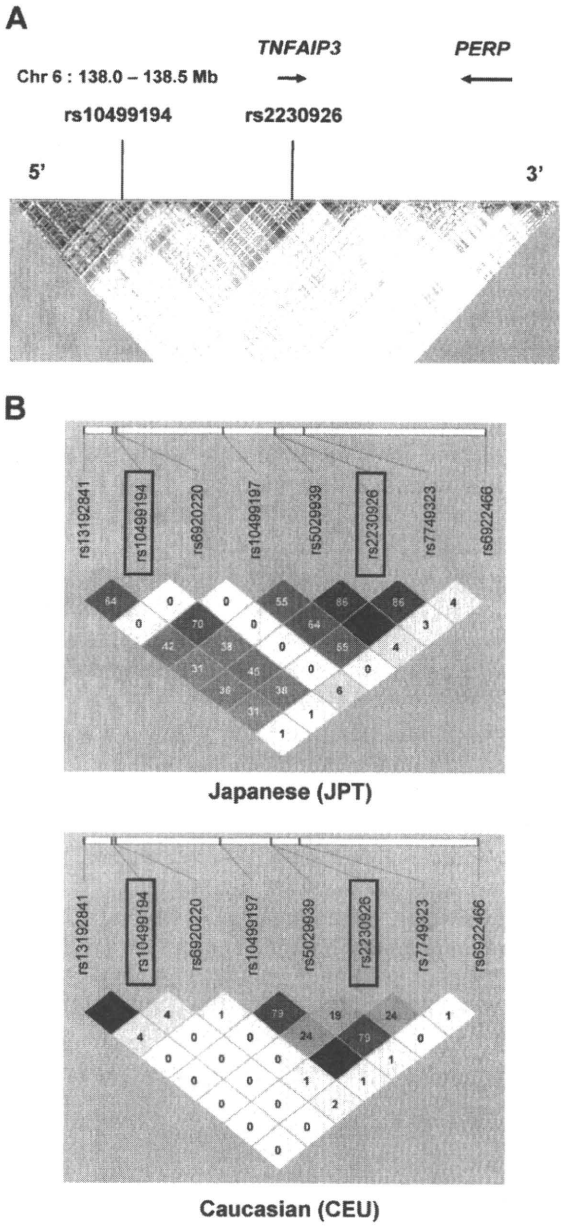

Figure 1. Pairwise linkage disequilibrium (LD) patterns for polymorphisms in the *TNFAIP3* region, according to HapMap phase II data. **A,** Pairwise LD pattern in the expanded *TNFAIP3* region derived from the HapMap data for Japanese patients, with r^2 values. *OLIG3* is located ~370 kbp away from *TNFAIP3* in the 5′ region and is not shown. **B,** Pairwise LD patterns for single-nucleotide polymorphisms (SNPs) in the *TNFAIP3* region that were significantly associated with systemic lupus erythematosus and rheumatoid arthritis in previous genome-wide association studies. The upper and lower panels were constructed using HapMap data for Japanese and Caucasian patients, respectively. The diagram shows pairwise LD values as quantified using the r^2 value. A stronger LD is depicted graphically by the densely shaded boxes. The boxed areas show the 2 SNPs genotyped.

that the variant was too rare (MAF <0.01) to be evaluated for associations (8).

Based on HapMap data for Japanese individuals, pairwise LD patterns for the SNPs in and near *TNFAIP3*, which were significantly associated with SLE and RA in the previous GWA studies, are presented in Figure 1 (for SLE, rs13192841, rs10499197, rs5029939, rs2230926, rs7749323, and rs6922466; for RA, rs10499194 and rs6920220).

Genotyping. We genotyped SNPs using TaqMan assays. For the selected SNPs, predesigned TaqMan SNP genotyping assays were used (probe ID: rs2230926, C.770116_10; rs10499194, C.1575581_10; Applied Biosystems, Foster City, CA). Fluorescence was detected using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Genotyping assessment was performed on >98% of the samples, for all of the polymorphisms genotyped. All of the SNPs were in Hardy-Weinberg equilibrium in control subjects, according to chi-square statistics ($P > 0.01$).

Case–control association tests. We first performed allele frequency comparisons of rs2230926 and rs10499194 in SLE cohort 1 and RA cohort 1. Then, further case–control association studies were conducted using SLE cohort 2 and RA cohorts 2 and 3, to validate the associations in the first cohorts. In the replication studies, we genotyped the SNPs with a P value less than 0.05 in either SLE cohort 1 or RA cohort 1 (the P value was determined after correction for conditional logistic analysis, as described below).

Measurement of autoantibodies. Sera from 1,104 patients in RA cohort 1 were available for the measurement of anti–cyclic citrullinated peptide (anti-CCP) antibodies and RF. Anti-CCP antibodies were measured using the Mesacup CCP test (Medical and Biological Laboratories, Woburn, MA), and RF was measured by enzyme-linked immunosorbent assay.

Statistical analysis. The case–control association of each SNP was tested with the Cochran-Armitage trend test. The genotype and allele frequencies for patients and control subjects were used to calculate the odds ratios (ORs) and 95% confidence interval (95% CIs) using Woolf's method. For the combined analysis, we used the Mantel-Haenszel test. We performed conditional logistic regression analysis to evaluate the effect of each polymorphism conditional on the remaining polymorphisms, using Statistica software (StatSoft, Tulsa, OK). We calculated pairwise LD indices between pairs of SNPs (the r^2 value), using HaploView software, version 4.0 (http://www.broad.mit.edu/haploview/ haploview). We calculated the population attributable risk (PAR) using the following formula: PAR = f(OR − 1)/(1 + f[OR − 1]), where f is the allele frequency in the control subjects. PAR is defined as the reduction in incidence that would be achieved if the population had been entirely unexposed. We calculated the statistical power of association using the R software program (http://www.r-project.org).

