NCR3/NKp30 contributes to pathogenesis in primary Sjögren's syndrome

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Abstract

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease characterized by a lymphocytic exocrinopathy. However, patients often have evidence of systemic autoimmunity and they are at markedly increased risk for the development of non- Hodgkin's lymphoma. Similar to other autoimmune disorders, a strong interferon (IFN) signature is present among subsets of pSS patients, though the precise etiology remains uncertain. NCR3/NKp30 is a NK-specific activating receptor regulating the cross-talk between NK and dendritic cells and type II IFN secretion. We performed a case-control study of genetic polymorphisms of the NCR3/NKp30 gene and found that rs11575837 (G>A) residing in the promoter was associated with reduced gene transcription and function as well as protection to pSS. We also demonstrated that circulating levels of NCR3/NKp30 were markedly increased among pSS patients compared with controls and correlated with higher NCR3/NKp30 but not CD16-dependent IFN-γ secretion by NK cells. Excess accumulation of NK cells in minor salivary glands correlated with the severity of the exocrinopathy. B7H6, the ligand of NKp30, was expressed by salivary epithelial cells. These findings suggest that NK cells may promote an NKp30-dependent inflammatory state in salivary glands, and that blockade of the B7H6/NKp30 axis could be clinically relevant in pSS.

Keywords

Sjögren's syndrome; autoimmunity; NK cells; innate immunity; NKp30/NCR3

Introduction

Although primary Sjögren's syndrome (pSS) affects up to 0.1–0.6% of the general population, few rigorous etiologic studies have focused on the initiating immunopathologic process occurring in the exocrine glands. Histologic examination of minor salivary glands obtained from pSS patients reveals persistent inflammatory foci consisting of mononuclear cells [primarily T lymphocytes, with some B lymphocytes and rare dendritic cells (DC)(1)] leading to progressive acinar epithelial cell atrophy and fibrosis. Interestingly, the severity of secretory dysfunction does not always correlate with the extent or degree of leukocytic infiltration and loss of acinar tissue, suggesting that immune dysregulation might impair exocrine gland secretion very early in the disease process (2, 3).

Gene expression profiling studies demonstrated activation of type I and type II IFN pathways in pSS patients (4–7). B cells are highly activated in this auto-immune disease through diverse pathways, including the overexpression of the BAFF cytokine (8), and T cell patterns are skewed towards a memory phenotype and a T helper 1 (Th1) polarization associated with high serum levels of IFN-γ (9). Recently, elegant studies reported data supporting the potential role of innate or cognate (double negative or conventional T cells) effectors in setting the stage of a Th17-based inflammation in pSS (10–13).

Animal models of pSS have provided insight into both genetic susceptibility as well as key pathologic molecules participating in the onset (preclinical phase) or development (clinical phase) of pSS (14). For example, studies performed in NOD.IFN-γ−/− or NOD.IFN- γR−/− mice indicate that IFN-γ is a crucial cytokine for pSS-like disease (15). More specifically, NOD.IFN-γ−/− and NOD.IFN-γR−/− mice fail to develop autoimmune responses, acinar cell apoptosis and leukocyte infiltration against salivary glands at the clinical stage of the disease (15). IFN-γ is central to salivary gland dysfunction in the Ro60 humanized model of Sjögren's syndrome (16). Recently, use of precise probes distinguishing type I from type II activity showed that the pSS-associated IFN signature could be assigned to IFN-γ (6). Moreover, IL-12 (a strong IFN-γ inducer) transgenic mice develop a pSS-like syndrome (17, 18). Lastly, a genetic polymorphism of IL-12A was recently shown to be associated with pSS in humans based on a genome wide association study (19), and our group recently showed that IL-12 is able to induce IFN-γ as well as IFN-α secretion in human pSS (20).

As a result of their interaction with various DC subsets, Natural Killer (NK) cells play a critical role as mediators of both type I and type II IFN biologic functions. Although animal models of pSS have not directly implicated NK cells in disease pathogenesis (21), recent work in mouse CMV-induced sialadenitis, which may be highly relevant to pSS, implicate a regulatory role for salivary NK cells (22). More importantly, flow cytometry analyses of salivary glands from pSS patients demonstrate the presence of a subset of NK cells expressing NKp44 and producing IL-22 which are associated with the severity of sialadenitis (11).

The aim of this study was to investigate the role of NK cells in pSS pathogenesis. We focused on *NCR3*/NKp30 effector functions for several reasons. First, NKp30 is selectively expressed by circulating NK cells and represents a surrogate marker of NK cell functions in

humans (23). Second, NKp30 is a crucial regulator of the DC/NK cell cross-talk, mediating the release of Th1 (IL-12, IFN-γ) cytokines (24). Third, transcription of the *NCR3*/NKp30 gene is subject to alternative splicing resulting in various isoforms endowed with distinct effector functions (25). Here we show that genetic polymorphisms within the promoter region of *NCR3*/NKp30 were associated with reduced levels of gene transcription and function as well as decreased susceptibility to pSS. Thus, NKp30 expression and functions were found up-regulated in pSS (compared with controls). NK cells correlate with the sialadenitis while salivary gland epithelial cells could express NKp30 ligands and trigger NKp30-dependent release of Th1 cytokines.

Results

Genetic polymorphisms within the NKp30 promoter region are associated with decreased susceptibility to pSS

Previous work has defined three isoforms of NKp30 with distinct effector functions that have prognostic relevance in GIST (gastrointestinal stromal tumor) malignancies (25). The NKp30 A and B isoforms, having high killing activity and secretory capacity for IFN-γ and TNF-α, respectively, are associated with a better prognosis than NKp30 isoform C. Thus, we performed quantitative Reverse Transcriptase PCR (qRT-PCR) using primers specific for NKp30A, NKp30B or NKp30C in 164 pSS patients from the French Assessment of Systemic Symptoms and Evolution in patients with pSS (ASSESS) cohort and compared the relative amounts of each isoform by calculating ΔCt ratios (A/B, B/C and A/C). The unsupervised hierarchical clustering of expression data defined 6 distinct groups (Supplemental Fig. 1) that did not appear to be associated with specific pSS phenotypes, such as systemic involvement, presence of auto-antibodies or history of lymphoma.

Then, we studied single nucleotide polymorphisms (SNPs) that could affect the transcription of the NKp30 gene. In the exploratory cohort, 574 French pSS patients and 451 independent French control subjects were genotyped for 9 SNPs within the 6p21.3 NKp30 locus (Supplemental Table S1). Due to the proximity of *NKp30* to the HLA- DR and TNF loci, which have previously been implicated in pSS (26, 27), 3 additional SNP proxies for HLA-DR2, HLA-DR3 and TNF −308 were investigated. Single-SNP analyses adjusting for ancestry (see methods) showed that 4 SNPs within the *NKp30* locus, including rs2736191 and rs11575837, were inversely associated with risk of pSS (Supplemental Table S2). These associations were neither stronger nor more statistically significant among subsets of patients with disease specific auto -antibodies (Supplemental Table S2).

Two *NKp30* SNPs (rs11575837, rs2736191) and the SNP proxy for *HLA-DR3* (rs2187668) were genotyped in the replication study that included 436 pSS patients from Sweden (n=244) and Norway (n=192) and 441 healthy controls (Sweden, n=232 and Norway, n=209). In the replication study, the *NKp30* variant rs11575837 was significantly associated with disease characterized by anti-SSA and/or -SSB positivity [p=0.011, OR= 0.060] (Table 1).

We then combined results from the cohorts via two meta-analysis methods. While an assumption of a single fixed effect across studies is appropriate in cases without evidence of

heterogeneity, the alternative of estimating random effects across studies is not well powered given our small number of studies and low frequency of the *NKp30* SNPs. Thus, we present both models in Table 1 and Table 2. These combined analyses indicated that both *NKp30* variants, rs11575837 and rs2736191, were inversely associated with risk of pSS, i.e. they were protective, with OR=0.48 (p=0.0039) and OR=0.56 (p=0.0019), respectively, assuming fixed effects (Table 1). The association between rs11575837 and pSS was stronger and more statistically significant among the anti-SSA/B subset of patients, OR=0.38 (p=0.0033) (Table 1).

In order to assess the independence of associations with the 2 NKp30 SNPs and the HLA-DR3 tag SNP, we also performed multi-SNP analyses modeling all three effects in a single logistic regression, again for both cohorts individually and then combined using meta-analysis methods (Table 2). NKp30 SNPs rs11575837 and rs2736191 are significantly associated in fixed-effects multiple-SNP models (thus adjusting for the HLA- DR3 tag SNP) with OR=0.50 (p=0.008) and OR=0.62 (p=0.014), respectively. However, there is significant heterogeneity and a lack of significant independence in the random effects models. With cases restricted to anti-SSA/B positive patients, there is homogeneity of association and a stronger and more significant effect, OR=0.36 (p=0.0036), regardless of the method used.

