isolated by flow cytometry, and were transfected with NK4 for 6 days in the presence of 50 nM CpG and 20 U/ml IL-2. Empty vector as control groups. (A) CD38 and CD20 expression on B cells from four groups were assessed after 6 days. Experiments using 8 healthy human showed similar results. (B and C) Total IgG and IgA assayed by ELISA on day 6th. mRNA was harvested, after memory B cells were transfected with empty vector or NK4 5-6 days. Real-time PCR was performed to measure the relative expression of switch circle transcript. Iγ-Cμ (D) or Iα-Cμ (E). Combined data (mean ± SD) from experiments using 8 healthy donors are presented separately. Data are from three independent experiments with triplicate assay. *, $P<0.05$, **$p<0.01$.

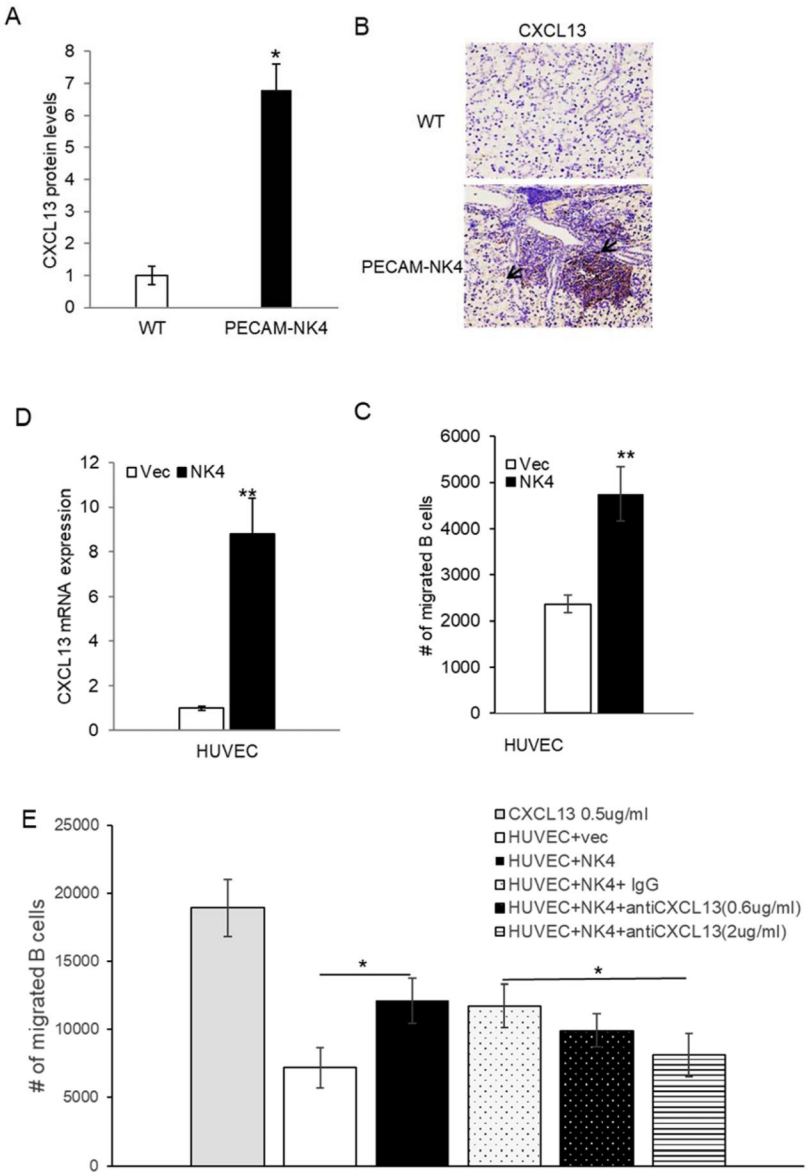

Figure 7.

NK4 promotes B cell migration through CXCL13.

(A) The protein array for immunofluorescence detecting chemokines/ cytokines (spots relevant to this study are in a circle, showing chemokine CXCL13) of sera from from 7-month-old PECAM-NK4 transgenic mice and littermate controls. CXCL13 protein levels are 6 times higher in serum from 7-month-old PECAM-NK4 transgenic mice than those from littermate controls. (B) Salivary gland tissues from three-month-old PECAM-NK4 mice and WT littermate controls were analyzed for CXCL13 expression by immunohistochemistry. Brown color marks CXCL13+ cells. Arrows point to blood vessels. Representative images are shown. (C) B cell migration was measured by a Transwell assay. Human CD19+ B cells and HUVECs were seeded into either the upper or bottom wells, respectively, with addition of empty vector or NK4 in the bottom chambers, and incubated for 24hrs. Migrated B cells in the bottom chamber were quantitated by flow cytometry for

CD19+cells (*p<0.05). (D) CXCL13 mRNA expression on HUVECs were measure using Real-time RT-PCR. (E) Identical conditions as in the Panel B were applied except with the addition of CXCL13 neutralizing antibody in the bottom chamber. Recombinant CXCL13 protein was used as a positive control, and isotype IgG was used as a negative control. Each experiment was performed in triplicate and repeated three times (*p < 0.05, **p<0.01).