RESULTS

Our results revealed a significant association between rs2230926 and both SLE and RA when comparing allele frequency in the patients and control subjects in the first cohort (for SLE, OR 1.92, 95% CI

Table 1. Association study of rs2230926 and rs10499194 with SLE in Japanese subjects*

dbSNP number, major/minor allele	No. of patients	No. of controls	Minor allele frequency		OR (95% CI)	*P*
			Patients	Controls		
rs2230926, G/T						
SLE 1	376	934	0.113	0.062	1.92 (1.43–2.58)	1.2×10^{-5}
SLE 2	341	428	0.116	0.064	1.91 (1.33–2.73)	3.0×10^{-4}
Combined analysis†	717	1,362	0.114	0.063	1.92 (1.53–2.41)	1.9×10^{-8}
rs10499194, T/C						
SLE 1	376	933	0.084	0.061	1.42 (1.03–1.95)	0.030

* SLE = systemic lupus erythematosus; dbSNP = Database of Single-Nucleotide Polymorphisms; OR = odds ratio; 95% CI = 95% confidence interval.
† By the Mantel-Haenszel method.

1.43–2.58, $P = 1.2 \times 10^{-5}$; for RA, OR 1.52, 95% CI 1.20–1.92, $P = 5.6 \times 10^{-4}$) (Tables 1 and 2). We also observed an association between rs10499194 and SLE patients in cohort 1 (OR 1.42, 95% CI 1.03–1.95, $P = 0.030$) (Table 1). However, the T allele appeared to represent a susceptibility allele in the SLE and RA patients in cohort 1, whereas the C allele appeared to be a risk allele for RA in Caucasians (3). We speculated that this association could be secondary to the moderate LD between rs2230926 and rs10499194 ($r^2 = 0.14$) according to data on control subjects in SLE cohort 1, and we subsequently performed a conditional logistic regression analysis to evaluate the effects of each polymorphism conditional on the remaining polymorphisms. The results of this analysis indicated that rs10499194 did not retain the statistically significant association when conditionally evaluated on rs2230926 ($P = 0.73$), while rs2230926 retained the significant association when conditionally evaluated on rs10499194 ($P = 3.4 \times 10^{-4}$). We concluded that rs2230926 was primarily associated with SLE located at this locus, and therefore genotyped only rs2230926 for replication studies in SLE (3–6).

The results of a case–control association study in SLE cohort 2 confirmed the significant association between rs2230926 and the risk of SLE (OR 1.91, 95% CI 1.33–2.73, $P = 3.0 \times 10^{-4}$). A combined analysis also confirmed a significant association (OR 1.92, 95% CI 1.53–2.41, $P = 1.9 \times 10^{-8}$, PAR = 0.055). In RA cohort 2 a statistically significant association between rs2230926 and a predisposition for RA was also replicated; however, this was not replicated in RA cohort 3 (for cohort 2, OR 1.39, 95% CI 1.07–1.81, $P = 0.013$; for cohort 3, OR 1.19, 95% CI 0.94–1.50, $P = 0.15$) (Table 2). In RA cohort 3, the statistical power required to detect an association at rs2230926 was 0.54 at a significance level of $\alpha = 0.05$ when we presumed that the OR for RA was 1.4 (the combined OR for RA cohorts 1 and 2 was 1.46). It was possible that the statistical power for RA cohort 3 may have been insufficient. A combined analysis on these data suggested a significant association (OR 1.35, 95% CI 1.18–1.56, $P = 2.6 \times 10^{-5}$, PAR = 0.024).

We observed no significant association of rs10499194 in RA cohort 1, but the statistical power to detect the association in this study was insufficient (1 –

Table 2. Association study of rs2230926 and rs10499194 with RA in Japanese subjects*

dbSNP number, minor/major allele	No. of Patients	No. of controls	Minor allele frequency		OR (95% CI)	*P*
			Patients	Controls		
rs2230926, G/T						
RA cohort 1	1,112	934	0.091	0.062	1.52 (1.20–1.92)	5.6×10^{-4}
RA cohort 2	825	655	0.100	0.074	1.39 (1.07–1.81)	0.013
RA cohort 3	1,478	747	0.087	0.075	1.19 (0.94–1.50)	0.15
Combined analysis†	3,415	2,326	0.092	0.069	1.35 (1.18–1.56)	2.6×10^{-5}
rs10499194, T/C						0.26
RA cohort 1	1,112	933	0.069	0.061	1.15 (0.90–1.48)	0.26
RA cohort 2	827	650	0.072	0.048	1.52 (1.11–2.08)	0.0090
RA cohort 3	1,472	716	0.073	0.059	1.32 (1.02–1.73)	0.038
Combined analysis†	3,411	2,299	0.071	0.056	1.30 (1.11–1.53)	8.4×10^{-4}

* RA = rheumatoid arthritis; dsSNP = Database of Single-Nucleotide Polymorphisms; OR = odds ratio; 95% CI = 95% confidence interval.
† By the Mantel-Haenszel method.