Reduced transcription of NKp30 in individuals carrying the promoter variant

Since both protective SNPs were located within the promoter region of NKp30, we next assessed whether they could influence transcription levels of NKp30. NKp30 mRNA levels were investigated in 102 pSS patients from the French ASSESS cohort. Among them, 24 carried the minor allele rs2736191C, 12 carried the minor allele for rs11575837A and 66 were homozygous for both major alleles (G and G respectively). Patients carrying the minor allele for rs11575837 had lower expression levels of NKp30 mRNA compared to pSS patients carrying the major allele (median ratio NKp30/TBP (housekeeping gene): 1.432 and 1.801 respectively, p=0.04, Fig. 1). Thus, the minor A allele of rs11575837, which is protective (underrepresented among pSS patients) is associated with decreased transcription levels of NKp30.

Elevated NKp30 expression and function in pSS

We performed phenotypic characterization of NK cells in 38 pSS patients [92% women, mean age (± standard deviation) 57 years (±15.3)] compared to 30 age-matched controls [43% women, mean age 58 years (±16.4)]. Among pSS patients, the mean European League Against Rheumatism Sjögren's Syndrome Disease Activity Index (ESSDAI) was 3.1 (± 4.1) (28), which is indicative of low to moderate activity. Twenty two patients (57.9%) had a history of systemic involvement, including lymphoma in 2 patients (5%).

Lymphopenia is a well-known abnormality in pSS and was also observed in our study [mean lymphocyte count 1421 (±541) and 1727 (±599) /mm3 in pSS patients and controls respectively, p= 0.0165, Fig. 2A, left panel]. The degree of lymphopenia was associated with disease activity [mean lymphocyte count 1106 (±508) and 1612 (±475)/mm3 in ESSDAI >4 or <4, respectively, p= 0.0064, Fig. 2A, right panel].

The proportion of circulating CD3-CD56+ NK cells was significantly reduced in pSS patients versus controls (Fig. 2B) but this decrease was not related to the severity of disease among pSS cases. Similar to many other autoimmune disorders, the proportion of NK CD56bright NK cells was increased among pSS patients compared to controls (mean % of 9.4 (±6.5) and 6 (±4.3) respectively, p= 0.0173, Fig. 2C), though this was not associated with disease activity among cases. Importantly, the expression pattern of most inhibitory or activating NK cell receptors was not significantly different in pSS cases compared with controls (Supplemental Fig. 2) except for the NKp30 activating receptor that was markedly overexpressed in pSS (mean MFI NKp30: 63.3 (±22.1) and 47 (±24.7) pSS versus controls respectively, p=0.0104, Fig. 2E). Similar conclusions were drawn comparing female pSS and female controls. A second series of pSS patients with autoantibodies (N=18) corroborated these data showing that the MFI of NKp30 in circulating NK cells from pSS with positive anti-SSA was higher than that of matched controls (N=16) (mean MFI 446 versus 263 respectively, p = 0.004).

To assess the functional relevance of high expression levels of NKp30 on NK cells, we performed a receptor cross-linking assay to analyze degranulation and IFN-γ secretion at various non-saturating concentrations of agonistic anti-NKp30 Ab (and isotype or anti-CD16 antibody (Ab) as controls). Eighteen individuals were assessed for correlation between the expression levels of NKp30 and CD107a, as a functional marker of degranulation, or IFN-γ release upon NKp30 cross-linking. At various concentrations of NKp30 antibodies, we observed a strong correlation between the proportion of NK cells expressing NKp30 with the percentage of NK cells secreting IFN-γ (Fig. 3), but not with the percentage of degranulating NK cells (Supplemental Fig. 3). No significant association was seen upon cross-linking with anti-CD16 Ab (Fig. 3, right panels).

Next, we addressed the functional relevance of harboring the minor allele of the rs11575837 (11575837A) for the NKp30-dependent IFN-γ release in four individuals. As expected, NK cells from these four carriers were hypo-responsive to NKp30 cross-linking despite normal to high expression levels of NKp30 (Fig. 3, red dots). Together, these data suggest that circulating NK cells are prone to exert potent NKp30– mediated IFN-γ release upon engagement of the respective ligands. Individuals harboring the minor allele of rs11575837 exhibit reduced IFN-γ secretion upon NKp30 triggering.

Accumulation of NK cells in minor salivary glands

A hallmark of pSS is the presence in minor salivary glands (MSG) of inflammatory foci. The focus score represents a standardized method for semi-quantifying this process, and involves counting the number of such foci (consisting of at least 50 mononuclear cells) per 4 mm2 of glandular tissue (29). We analyzed the degree and topography of the NK cell infiltration in MSG biopsies harvested from 7 controls (sicca syndrome with normal salivary gland biopsy or non-specific sialadenitis and without auto-antibodies) and 20 pSS patients. Staining of paraffin-embedded tissue sections using anti-NKp46 Ab revealed increased numbers of tissue- resident NK cells (25, 30) (p= 0.026 comparing pSS and controls, Fig. 4 A–C). In pSS patients, these NK cells accumulated mainly outside of the inflammatory foci in the same areas as plasmacytoid DC-like cells (5). Moreover, we observed a correlation

between the grading of focus score and the number of NKp46 positive cells outside of the inflammatory foci (r^2=0.425; p<0.0001, Fig. 4B).

NKp30-dependent cross-talk between NK cells and epithelial cells within salivary glands

Interestingly, the levels of NKp30 expression were lower in NK cells residing in salivary glands of pSS compared with circulating NK cells (Supplemental Fig. 4A), suggesting a local NKp30 engagement. However, the transcription of the NKp30 gene remained unaltered (Supplemental Fig. 4B). Therefore, we assessed the expression of B7-H6, one of the NKp30 ligands, on salivary gland epithelial cells, the HSG (Human Salivary Gland) cell line and primary cultures of salivary glandular epithelial cells (SGEC) derived from minor salivary gland biopsies. B7-H6 mRNA was detectable in both pSS and sicca salivary glands at diagnosis (Fig. 5A). HSG were incubated with different pro-inflammatory cytokines known to play a role in pSS (31). TNF-α and IFN-γ were of special interest as they are secreted by NK cells upon engagement of NKp30. Incubation for 6 hours with TNF-α or poly (I:C) (but not IL-1, IL-17, IL-22, IL-23 (not shown) nor IFN-γ) significantly up-regulated B7-H6 mRNA levels on HSG and some cases of SGEC (Fig. 5B). Next, we assessed whether the interaction between NKp30 and B7-H6 was functional. HSG cells stimulated for 24 hours with TNF-α or Poly (I:C) were incubated with Jurkat cells genetically modified to express one of the three major NKp30 isoforms or a control vector. The secretion of IL-2 was used as a marker of Jurkat cell activation. TNF-α promoted the cross-talk between HSG and Jurkat-NKp30A (and to a lesser extent with NKp30B) in a NKp30 (but not NKp44 or DNAM)-dependent manner (Fig. 5D). We assessed the TH1/TH17 polarization of the effector cells contained in salivary glands of six pSS and sicca patients using qRT-PCR detecting cytokines (IFN-γ, IL-17, IL-22) and transcription factors (Rorγt, T-bet). There was a trend towards a Th1 pattern of gene expression in pSS patients (Supplemental Fig.5).

Discussion

Our findings implicate the activating NK cell specific receptor NKp30 and its interaction with B7-H6 epithelial cells in the pathogenesis of pSS. Four lines of evidence support this assumption. First, a rare genetic variant residing in the promoter region is associated with reduced NKp30 mRNA expression levels and function and appears to protect against the development of pSS. Second, NK cells from pSS patients overexpress NKp30 receptor, which are associated with higher IFN-γ secretion levels upon engagement. Third, while NK cells are quite reduced in the blood, they are enriched in MSG outside the inflammatory foci and correlate with the sialadenitis score. Last, B7-H6, the ligand for NKp30, is expressed by salivary epithelial cells and regulated by TNF-α or ploy (I:C), triggering NKp30 mediated-effector functions.