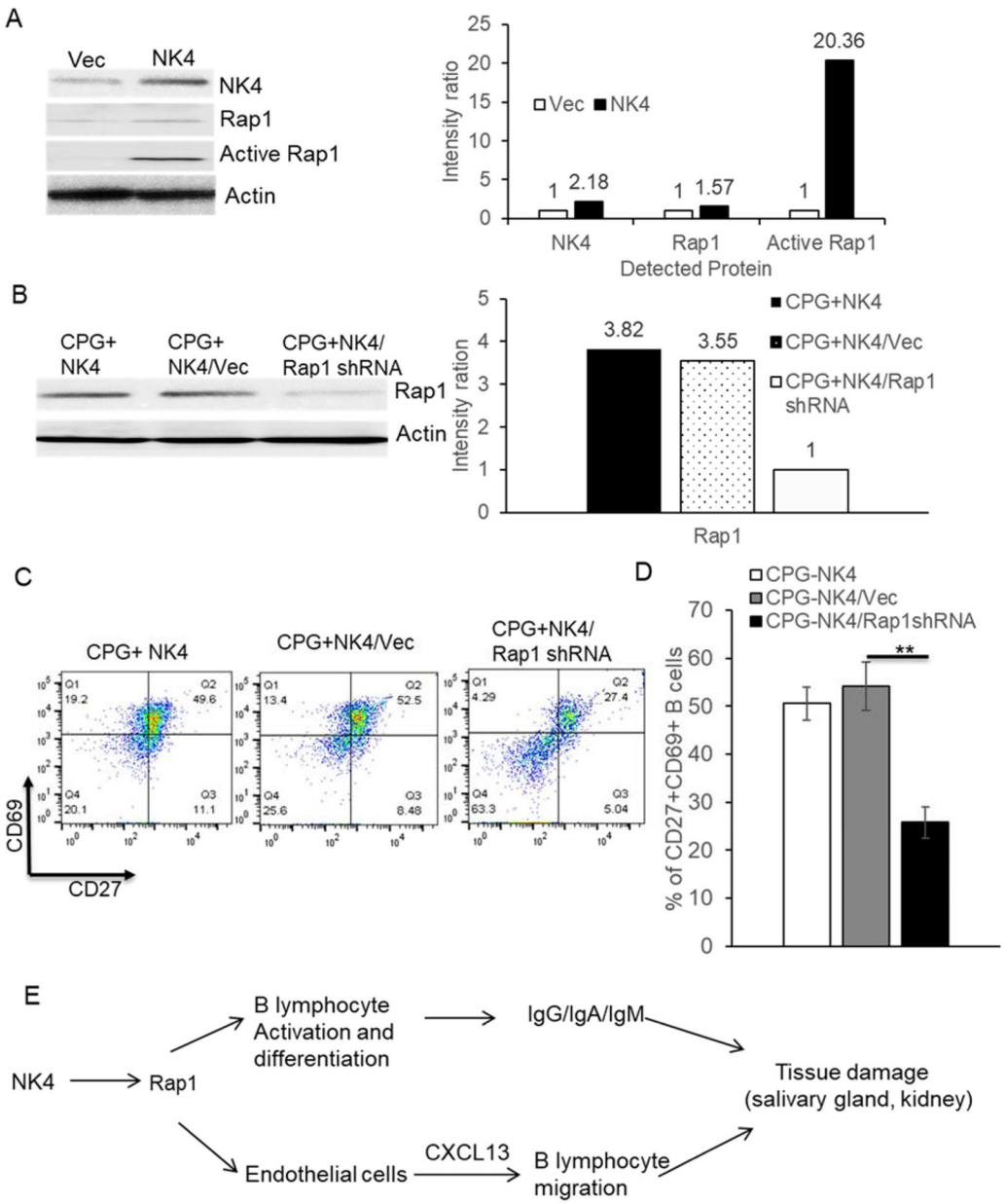

Figure 8.

NK4 regulates naïve B cells activation through upgrading Rap1 activity.

(A) Naïve B cells were transfected with empty vector or NK4, NK4 and Rap1 protein were measured using western blotting. For detection of Rap1 activity, the protein was measured with activated Rap1 antibody, after IP, according to the Rap1 activity measuring protocol. The band intensities of WB were analyzed using imageJ software. The intensity ratios were calculated. (B) Rap1 protein was detected via western blotting, after naïve B cells were transfected with NK4 in the presence of empty shRNA or Rap1a shRNA for 6 days. Both representative WB results and their band intensity ratios were shown. (C) CD69 and CD27 expression on B cells above (B) were also measured. (D) graph data are from three independent experiments. *, P<0.05, **p<0.01. (E) One diagram of putative signaling

pathway, by which NK4 regulates B cell differentiation and further induce autoimmune phenotypes such as tissues damage via upgrading Rap1 activity.

Table I.

Comparison of splenic B cell subpopulations in 7-month-old PECAM-NK4 transgenic mice and littermate control[a]

	Splenic B cells	Marginal zone B cell	Germinal center B cell	Plasma cells
Wild-type	30.17±3.26	1.94±0.17	3.31±0.86	0.53±0.16
PECAM-NK4	31.23±6.12	8.61±1.01 *	6.35±1.13 *	1.27±0.39 *

[a]. Splenic B cells were $CD19^+$, marginal zone B cells $CD19^+/CD21^{hi}/IgM^+$, germinal center B cells $CD19^+$/B220+/GL-$7^+/IgD^{lo/-}$, and plasma cells $CD19^-$ /$CD138^+$.

*P<0.05. four mice in each group