$\beta = 0.31$) considering the previously reported OR of 0.75 and a significance level of $\alpha = 0.05$ (3). Therefore, we genotyped rs10499194 in RA cohorts 2 and 3 for confirmation. Unlike in RA cohort 1, a significant association of rs10499194 was observed in RA cohorts 2 and 3 (for cohort 2, OR 1.52, 95% CI 1.11–2.08, $P = 0.0090$; for cohort 3, OR 1.32, 95% CI 1.02–1.73, $P = 0.038$) (Table 2). However, the risk allele for Caucasian patients with RA was protective against RA in our population, just as was observed in SLE cohort 1. The combined analysis showed a significant association of rs10499194 with RA (OR 1.30, 95% CI 1.11–1.53, $P = 8.4 \times 10^{-4}$).

We stratified patients in RA cohorts 1 and 3 according to the presence of anti-CCP antibodies and RF and examined for the association between *TNFAIP3* polymorphisms (rs2230926 and rs10499194) and RA susceptibility (see Supplementary Table 1, available in the online version of this article at http://www3.interscience.wiley.com/journal/76509746/home). When the patients were stratified according to anti-CCP antibody status, the G allele of rs2230926 was found to confer increased risk for RA in anti-CCP antibody–positive patients relative to anti-CCP antibody–negative patients (for anti-CCP antibody–positive patients, OR 1.36, 95% CI 1.15–1.62, $P = 4.0 \times 10^{-4}$; for anti-CCP–negative patients, OR 1.16, 95% CI 0.83–1.61, $P = 0.39$ in the combined analysis). A similar trend was observed when patients were stratified according to RF status. A stratified analysis on rs10499194 also showed that the disease susceptibility allele in Japanese patients with RA (the T allele) conferred higher risk in autoantibody-positive patients than in autoantibody-negative patients.

DISCUSSION

In the current study, rs2230926, located in exon 3 of *TNFAIP3,* was shown to be significantly associated with a predisposition to both SLE and RA in 2 and 3 independent cohorts of subjects, respectively. Our results confirmed that *TNFAIP3* is one of the common genetic risk factors for both SLE and RA, similar to *STAT4* and *IRF5*, in the Japanese and Caucasian populations (2). In addition, recent studies in Caucasian patients with RA have demonstrated that the *TNFAIP3* variant conferred an increased risk of RA in anti-CCP antibody– and RF-positive patients compared with anti-CCP antibody– and RF-negative patients (12,13). Our analysis stratified according to the autoantibodies confirmed this observation in Japanese patients with RA.

TNFAIP3 encodes a cytoplasmic zinc finger protein that is also known as the A20 protein. The A20 protein is required for negative regulation of the NF-κB signaling pathway, which is mediated by innate immune receptors such as TNF receptors and Toll-like receptors, and it prevents overstimulation of the innate immune response (7,14). The disease-associated variant, rs2230926 (T/G), is a nonsynonymous variant that results in a phenylalanine-to-cysteine change at residue 127 of the A20 protein (5). The risk allele is known to be the G allele that encodes Cys. Musone et al have reported that Cys^{127} A20 protein was only modestly, but consistently, less effective at inhibiting TNF-induced NF-κB activity than the Phe^{127} protein (5). This result suggests that reduced negative regulatory activity of A20 protein may allow excessive immune activity, leading to enhanced autoreactivity.

GWA studies of SLE patients in Caucasian populations have suggested that several polymorphisms in the *TNFAIP3* region, including the nonsynonymous SNP rs2230926, are associated with a predisposition to the disease. The genetic significance of rs2230926 was evident in the Japanese patients with SLE or RA entered into our study, although its precise role in Caucasian patients with RA remains unclear. The intergenic SNP rs10499194 is one of the landmark polymorphisms identified in Caucasian patients with RA (3,15), although the significant association with RA could not be replicated in several Caucasian populations (3,12). Because rs10499194 is also associated with RA susceptibility and autoantibody status in our population, rs10499194 could be a landmark for disease causal variants in Japanese patients with RA. However, considering the inverted susceptibility allele of rs10499194 between Japanese patients (T allele) and Caucasian patients (C allele), this association of rs10499194 would appear to be secondary, as a result of LD between rs10499194 and the disease causal variants. This finding is further supported by the lack of independent association at rs10499194 in SLE when conditioned with the rs2230926 genotype, suggesting that the association observed in rs10499194 may be partially influenced by rs2230926.

Taking into account the biologic impact of rs2230926 demonstrated by Musone et al (5), rs2230926 seems likely to be an important candidate for a causal variant in *TNFAIP3* (5). However, additional polymorphisms that are located in the intergenic region of *OLIG3* and *TNFAIP3* as well as that of *TNFAIP3* and *PERP* may also independently exercise an effect on disease susceptibility, a hypothesis that was previously raised by Musone et al (5) and Graham et al (6). Further mapping of the *TNFAIP3* region in Asian and Caucasian

populations is required for the precise determination of the additional causal polymorphisms present in patients with RA or SLE.

In conclusion, we confirm that *TNFAIP3* is a genetic risk factor for the development of both SLE and RA in the Japanese population. Although the nonsynonymous SNP rs2230926 is a strong causal variant candidate in this region, a search for additional causal variants in *TNFAIP3* is required.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kochi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Shimane, Kochi, Horita, Ikari, Yamada, Atsumi, Koike, Momohara, Yamanaka, Nakamura, Yamamoto.