Classically, autoimmune diseases (AID) represent syndromes caused by an exaggerated activation of the adaptive arm of immunity (T and B cells), aimed at recognizing antigen-specific targets. Alternatively, AIDs could be conceived as abnormal states in which self and damaged tissues are abnormally released into the periphery, processed and presented by DC to T cells that then migrate to the diseased organ. Thus, NK cells could play a crucial role in

this process. Primary SS typifies this model. In inflamed salivary glands from pSS patients, apoptotic epithelial cells may release auto-antigens such as Ro/La ribonucleoprotein complexes (32). These auto-antigens participate in the activation of B and T cells that lead to tissue damage. In this model, NK cells would be activated by ligation of their activating receptors recognizing stress-induced molecules in the damaged tissues, thereby activating immature DCs in the lymph node and eliciting the priming of effector T cells that can destroy peripheral target tissues. To date, research focusing on NK cell numbers, phenotypes and functions in AID has been mostly correlative and characterized by a number of pitfalls and limitations. First, circulating NK cells rather than tissue residing NK cells have been studied, due to limited access to inflamed tissues. Second, some analyses of NK cells have failed to distinguish bona fide NKp46 positive NK cells from NKT cells and T cells expressing NK receptors. Third, antibodies and technical methods specifically recognizing NK cells in paraffin-embedded tissues have not been available until very recently (25, 30). Fourth, there is still no perfect animal model of pSS and those recently described (33–35)have not specifically studied the role of NK cells. Moreover, mouse and human NK cells may diverge at several levels (including NKp30, which is a pseudo gene in mice). Finally, improved methods for pSS classification (36) and activity assessment (28) now allow for more consistency in disease characterization and status across studies. In spite of these limitations, direct involvement of NK cells has been found in some human immunopathologies such as macrophage activation syndromes, antigen processing (TAP) deficiencies, multiple sclerosis and psoriatic arthritis (37–43).

Results of our case-control genetic study support a role for NK cells in pSS since genetic polymorphisms residing within the promoter region of NKp30 are associated with reduced risk of disease. The association between 11575837A and pSS was even stronger among patients whose disease is characterized by specific autoantibody production. This could result from the central role of NKp30 in DC maturation (44), thus promoting the priming process. Interestingly, the rs2736191 SNP was found to be associated with a more severe form of malaria (45). The GGTCCT sequence containing the rs2736191 polymorphic site is a RREB1/LZ321 binding motif. The polymorphism could cause the loss of this binding site leading to decreased levels of transcription. In the context of acute malaria, this decrease in NKp30 expression is deleterious. Conversely, due to the possible deleterious role of NKp30 expressing NK cells in AID, such a variant could be protective in the context of chronic inflammation. Although there was no decrease of NKp30 mRNA in patients carrying the rs2736191C allele, this was the case among patients carrying the minor A allele of the rs11575837 SNP. Thus, presence of the major G allele, which was more frequent in pSS patients than in controls, could lead to increased levels of NKp30 mRNA expression favoring IFN-γ secretion upon triggering by ligands.

Our data support the fact that a functional interaction of B7-H6 or other NKp30 ligands with NKp30 receptors is relevant in salivary glands of pSS patients. First, B7-H6 is expressed by freshly isolated epithelial cells of pSS patients. The transcriptional activity of the B7-H6 gene can be up-regulated by Poly (I:C) or TNF-α. Second, NKp30 is down-regulated in NK cells from the pSS glands (but at the transcriptional level, NKp30 transcripts remain detectable) compared with circulating NK cells. Third, pSS glands are markedly enriched in NK cells compared with non pSS glands and located outside of the inflammatory foci, where

DC-SIGN[+]DC and pDC have been described ((5) from our group, (1)). Our results suggest a positive feedback loop between TNF-α and IFNγ-producing NK cells and B7-H6 expression on epithelial cells in the neighbouring acini. This cross-talk could be a key early event in the pathogenesis of the disease leading to an increase of all the cytokines (INF-γ, IL-17, BAFF) who are going to secondarily promote migration of T and B cells.

The pathogenic role of immune infiltrates in pSS development has been addressed in mouse and human studies. Several reports in mouse models of autoimmune exocrinopathy mimicking human pSS supported the role of the Th17/IL-23 axis in the exocrinopathy (12, 46) while IL-27 ameliorated the syndrome (47). In human pSS, the cellular origin (Th17, double negative T cells) of IL-17 remains controversial (10, 13). The recent work from Triolo's group elegantly showed using IHC, qRT-PCR and flow cytometry that NK cells are the major sources of IL-22 in minor salivary glands from pSS patients (11). In our small series of 6 patients, the qRT-PCR profile of pSS glands indicated high levels of transcription of Th1 cytokines (TNF-α, IFN-γ, Tbx21) in pSS compared with sicca syndrome, while RORγt transcription was elevated but non-different between the two groups. Our flow cytometry analyses of leukocytic infiltrates of salivary glands failed to reveal Th17 cells but identified CD3^{−}CD56^{−}CD45^{+} cells as potential IL-22 producers (not shown). Last, should IL-17 and Il-22 be present in salivary glands, they do not play a major role in the cross-talk between epithelial cells and NK. Indeed, in contrast to TNF or poly (I:C), most cytokines of the IL-17/IL-23 axis failed to up-regulate B7-H6 in the HSG salivary epithelial cell line. Such variabilities in the reported findings might stem from the precise kinetics of the NK and T cell homing and cytokine release in the inflamed tissues at the different stages of pathogeny of the disease (as exemplified in the mouse C57BL/6.NOD-Aec1Aec2, (12).

Altogether, these results strongly implicate the NKp30/B7-H6 axis in the pathogenesis of pSS. Increased NKp30 signaling based on genetic predisposition could contribute to the IFN signature and the characteristic exocrinopathy. More work will be needed to elucidate the causal mechanism of NK cells in the induction phase of this AID and to determine whether the NKp30/B7-H6 interaction is drug targetable.

Material and methods

Patients and controls

NCR3/NKp30 polymorphisms were studied among patients from the French ASSESS and Hôpitaux Universitaires Paris-Sud cohorts and French healthy blood donors (574 patients and 451 control subjects) (exploratory cohort) and among a Scandinavian cohort of pSS patients and controls of Caucasian ancestry (replication cohort). pSS patients fulfilled American-European Consensus Group criteria (36). The Scandinavian cohort includes a total of 436 Caucasian pSS patients from Sweden (n=244) and Norway (n=192) and 441 healthy controls (Sweden=232 and Norway, n=209). The study was approved by the local research ethics committee, and informed written consent was obtained from all patients and controls.

We obtained blood samples from 38 pSS patients to perform phenotypic characterization, and 16 pSS patients to perform functional assays. Patients were referred to the Department

of Rheumatology of Hôpitaux Universitaires Paris-Sud between October 2010 and April 2013. Patients treated with immunosuppressive drugs and/or corticosteroid therapy ≥10 mg per day were excluded. For functional assays, exclusion criteria were more rigorous as drugs could impair NK cell function (48). Thus, patients treated with non-steroidal anti-inflammatory drugs or corticosteroid therapy at any dose were excluded. Disease activity was assessed using the European League Against Rheumatism Sjögren's Syndrome Disease Activity Index (ESSDAI) (28), and a score ≥4 defined active disease. Patients referred to the department for mechanical back pain were used as controls, including 30 age-matched patients for phenotypic characterization and 10 patients for functional assays. Exclusion criteria for controls were history of autoimmune disorders or recent neoplasia (in the last 5 years), current biological inflammatory syndrome and current corticosteroid therapy or NSAID current biological inflammatory syndrome and current corticosteroid therapy or NSAID.

Genotyping and Quality Control

After isolating genomic DNA from PBMCs using EZ1 DNA blood kits (Qiagen,), the following SNPs were genotyped (Table S1): 9 SNPs encompassing the *NCR3 locus (6p23)* and 3 additional SNP proxies for HLA–DR2, HLA- DR3 and TNF, given the extensive LD (linkage disequilibrium) within the MHC region and known association of pSS with MHC region variants (23, 24). Genotyping employed a predesigned TaqMan assay from Applied Biosystems (assay no. 26882391-1) using a competitive allele-specific PCR system (KASpar genotyping, http://www.kbioscience.co.uk/).

Subjects with individual genotyping call rates <0.85 were removed as part of quality control measures. Duplicate samples/individuals were also removed. Principal component (PC) analysis based on 47 Ancestry Informative Markers (AIMs) was used to detect population outliers in the French cohort. After using data from AIMs, applying quality control (QC) measures and outlier removal, the exploratory cohort consisted of 574 pSS patients from the French ASSESS Cohort or followed at Bicêtre's teaching hospital and 451 independent control subjects.

The Scandinavian cohort was genotyped by single base extension with Fluorescent Polarization Template Dye Incorporation. The replication study utilized PC analysis with 3410 independent genetic markers from the Immunochip (r2 < 0.2) to remove subjects with evidence of non-European ancestry, resulting in 436 Caucasian pSS patients and 441 healthy controls available for analysis. Both cohorts used EIGENSTRAT (49) for principal component analysis and outlier detection, which was also used for ancestry adjustment in logistic regression analyses (see statistical analysis).

Quantitative reverse-transcription PCR and nonhierarchical clustering

Total cellular RNA was isolated from PBMCs with the RNeasy kit. First-strand cDNA was synthesized from 5 μg of total RNA using SuperScript III reverse transcriptase and random primers according to Invitrogen's instructions. The PCR primers and TaqMan probes for the 6 NKp30 transcripts and the β2 microglobulin housekeeping transcript were designed with Primer Express software version 1.0 (Life Technology®) as previously described (25). For

each NKp30 isoform, standard curves based on six data points of NK92 cDNA dilutions in triplicate were established to estimate the efficiency of PCR amplification with StepOne software version 2.0. The efficiencies of NKp30a, NKp30b and NKp30c PCR were 88.8%, 90.6% and 93.8%, respectively. The qRT-PCR data were analyzed using the $2-(\Delta^{\wedge}\Delta Ct)$ method (50). Proportions of the distinct NKp30 isoforms were determined as the ratio of the relative quantities of each isoform and the total quantity of the three isoforms. Unsupervised hierarchical clustering was applied to data that had been log-transformed and median-centered using the Cluster and TreeView programs (average linkage clustering using Pearson's centered correlation as similarity metric) (51). The Chi2 test was used to assess association between isoform profiles and pSS phenotypes. Total NKp30 mRNA levels were determined with Bio-Rad real Time PCR systems. TBP (TTA Box Binding Protein) was used as a housekeeping gene. PCR primers and Taqman probes were designed by Life Technology®. Primers for *NCR3* were specific to exon 1 and exon 2, which allowed for the amplification of all 3 NKp30 isoform transcripts leading to the assessment of the global quantity of NKp30 transcripts irrespective of isoform profiles.

NK cell functional characterization

Phenotyping—Nine-color flow cytometry analysis was performed on whole blood cells and on peripheral blood mononuclear cells (PBMCs). The following mouse anti-human antibodies were used (Supplemental Table S3): From Miltenyi®: anti-CD8-FITC, anti-NKp306-PE, anti-CD4-PerCP, anti-NKG2D-APC, anti-NKp44-PE, anti-NKp46-APC, anti-CD158b (KIR 2DL2)-PerCP, anti-CD158e/k (KIR 3DL1)-PE, anti-CD158i (KIR 2DS4)-APC and anti-CD158a/h (KIR2DL1)-FITC. From Beckman Coulter®: anti-CD3-APC750, anti-CD56-PECy7, anti-CD56-APC, anti-CD16-PB, anti-CD117-PECy7. From R&D®: anti-IL23-R-FITC, anti-DNAM-1-FITC, anti-NKp80-FITC, anti-LAG 3-FITC, anti-NKG2A-FITC. From BD®: anti-CD45-PECy7, anti-CCR6-PerCP Cy5.5. Dead cells stained by Vivide Yellow (Molecular Probe, Invitrogen®) were excluded prior to analysis. The following isotype controls were used: mouse IgG1 FITC, PE and APC. Prior to the staining, PBMC were FcR blocked (Miltenti®) for 30 minutes. Stained cells were acquired within 24 hours on a Cyan Flow Cytometer (Beckman Coulter) and analyses were performed using FlowJo software (Tree Star). Results were expressed as percentage of cells positive for each marker and/or MFI to assess the density of receptors expressed on the cell surface.

IFN-γ production and CD107a degranulation assays—NK cells were stimulated by NKp30 cross linking without purifying NK cells from PBMC as previously described (52). Briefly, PBMCs from patients and controls were prepared according to standard procedures on Ficoll-Hypaque. Monoclonal antibodies anti-NKp30 agonist and mouse IgG2a isotype control (R&D®) were coated onto flat-bottomed 96 well-Maxisorb plates (Nunc®) overnight at 4°C. Three coating concentrations of anti- NKp30 were used: 2.5, 0.8 and 0.3 µg/ml. The specificity of the responses observed in our assay was tested using isotype control reagents as background controls and an additional cross linking assay using anti-CD16 agonist (R&D®). Isotype control and anti- CD16 were coated at the maximal coating concentration (2.5 µg/ml). To assess CD107a degranulation, 2×10^5 PBMC/well in RPMI and 10% FCS were added onto anti-NK cell receptor antibody coated plates in the presence of low dose IL2 (10 UI/ml). Triplicate wells were set up for each test condition. Following

one hour of incubation with anti- CD107a-FITC, the protein transport inhibitor Golgi Stop (BD Biosciences®) was added at a final dilution of 6 µg/ml. Cells were then incubated for an additional 4 h at 37°C. Cells were then recovered, washed and re-suspended with PBS into 5 ml polystyrene round bottomed FACS tubes and stained for flow cytometry analysis (anti-CD45-APC Cy7, anti-CD56- PE Cy7, anti-CD3-PB and viability marker vivide yellow). To assess IFN-γ secretion, PBMC were pre activated in the presence of IL2 (100 UI/ml) overnight. The following day, 2×105 cells/well of pre-activated PBMC were seeded in triplicate onto Maxisorb plates coated with anti-NK cell receptor antibody. After a total of 5 hours, NK cells were recovered and stained for cell surface phenotypic analysis as described earlier. For intracellular cytokine staining, cells were fixed, permeabilized and labeled with anti-IFN-γ PerCp following manufacturer's protocol (BD Biosciences®). Stained cells were acquired within 24 hours on Cyan Flow Cytometer (Beckman Coulter®) and analyses were performed using FlowJo software (Tree Star®).

Immunohistochemistry staining of minor salivary gland biopsies (MSGB)

The most widely accepted grading system of MSGB, termed Focus Score, records the number of foci of lymphoid tissue, defined as collections of 50 or more lymphocytes per 4 mm2 (29, 53). In the Chisholm-Mason scale (54), a grade III (equivalent to 1 focus/4mm2) or IV (equivalent to >1 focus/4 mm2) are suggestive of the diagnosis of pSS. MSGB from 20 pSS patients and 7 controls were graded according to the Focus Score and Chisholm-Mason scale and stained with anti-NKp46 to assess NK cell infiltration. 3µm-thick sections of formalin-fixed, paraffin embedded MSGB were mounted on poly-L- lysine-coated slides, deparaffinized and hydrated through graded alcohols to water. Sections were pre-treated with Tris-EDTA buffer (10mM Tris, 1mM EDTA, pH 9) for 30 minutes in a 98°C water bath. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxidase (DAKO) for 10 minutes. The primary antibody, mouse IgG2b anti- human NKp46 monoclonal antibody (clone 195314, 5µg mL-1, R&D) was incubated for 1 hour, followed by the secondary antibody, Biotin F(ab')2 Donkey anti-Mouse IgG (H+L) (Jackson Immunoresearch) for 30 minutes, and detected with 3-amino-9-ethylcarbazole substrate (AEC, Vector Laboratories), and the sections were counterstained with Harris's hematoxylin and the slides were mounted using Glycergel (DAKO). Negative controls were made by substituting primary Ab with isotype controls.

Cell lines

HSG is a cell line derived from neoplastic epithelial duct cells of the human salivary gland [a gift of Bruce Baum and Marc Kok (U.S. National Institutes of Health)], grown in DMEM/ F-12 supplemented with 10% FCS, penicillin (100 IU/ml) and streptomycin (100 µg/ml). Jurkat cells, transduced with adenovirus containing the sequence for NKp30 a, b and c isoforms or with vector control (kindly provided by David Klatzman), were cultured in RPMI plus 10% FCS, 1% Sodium pyruvate (Gibco) and 1% Pen/Strep.

Cultures of salivary glands epithelial cells (SGECs)

Primary cultures of SGECs were established from minor salivary glands from 8 patients (3 pSS and 5 SICCA) as described (55). In brief, each lobule was cut into small fragments and set in six 75 cm2 flasks with basal epithelial medium (a 3:1 mixture of Ham's F-12 and

DMEM) supplemented with 2.5% FCS, epidermal growth factor (10 ng/ml), hydrocortisone (0.4 μg/ml), insulin (0.5 μg/ml), penicillin (100 IU/ml) and streptomycin (100 μg/ml) and incubated at 37°C under 5% CO_2. After 4 to 5 weeks of culture, at 70 to 80% confluence, cells were tested for the expression of B7H6.

Assessment of NKp30/B7H6 interaction within salivary glands

Co-culture between HSG and Jurkat cells transduced with NKp30 isoforms—A
HSG cell line was seeded overnight at 5×104 cells per well in 96-flat bottom culture plate in DMEM/F-12 medium supplemented with 10% Fetal Calf serum (FCS, PAA) and 1% Penicillin/streptomycin antibiotic (Gibco). HSG and transduced Jurkat T cells were co-cultured at a ratio of 1:1 for 24 hours in complete RPMI medium at 37°C. In some experiments, the NKp30-transduced Jurkat T cells were pre-incubated with anti-NKp30 (IgG1 or IgM), anti-NKp44 (IgM) or anti-PVR (IgG1, all used at 10% v/v, kindly provided by Cristina Bottino) for 2 hours at 37°C prior to the co-culture. Supernatants were harvested to measure IL-2 cytokine levels using commercial ELISA (BD OpTIEA, Cliniscience).