Acquisition of data. Shimane, Kochi, Horita, Ikari, Amano, Hirakata, Okamoto, Myouzen, Suzuki, Kubo, Takasaki.

Analysis and interpretation of data. Shimane, Kochi, Horita, Ikari, Yamamoto.

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Complement activation in patients with primary antiphospholipid syndrome

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ABSTRACT

Objective: To investigate the significance of complement activation in patients with primary antiphospholipid syndrome (APS).

Methods: Thirty-six patients with primary APS, 42 control patients with non-systemic lupus erythematosus (SLE) connective tissue diseases, and 36 healthy volunteers were analysed retrospectively. Serum complement levels (C3, C4, CH_{50}) and anaphylatoxins (C3a, C4a, C5a) were examined in all subjects, and serum complement regulatory factors (factor H and factor I) were measured in patients with primary APS. Plasma anticoagulant activity was determined in a mixing test using the activated partial thromboplastin time.

Results: Serum complement levels were significantly lower in patients with primary APS than in patients with non-SLE connective tissue diseases (mean (SD) C3: 81.07 (17.86) vs 109.80 (22.76) mg/dl, $p<0.001$; C4: 13.04 (8.49) vs 21.70 (6.96) mg/dl, $p<0.001$; CH_{50}: 31.32 (8.76) vs 41.40 (7.70) U/ml, $p<0.001$) or healthy volunteers. Only two healthy subjects with low serum C4 levels showed hypocomplementaemia, whereas most patients with primary APS showed raised serum C3a and C4a. No subjects showed raised C5a. Patients with primary APS with low serum C3 or C4 had significantly higher levels of C3a or C4a than healthy controls. No patients had low serum complement regulatory factors. Among patients with primary APS, hypocomplementaemia was significantly more common in those with high anticoagulant activity than in those with low or normal activity.

Conclusion: Hypocomplementaemia is common in patients with primary APS, reflecting complement activation and consumption, and was correlated with anticoagulant activity, suggesting that antiphospholipid antibodies may activate monocytes and macrophages via anaphylatoxins produced in complement activation.

Antiphospholipid syndrome (APS) is a clinical condition characterised by recurrent venous/arterial thrombosis and pregnancy morbidity in the presence of antiphospholipid antibodies (aPL).[1] Despite the strong association between aPL and clinical manifestations, the pathogenic role of aPL has not been fully elucidated[2]; however, this role is possibly multifactorial in nature. The engagement of aPL on cell surfaces promotes intracellular signalling[3 4]; in endothelial cells, this leads to expression of procoagulant activity and adhesion molecules[5] that in turn increases leukocyte adherence to endothelial cells.[6] In monocytes, it leads to the upregulation of tissue factor[7 8] that can enhance thrombin-induced platelet activation/aggregation.[9] Thrombosis is also favoured by aPL-induced depression of fibrinolysis[10] and by aPL interference with natural anticoagulant activities.[11–13]

Complement is part of the innate immune system and represents one of the effector arms of antibody-mediated immunity.[14] The complement system is commonly activated in systemic lupus erythematosus (SLE) and is strongly associated with the physiopathology of inflammation, as suggested by the low serum complement concentration with increased deposition at sites of tissue damage. Complement-derived inflammatory mediators (anaphylatoxins) such as C3a, C4a and C5a increase vascular permeability, activate platelets[15] and neutrophils,[16] and promote release of cytokines such as tumour necrosis factor (TNF) α from monocytes,[17] with simultaneous induction of systemic inflammation and coagulation.

A number of studies on murine models have highlighted how complement activation is essential for aPL-induced pregnancy morbidity.[18 19] C5a, the most powerful inflammatory anaphylatoxin, seems to be crucial in clinical manifestation in these models.[18] These findings have provided a new insight, suggesting that tissue injury in APS may be caused by a complement-mediated inflammatory process, rather than by thrombosis alone.[20] Since complement activation in aPL-related thrombosis has not been examined clearly, this study was performed to evaluate the prevalence and relevance of hypocomplementaemia in patients with primary APS.

PATIENTS AND METHODS

Patients

This study was performed with a retrospective and cross-sectional design and included 36 patients with primary APS treated at Hokkaido University Hospital from 1996 to 2006. Primary APS was diagnosed according to the classification criteria for APS,[1 21] with exclusion of patients who fulfilled criteria for SLE.[22] Thirty-six age and gender-matched healthy volunteers and 42 non-SLE patients with connective tissue disease (15 systemic sclerosis, 9 Sjögren's syndrome, 11 polymyositis/dermatomyositis, 5 mixed connective tissue disease, 1 overlap syndrome and 1 allergic granulomatous angiitis) were enrolled as controls. The non-SLE connective tissue disease control group comprised consecutive patients attending our autoimmune disease and rheumatology clinic who matched as controls and agreed to join this study. None of the participants had complications associated with infection, malignancy, impaired circulation or tissue ischaemia at the time of blood collection and all were negative for C-reactive protein.

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Table 1 Characteristics of the patients

Characteristics	Primary APS (n = 36)	Control* (non-SLE) (n = 42)
Gender (F:M)	27:9	37:5
Age (years), mean (range)	46 (18–75)	52 (13–77)
Number of SLE criteria fulfilled†, mean (SD)	2.08 (0.78)	2.00 (0.83)
Manifestation		
Venous thrombosis (n)	16	2
Arterial thrombosis (n)	19	4
Pregnancy morbidity (n)	3	
Time since last thrombotic event (years), mean (SD)	4.2 (3.6)	

*Control group includes cases of systemic sclerosis (n = 15), Sjögren's syndrome (n = 9), polymyositis/dermatomyositis (n = 11), mixed connective tissue disease (n = 5), overlap syndrome (n = 1) and allergic granulomatous angiitis (n = 1); †revised American College of Rheumatology SLE Classification Criteria (1997). APS, antiphospholipid syndrome; SLE, systemic lupus erythematosus.

Clinical records were reviewed retrospectively or patients were interviewed at the time of blood sample collection, or both. Arterial events such as stroke, myocardial infarction and iliac artery occlusion were confirmed by CT scan, magnetic resonance imaging (MRI) or angiography, as required. Deep vein thrombosis and pulmonary thrombosis were defined as venous thrombosis and confirmed by angiography or scintigraphy. Clinical manifestations of APS were diagnosed by rheumatologists with reference to imaging tests and clotting assays.

Plasma and serum sample collection

Blood was drawn by venepuncture and collected into different tubes. Blood samples for clotting assays were collected in plastic tubes containing 0.105 M citrate, immediately centrifuged at 3000 rpm for 15 min at 4°C, filtered through a 0.22 μM pore size membrane to obtain platelet-free plasma, and stored at −80°C until use. In the patients with primary APS, the plasma levels of D-dimer and fibrin degradation product (FDP) were determined using LPIA ace D-dimer II (Mitsubishi Kagaku Iatron, Tokyo, Japan) and Nanopia P-FDP (Daiichi Pure Chemical Co, Tokyo, Japan) kits, respectively. Blood samples for anaphylatoxin determination were collected in EDTA tubes containing nafamostate mesilate and centrifuged immediately to avoid cold in vitro complement activation.[23] Blood samples for measurement of serum complement were drawn in plain tubes, allowed to clot and then centrifuged before measurement.

The study was done in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice. Approval was obtained from the local ethics committee, and informed consent was obtained from each subject before enrolment.

aPL determination

The levels of IgG/M anticardiolipin (aCL) and IgG/M anti-β_2-glycoprotein I (β_2GPI) antibodies were measured using a standard aCL ELISA[24] and an in-house ELISA assay,[25] respectively. The positive cut-off values of the assays were set at the 99th centile for 134 healthy controls, according to laboratory criteria for APS.[1] IgG/M phosphatidylserine-dependent antiprothrombin (aPS/PT) antibodies were assayed as previously described.[26] Lupus anticoagulant (LA) was determined by two clotting assays using an opto-mechanical coagulation analyser (Start4, Diagnostica Stago, Asnières, France) based on the guidelines of the Subcommittee on Lupus Anticoagulant/Phospholipid-Dependent Antibody.[27] For the activated partial thromboplastin time (aPTT), a sensitive reagent with a low phospholipid concentration (PTT-LA, Diagnostica Stago) was used for screening, with confirmation using a Staclot LA kit (Diagnostica Stago). The dilute Russell's viper venom time was determined using a Gradipore LA test (Gradipore, Frenchs Forest, NSW, Australia). LA was considered positive when at least one of these tests was positive for LA, and was arbitrarily classified into either strong or weak LA according to the anticoagulant potential, as follows. One volume of sample plasma was mixed with four volumes of normal pooled platelet-free plasma, and the clotting time of the mixture was measured using PTT-LA. LA was defined as strong if the aPTT ratio (1:4 mixed plasma/normal plasma) was >1.28 and weak if this ratio was <1.28.

Serum complement and anaphylatoxin determination

Complement components C3, C4 and C5 were determined by a nephelometric method that gives normal ranges of 86–160, 17–45 and 9–13 mg/dl, respectively. CH_{50} activity was determined by the Mayer method, with a normal range of 30–45 U/ml. Serum anaphylatoxin levels were determined by radioimmunoassay (complement C3a des-Arg [^{125}I], complement C4a des-Arg [^{125}I], complement C5a des-Arg [^{125}I], Human Assay, GE Healthcare Bioscience, London, UK), with normal ranges of

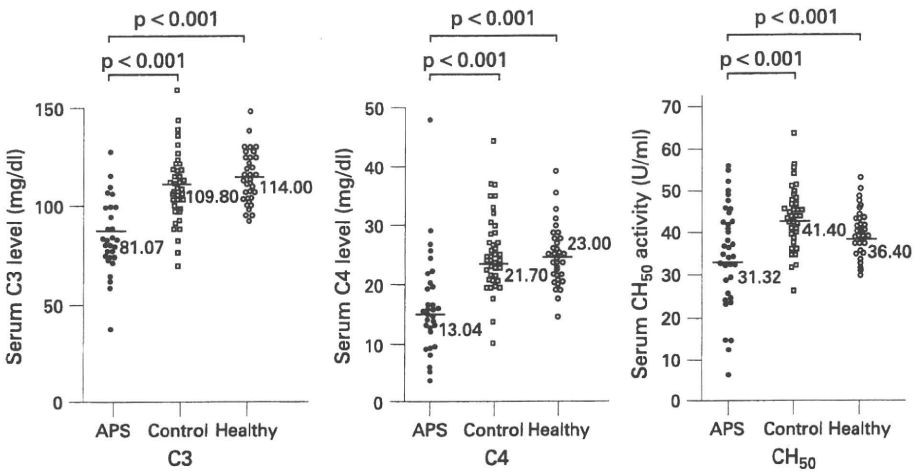

Figure 1 Serum complement levels. C3, C4 and CH_{50} levels in patients with primary antiphospholipid syndrome (APS), patients with non-systemic lupus erythematosus (SLE) connective tissue diseases and healthy volunteers. Control, patients with non-SLE connective tissue diseases; healthy, healthy volunteers. Statistical analysis by Student *t* test.