Measurement of B7-H6 mRNA levels by qRT-PCR

First-strand cDNA was synthesized as described earlier. The PCR primers and TaqMan probes for the B7-H6, Bat3 and PPIA housekeeping transcripts (TaqMan Probe) were designed with Primer Express software version 1.0 (Life Technology) or by Life Technology® (Supplemental Table S4). The qRT-PCR were performed on Step One following manufacturer's recommendations (Life Technology) and were analyzed using the 2−(Δ^ΔCt) method (50).

Statistical analysis

Results are shown as mean (± standard deviation). The Mann Whitney test was used to compare independent samples. The Wilcoxon signed-rank test was used to compare two related samples or for repeated measurements of a single sample. Linear regression was performed. Statistical comparisons were performed with StatView, version 5.0 (SAS institute Inc). A p-value <0.05 was considered significant. Analysis of single SNP associations were performed in PLINK and multivariate associations were performed in Stata 11. First, principal components (PCs) reflecting ancestry were generated (after outlier removal) using EIGENSTRAT (49), from 47 AIMs in the exploratory cohort and 3410 independent SNPs in the replication cohort. Each cohort (French and Scandinavian) was analyzed separately via logistic regression, adjusting for the top two ancestry PCs. Results were then combined using fixed- and random-effects meta-analysis in the Stata metan function, which also produced the chi2 test for homogeneity. SNPs were first analyzed individually in single-SNP analyses (one regression per SNP) and then together in multi-SNP analyses (one regression with all 3 SNPs) to investigate independence of effects.

Online supplemental material

Fig. S1 shows the absence of qualitative differences in the NKp30 isoform patterns of pSS and controls. Fig. S2 shows the expression pattern of inhibitory and activating NK cell receptors in pSS and controls. Fig. S3 shows that the NKp30-dependent degranulation of

PBMC is not correlated with NKp30 expression. Fig. S4 depicts the down-regulation of NKp30 expression in salivary glands. Fig. S5 shows the salivary gland cytokine/ transcription factors analysis by qRT-PCR. Table S1 lists the SNPs subjected to genotyping. Table S2 shows the logistic regression of 12 SNPs in the exploratory cohort. Table S3 lists the antibodies used for flow cytometry analysis. Table S4 shows PCR Primers and TaqMan probes for the B7-H6 transcripts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

AID	Autoimmune Disease
AIM	Ancestry Informative marker
ASSESS	Assessment Of Systemic Symptoms and Evolution Of patients with primary Sjögren's Syndrome
DC	Dendritic Cells
GIST	Gastro-Intestinal Stromal Tumor
HSG	Human Salivary Gland
IFN	Interferon
IFNR	Interferon receptor
Ig	Immunoglobulin

IL	Interleukin
LD	Linkage Disequilibrium
LT	Lymphotoxin
MHC	Major Histocompatibility Complex
MSG	Minor Salivary Glands
MSGB	Minor Salivary Gland Biopsy
NCR3	Natural Cytotoxicity Receptor 3
NK	Natural killer
NOD	Non Obese Diabetic
PBMC	Peripheral Blood Mononuclear Cells
PC	Principal component
pDC	Plasmacytoid Dendritic Cells
pSS	Primary Sjögren's syndrome
QC	Quality Control
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
SGEC	Salivary Glands Epithelial Cells
SICCA	Sicca syndrome without any immunological features of Sjögren's syndrome
SNP	Single Nucleotide Polymorphism
TBP	TATA Box binding Protein
TCR	T Cell Receptor
TH	T cell helper

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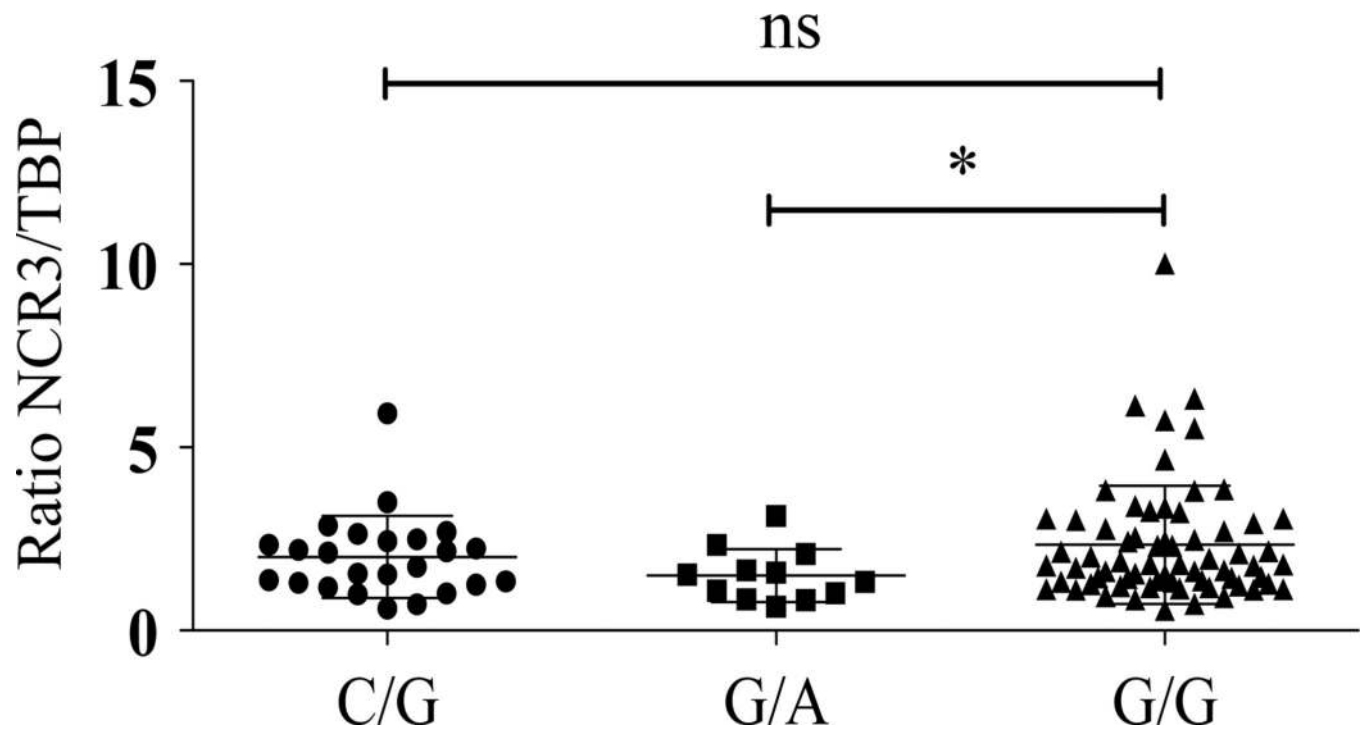
Figure 1. The rs11575837 minor allele (A) is protective for pSS risk and is associated with reduced NKp30 expression levels