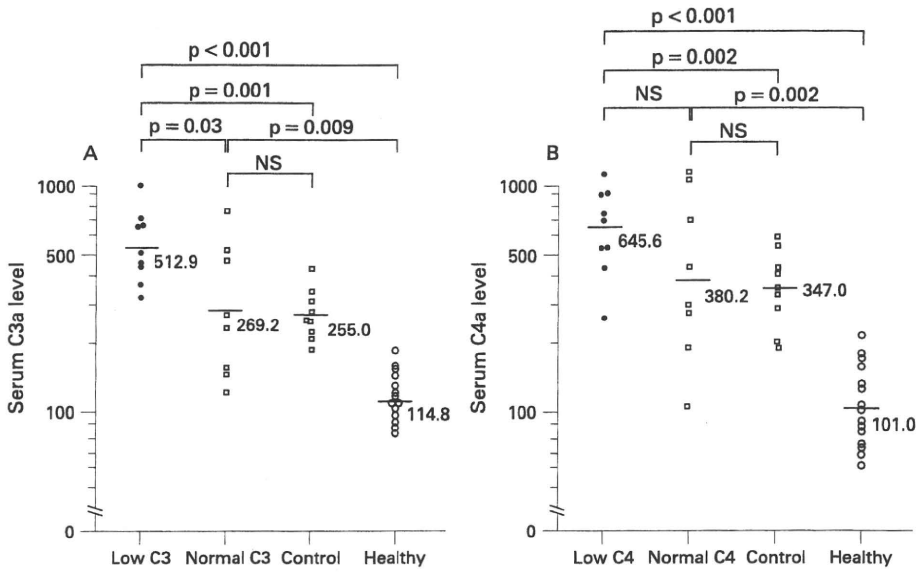

Figure 2 Serum anaphylatoxin levels. Serum C3a and C4a levels were measured in 17 patients with primary antiphospholipid syndrome (APS), 9 patients with non-systemic lupus erythematosus (SLE) connective tissue diseases and 15 healthy volunteers. The bars and figures in the graphs represent the mean levels of anaphylatoxins in each groups. (A) Comparison of C3a levels between patients with primary APS with low or normal C3, control patients and healthy volunteers. (B) Comparison of C4a levels between patients with primary APS with low or normal C4, control patients and healthy volunteers. Control, patients with non-SLE connective tissue diseases; healthy, healthy volunteers. Statistical analysis by Student *t* test.

50–200 ng/ml for C3a, 50–250 ng/ml for C4a and <10 ng/ml for C5a. C3, C4 and CH_{50} were measured in all participants, C5 was determined in 10 patients with primary APS, and anaphylatoxins were examined in 17 patients with primary APS, 9 control non-SLE patients and 17 healthy controls.

Plasma TNFα level determination

Plasma TNFα levels were examined in 22 patients with APS using sandwich ELISA (Endogen Human TNFα ELISA kit: Pierce Biotechnology, Rockford, Illinois, USA).

Measurement of serum complement regulatory factor

Serum levels of complement regulatory factor H (C3b-related C5 activation inhibitor) and factor I (C4b-related C3 activation inhibitor) were measured in 16 and 13 patients with APS, respectively, by radioimmunoassay (Monoclonal Antibody to Human Factor H, Monoclonal Antibody to Human Factor I, Quidel Corporation, San Diego, California, USA). The normal ranges are 22.8–41.7 mg/dl for factor H and 3.3–14.4 mg/dl for factor I, according to the manufacturer's instructions.

Measurement of serum immune complex

Serum levels of immune complex were measured in 33 patients with primary APS and 22 patients with non-SLE connective tissue disease, by enzyme immunoassay (Immunocomplex mRF Nissui, Nissui Pharmaceutical, Tokyo, Japan). The normal range is <4.2 μg/ml, according to the manufacturer's instruction.

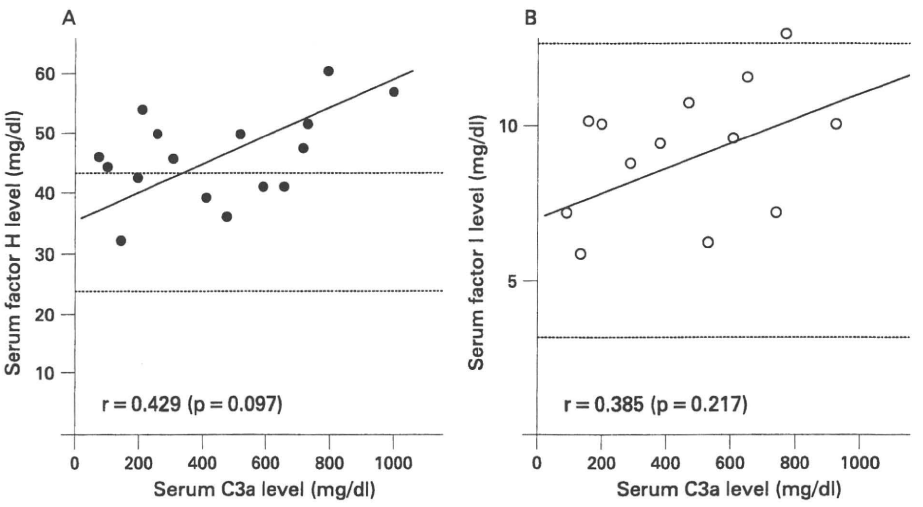

Figure 3 Complement regulatory factor levels in patients with primary antiphospholipid syndrome (APS). Serum factor H (A) and factor I (B) were measured in 16 and 13 patients with primary APS, respectively. Dotted lines represent the upper and lower limits of the normal ranges. Statistical analysis by Pearson correlation coefficient.