Relative NKp30 mRNA expression in peripheral blood mononuclear cells from patients with pSS, according to genotype: C/G: carriers of rs2736191C minor allele and rs11575837G major allele, G/A: carriers of rs2736191G major allele and rs11575837A minor allele, G/G: carriers of both major alleles. Results represent the mean ± standard error of the mean (SEM). ns: non- significant, * p<0.05
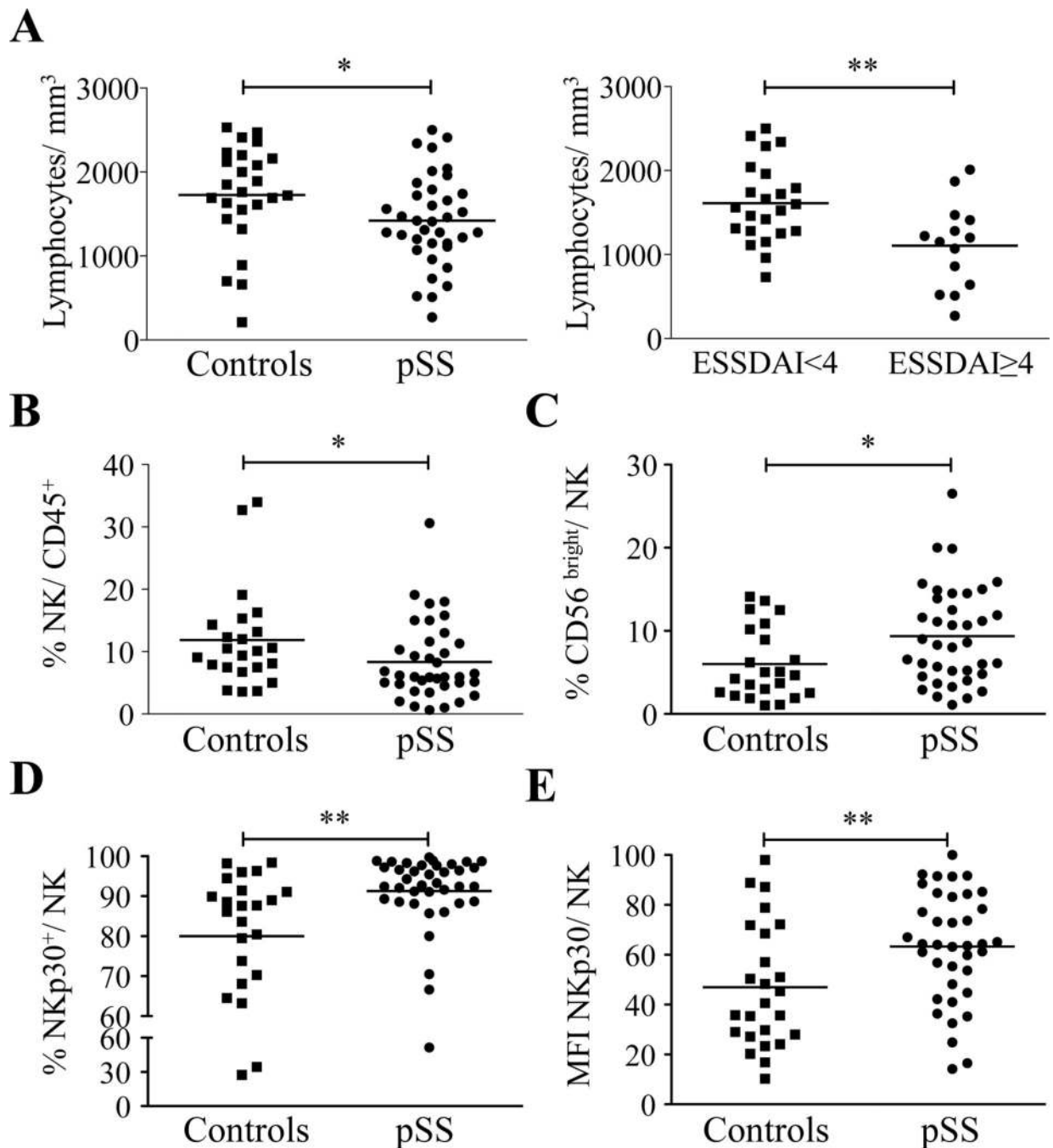
Figure 2. Elevated expression of NKp30 among pSS patients

Phenotypic characterization using flow cytometry analyses of 38 pSS patients and 30 control donors. pSS patients are lymphopenic (A, left) and this lymphopenia is more severe in patients with active disease defined by an ESSDAI ≥4 (A, right panel). The proportion of circulating CD3⁻CD56⁺ NK cells (gated on CD45⁺ cells) is significantly reduced in pSS patients versus controls (B) but the proportion of CD56^{bright} cells (gated on NK cells) increased in patients (C). The NKp30 activating receptor is markedly overexpressed in NK cells [in percentages (D), and in mean fluorescence intensity (E)] in pSS patients. Results