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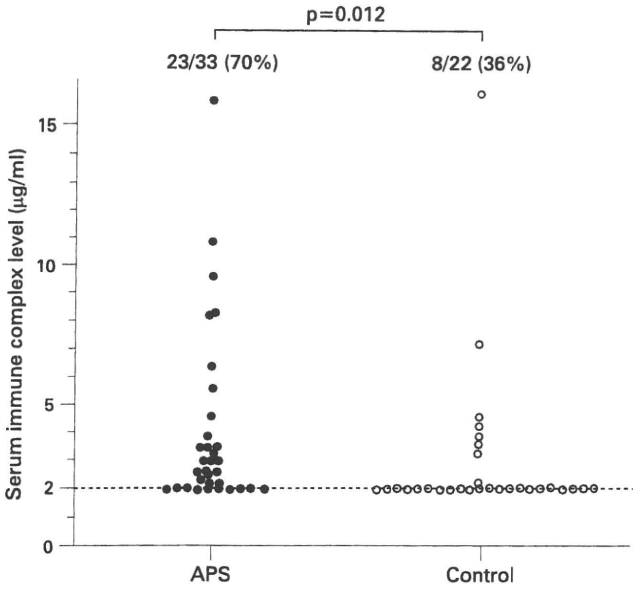

Figure 4 Serum immune complex levels. Serum immune complex levels were examined in patients with primary antiphospholipid syndrome (APS) and non-systemic lupus erythematosus (SLE) connective tissue disease controls. Figures at the top of the scatter diagram represent the ratio of the patients with detectable serum immune complex level. The dotted line represents the detection limit (2.0 μg/ml) of serum immune complex level. Control, patients with non-SLE connective tissue diseases. Statistical analysis by Fisher's exact test.

Statistical analysis

A Student *t* test, Mann–Whitney non-parametric test, Pearson correlation coefficient or Fisher's exact test was used as appropriate. In categorical analysis, the relative risks were expressed as odds ratios with 95% confidence intervals (95% CI). SPSS II for Windows was used for all calculations.

RESULTS

Serum complement levels and prevalence of hypocomplementaemia in primary APS

Criteria for SLE were investigated in the subjects by chart review or interview of the patients. The number of lupus criteria met by the primary APS group did not differ from that for patients with non-SLE connective tissue diseases (table 1). Signs of acute thrombosis were not detected in any patients with primary APS at the time of blood collection, and no significant increase of plasma D-dimer or FDP was found in these patients. The mean (SD) period after the last manifestation in patients with primary APS was 4.2 (3.6) years (range 0.3–10) (table 1). The patients with primary APS showed a higher prevalence of reduced levels of C3, C4 and CH_{50} than patients with non-SLE connective tissue diseases (C3: 69.4% vs 9.52%, OR = 21.59, 95% CI 6.18 to 75.42; C4: 61.1% vs 7.1%, OR = 15.32, 95% CI 4.48 to 52.31; CH_{50}: 47.2% vs 2.4%, OR = 36.68, 95% CI 4.54 to 296.26). No healthy volunteers had a reduced complement level, but two had a low serum C4 level. The mean levels of C3, C4 and CH_{50} were lower in patients with primary APS than in patients with non-SLE connective tissue disease or healthy volunteers (fig 1). Additionally, serum complement levels of patients with non-SLE connective tissue disease with a past history of thrombosis (C3: 112 (13.5); C4: 20.0 (6.32); CH_{50}: 40.4 (4.54)) did not show significant differences with the patients without a past history of thrombosis.

Serum anaphylatoxin levels

Most patients with primary APS showed raised serum C3a and C4a levels (C3a: 13/17, 76%; C4a: 14/17, 82%), but none showed raised C5a (0/17). Patients with primary APS with a low serum C3 concentration had significantly higher levels of C3a than those with normal C3, but there was no significant difference in C4a levels between patients with low and normal C4 (fig 2). The non-SLE patients had significantly lower levels of C3a and C4a than patients with primary APS with low serum C3 and C4 concentrations. However, there were no significant differences in C3a and C4a levels between control patients and patients with primary APS with normal C3 and C4 levels. C3a and C4a levels of control patients with past history of thrombosis (n = 4; C3a: 2 68 (73.1); C4a: 362 (62.1)) did not show significant differences from patients without a past history of thrombosis. No healthy volunteers showed raised serum anaphylatoxin levels.

Plasma TNFα levels in patients with primary APS

Raised plasma TNFα was found in 7/22 (32%) of patients with primary APS. The prevalence of raised TNFα was greater in patients with hypocomplementaemia than in those with the normal serum CH_{50} activity (63% vs 14%, OR = 10, 95% CI 1.26 to 79.34).

Serum complement regulatory protein levels

Serum complement regulatory factor H and factor I were measured in 16 and 13 patients with primary APS, respectively. These patients did not have reduced levels of factor H or factor I (fig 3). Serum factor H levels tended to be raised in patients with high C3a serum levels, but the increase was not statistically significant (fig 3).