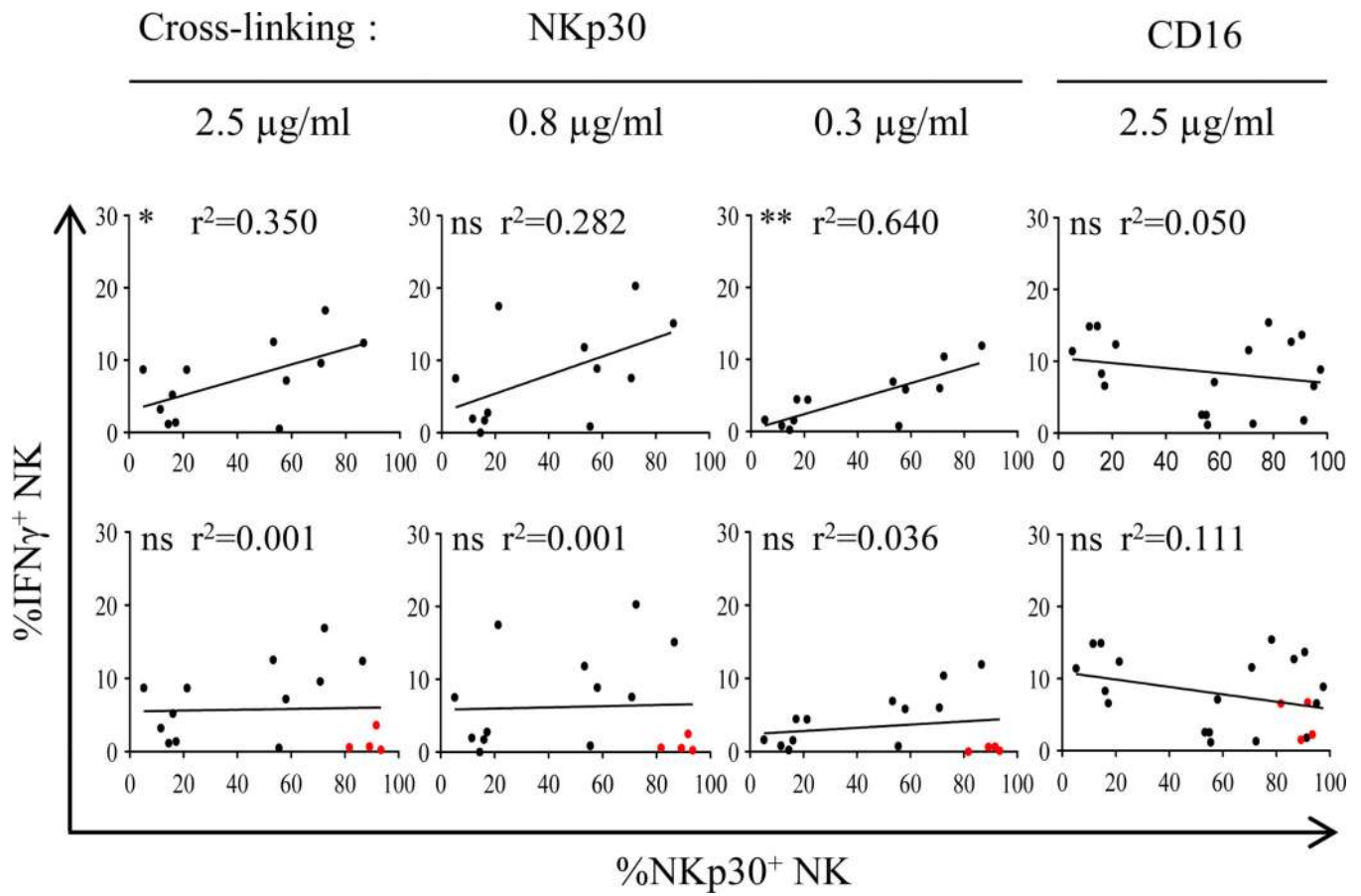represent the mean ± standard error of the mean (SEM). ns: non-significant, * $p < 0.05$, ** $p < 0.001$
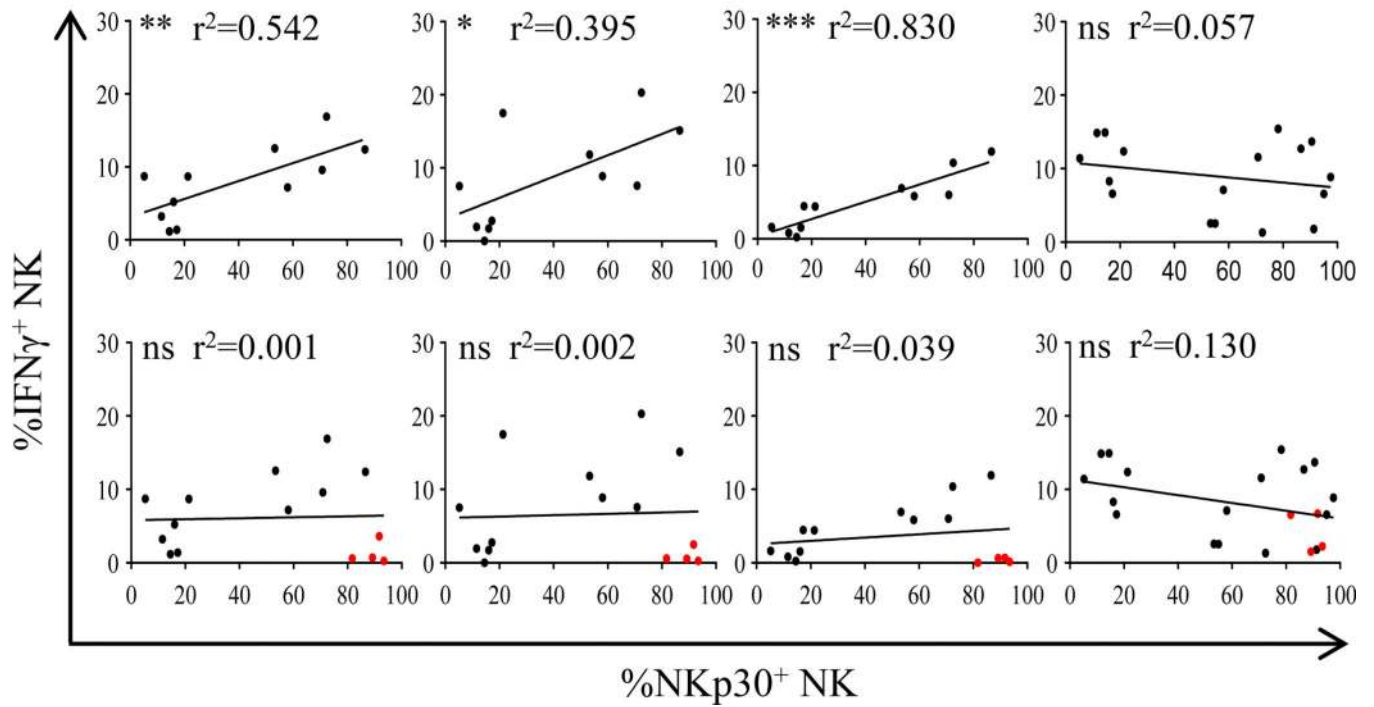
Figure 3. Functional relevance of a high expression level of NKp30 on NK cells
Proportions of IFN-γ positive NK cells determined using intracellular stainings in flow cytometry analyses after a 24hr- stimulation of PBMC with NKp30 or FcγRIII (CD16) (right panels) antibodies. Linear regression with NKp30 expression levels. A dose response (using 2.5, 0.8 and 0.3 μg/ml of anti -NKp30 Ab coated onto plastic dishes) is depicted, each dot representing one patient or control. Four individuals carrying the minor allele of the rs11575837 (11575837A) were assessed in parallel and the results are indicated in red dots. ns: non-significant, * p< 0.05, ** p< 0.001
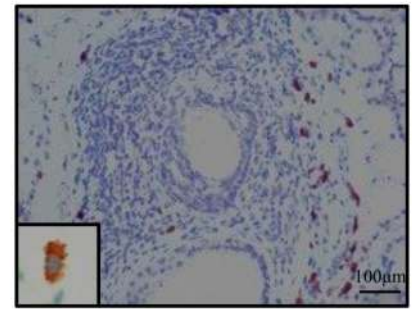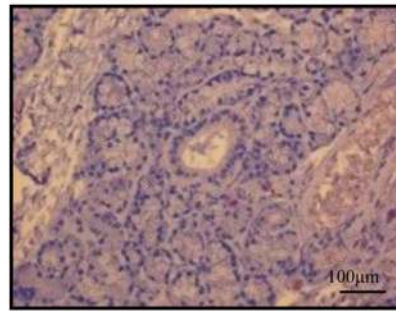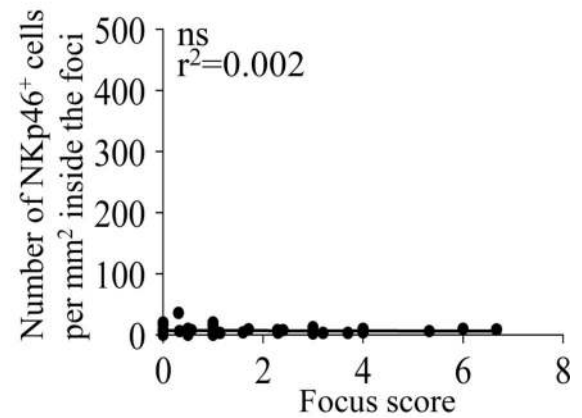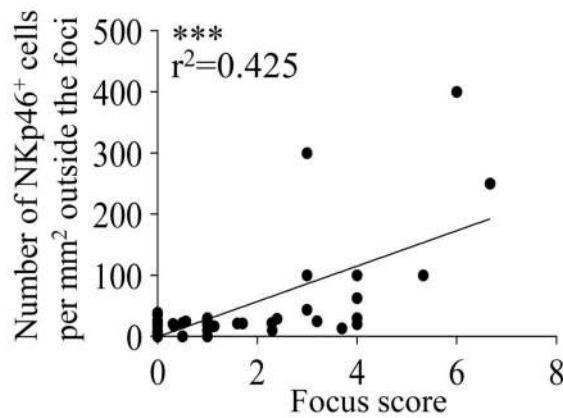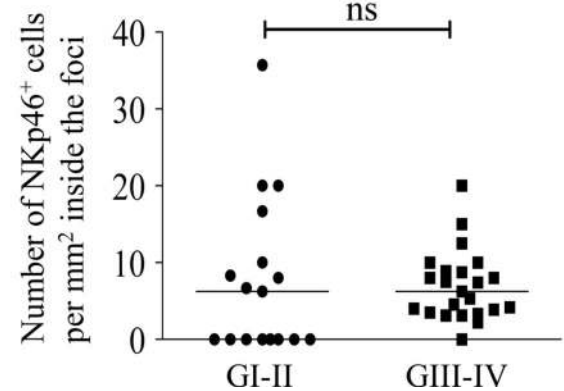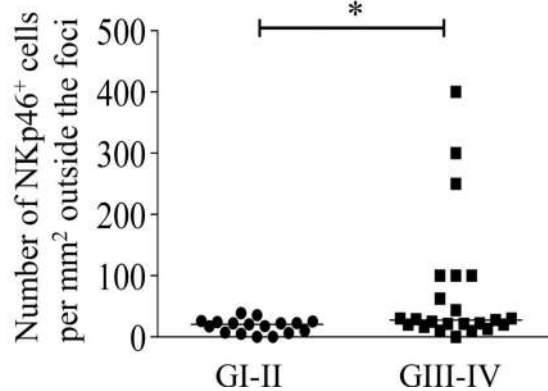
Figure 4. Accumulation of NK cells in minor salivary gland biopsy (MSGB)
A representative NKp46 staining of a paraffin-embedded specimen from pSS and sicca patients. pSS (right micrograph pictures) and sicca controls (left panel): magnification 250X and 945X (inset) (right panel) or 200X (left panel). Scale bars indicated. B-C. Correlation between NK cell numbers and focus score (B) or grade (C) in different areas of the glands. In the Chisholm-Mason scale (54), a grade III (equivalent to 1 focus/4 mm^2) or IV (equivalent to >1 focus/4 mm^2) are suggestive of the diagnosis of pSS. The numbers of NKp46 positive cells within (right panels) versus outside the foci (left panels) were

evaluated in 40 fields observed at x200 power of magnification in 23 MSGB grade GIII and IV and 17 MSGB GI and II. Each dot represents one MSBG. ns: non-significant, * p< 0.05, Mann-Whitney t-test.
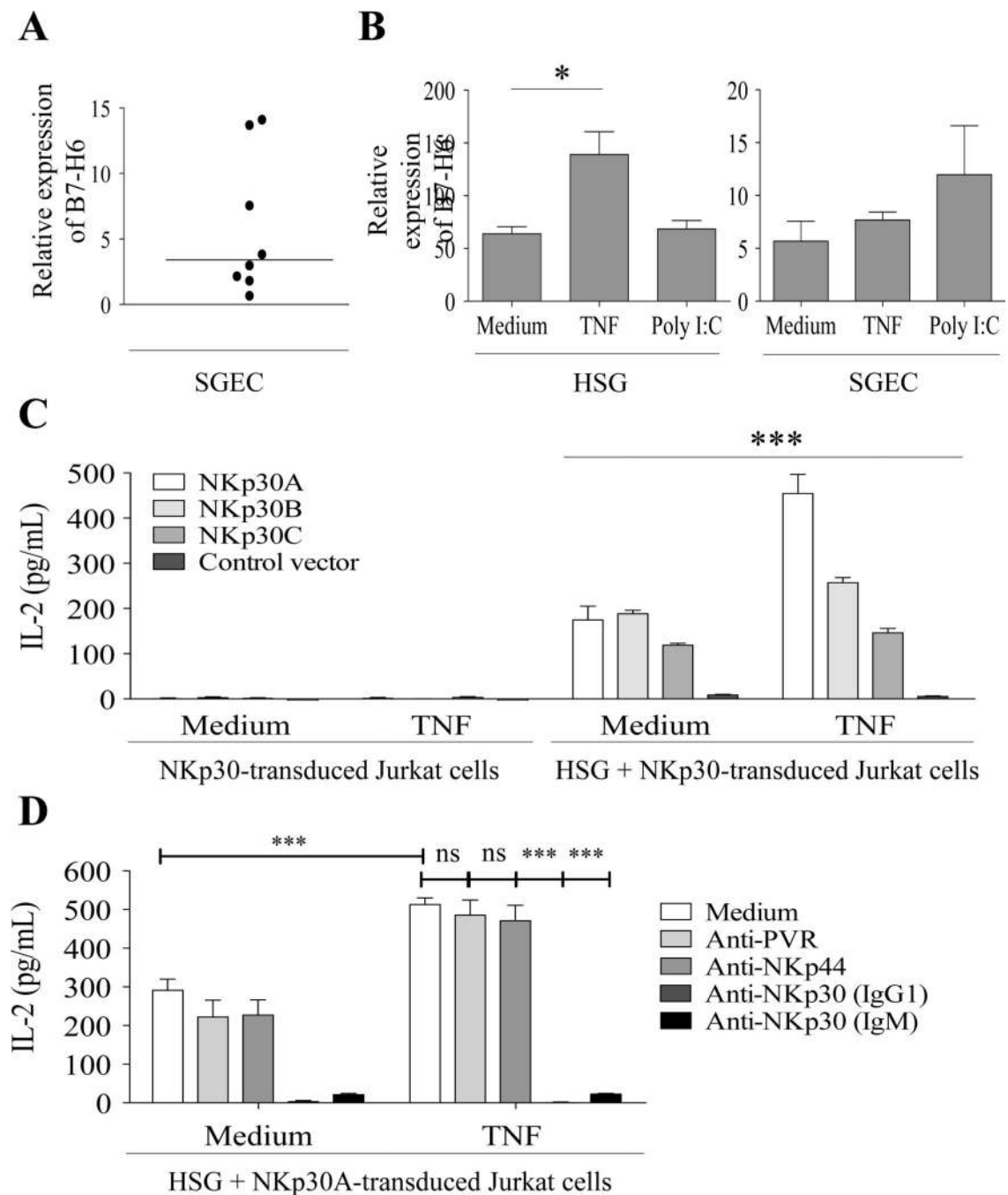
Figure 5. TNF-α induces the cross-talk between salivary glands and NK cells in an NKp30-dependent manner

A-B. Transcriptional levels of B7-H6 in salivary glands and regulation. B7-H6 mRNA expression was assessed by quantitative RT-PCR. B7-H6 mRNA expression was assessed from HSG cell line or salivary glandular epithelial cells derived from minor salivary gland biopsy (SGEC), at baseline (biopsy of 8 glands, A) and after a 6 hours-incubation with TNF-α (1 ng/ml), poly I:C (30 μg/ml), IL-17, IL-22 or IL-23 (10ng/mL) (B). C. Functional cross-talk between HSG and NKp30 overexpressing Jurkat cells stimulated by TNF-α. TNF-α -stimulated HSG cells were incubated with Jurkat cells transduced with the three major

NKp30 isoforms or with the control vector for 24 hours. The secretion of IL-2 was monitored by ELISA and a histogram of two independent experiments is shown. D. Role of NKp30 ligands/NKp30 interaction in the dialogue between HSG and transduced Jurkat cells. NKp30A–transduced Jurkat cells were incubated with anti-NKp30 or irrelevant blocking antibody for 2 hours prior to stimulation with HSG (as described in B). A representative histogram of two experiments is shown. ** p< 0.001, *** p<0.0001, Two-way Anova.

Table 1

Single-SNP logistic regression results adjusted for top 2 principal components, by cohort and combined via meta-analysis: (A) pSS cases versus healthy controls, (B) SSA/B+ pSS cases versus controls, and (C) SSA/B+ pSS cases versus SSA/B− pSS cases. SSA/B+ cases are SSA positive or SSB positive. SSA/B− cases are SSA negative and SSB negative.

	SNP	Exploratory (French) cohort				Replication (Scandinavian) cohort				Meta-analysis of 2 cohorts				homogeneity
		574 pSS cases (340 SSAB+, 233 SSAB−) and 451 controls				436 pSS cases (329 SSAB+, 105 SSAB−) and 441 controls				fixed effect		random effects		
		MAF cases	MAF controls	OR	P	MAF cases	MAF controls	OR	P	OR	P	OR	P	P
A. all pSS cases versus healthy controls	rs11575837	0.017	0.041	0.41	0.0021	0.0080	0.010	0.80	0.66	0.48	0.0039	0.5	0.026	0.25
	rs2736191	0.025	0.058	0.46	0.0013	0.025	0.032	0.75	0.31	0.56	0.0019	0.57	0.019	0.2
	rs2187668*	0.24	0.12	2.46	1.73E−11	0.33	0.13	4.91	2.9E−27	3.36	1.7E−34	3.46	0.0003	0.0005
B. SSA/B+ cases versus healthy controls	rs11575837	0.018	0.041	0.42	0.012	0.0015	0.010	0.15	0.075	0.38	0.0033	0.38	0.0033	0.36
	rs2736191	0.025	0.058	0.47	0.0083	0.029	0.032	0.88	0.67	0.63	0.028	0.64	0.16	0.13
	rs2187668*	0.30	0.12	3.93	1.3E−19	0.38	0.13	7.60	1.6E−34	5.3	1.5E−50	5.45	2.8E−07	0.003
C. SSA/B+ cases versus SSA/B− cases	rs11575837	0.018	0.015	1.18	0.73	0.0015	0.024	0.060	0.011	0.73	0.48	0.31	0.43	0.01
	rs2736191	0.025	0.026	0.98	0.96	0.029	0.014	2.20	0.22	1.22	0.55	1.25	0.55	0.28
	rs2187668*	0.30	0.14	3.59	1.1E−12	0.38	0.18	4.56	1.3E−10	3.92	1.2E−21	3.92	1.2E−21	0.42

MAF: Minor Allele Frequency, OR: Odds Ratio, P: p-value,

*
rs2187668 is a SNP proxy for HLA-DRB1*0301 allele.

Table 2

Multi-SNP logistic regression adjusted for top 2 principal components, by cohort and combined via meta- analysis: (A) pSS cases versus healthy controls, (B) SSA/B+ pSS cases versus controls, and (C) SSA/B+ pSS cases versus SSA/B- pSS cases. SSA/B+ cases are SSA positive or SSB positive. SSA/B- cases are SSA negative and SSB negative.

| | SNP | Exploratory (French) cohort 574 pSS cases (340 SSAB+, 233 SSAB-) and 451 controls | | Replication (Scandinavian) cohort 436 pSS cases (329 SSAB+, 105 SSAB-) and 441 controls | | Meta-analysis of 2 cohorts | | | | homogeneity |
| | | | | | | fixed effect | | random effects | | |
		OR	P	OR	P	OR	P	OR	P	P
A. all pSS cases versus healthy controls	rs11575837	0.38	0.001	1.22	0.7	0.5	0.008	0.63	0.4	0.06
	rs2736191	0.43	0.001	1.05	0.9	0.62	0.014	0.66	0.35	0.001
	rs2187668*	2.47	2.0E-11	4.93	8.2E-27	3.37	5.0E-34	3.48	0.0003	0.001
B. SSA/B+ cases versus healthy controls	rs11575837	0.37	0.007	0.29	0.27	0.36	0.0036	0.36	0.0036	0.8
	rs2736191	0.40	0.004	1.44	0.28	0.72	0.15	0.75	0.7	0.006
	rs2187668*	4.00	1.3E-19	7.67	1.1E-33	5.36	1.6E-49	5.52	1.6E-07	0.004
C. SSA/B+ cases versus SSAB- cases	rs11575837	1.1	0.8	0.06	0.013	0.69	0.4	0.31	0.4	0.02
	rs2736191	1.17	0.7	3.12	0.09	1.52	0.2	1.66	0.28	0.2
	rs2187668*	3.56	1.6E-12	4.85	7.1E-11	3.98	1.4E-21	3.98	1.5E-20	0.3

MAF: Minor Allele Frequency, OR: Odds Ratio, P: p-value.

*
rs2187668 is a SNP proxy for HLA-DRB1*0301 allele