Serum immune complex levels

The positive ratio of serum immune complex in patients with primary APS was 23/33 (70%), which was significantly increased compared with ratio of patients with non-SLE connective tissue disease (8/22 (36%), OR = 4.03, 95% CI 1.28 to 12.6) (fig 4).

Correlation between clinical manifestations and hypocomplementaemia

Clinical manifestations of APS occurred as venous thrombosis, arterial thrombosis and pregnancy morbidity in 16/36 (44.4%), 19/36 (52.8%) and 3/27 (11.1%) cases, respectively. No particular manifestation was correlated with hypocomplementaemia. The relative risks for having those manifestation in patients with low CH_{50} were 1.28 (95% CI 0.34 to 4.82), 1.55 (0.41 to 5.78), 1.88 (0.16 to 22.8), respectively. All patients received warfarin and/or antiplatelet agents, but none received heparin or its derivatives.

Six patients with non-SLE connective tissue diseases had past histories of thrombosis (one venous thrombosis and three arterial thrombosis) and no patients showed hypocomplementaemia.

Correlation between aPL levels and hypocomplementaemia

The prevalences of IgG-aCL, IgM-aCL, IgG-anti-β_2GPI, IgM-anti-β_2GPI, IgG-aPS/PT, IgM-aPS/PT and LA were 26/36 (72%), 9/36 (25%), 21/36 (58%), 8/36 (22%), 21/36 (58%), 18/36 (50%)

Table 2 Correlation between aPL and hypocomplementaemia (low CH_{50})

aPL	n	Normal CH_{50}	Low CH_{50}	OR (95% CI)
aCL IgG*				
Positive	26	12	14	0.86 (0.20 to 3.69)
Negative	10	5	5	
aCL IgM*				
Positive	9	4	5	0.86 (0.19 to 3.92)
Negative	27	13	14	
Anti-β_2GPI* IgG				
Positive	21	11	10	1.65 (0.43 to 6.31)
Negative	15	6	9	
Anti-β_2GPI* IgM				
Positive	8	3	5	0.6 (0.12 to 3.01)
Negative	28	14	14	
aPS/PT* IgG				
Positive	21	14	7	8.0 (1.69 to 38.0)
Negative	15	3	12	
aPS/PT* IgM				
Positive	18	11	7	3.14 (0.80 to 12.3)
Negative	18	6	12	
LA				
High	14	3	11	6.42 (1.37 to 30.1)
Low/normal	22	14	8	

*IgG and IgM were tested in all 36 samples.
aCL, anticardiolipin antibody; aPL, antiphospholipid antibodies; aPS/PT, phosphatidylserine-dependent antiprothrombin antibody; anti-β_2GPI, anti-β_2-glycoprotein I antibody; CI, confidence interval; LA, lupus anticoagulant; OR, odds ratio.

and 30/36 (83%), respectively, in patients with primary APS. Neither IgG/M-aCL nor IgG/M-anti-β_2GPI was correlated with hypocomplementaemia (low CH_{50}), but the presence of IgG/M-aPS/PT positively correlated with hypocomplementaemia (table 2), which occurred significantly more often in patients with primary APS with high LA than in those with low or negative LA (11/14 (79%) vs 8/22 (36%), OR = 6.42, 95% CI 1.37 to 30.1; table 2).

DISCUSSION

Our results show that hypocomplementaemia is frequently found in patients with primary APS. The high serum C3a and C4a levels and the correlation between serum C3a concentrations and low C3 suggests that hypocomplementaemia in these patients is due to complement activation rather than complement deficiency. None of the patients with primary APS had reduced factor H or factor I levels, indicating that complement activation is not caused by deficiency of these factors but presumably by enhanced immune complex formation. In primary APS, immune complex formation might have a bearing on the anticoagulant potential of aPL, given the positive correlation between strong LA and hypocomplementaemia. This relationship is also related to plasma TNFα released by procoagulant and proinflammatory cells, further supporting a role for complement activation in some manifestations in patients with APS.

Concurrent reduction of C3, C4 and CH_{50} was the most common profile in our patients, reflecting activation of the classical complement pathway. The proinflammatory effect of C5a is relevant to the pathogenesis of miscarriages in the APS animal model, but none of the patients had raised serum C5a. The finding of lower serum C5a compared with C3a and C4a is consistent with reports in other diseases, including SLE.[28–30] C5a is a strong inflammatory mediator and regulatory factors such as factor H or factor I inhibit C3b-dependent activation of C5. In this study, patients with primary APS with high serum C3a levels tended to have raised factor H, and persistent C3 activation may lead to upregulated production of regulatory factors; however, none of the patients were in the acute phase of thrombosis at the time of blood collection. It is likely that the behaviour of C5a in primary APS is similar to that in SLE flares,[30] but complement activation is common during pregnancy[31] and C5 activation may occur in APS pregnancy. A recent report provides evidence to show that serum of patients with APS can induce tissue factor production on neutrophils and this effect was shown to be due to C5a activation.[32] Anaphylatoxins, especially C5a, are extremely labile and are quickly degraded by serum protease, and thus blood was examined immediately; however, it is possible that C5a was degraded in some cases.

Infection, injury or other biological stresses can activate the complement system.[33] Accumulating evidence indicates that tissue ischaemia or platelet aggregation can induce complement activation,[34 35] therefore, thrombosis itself is one of the incidents which might induce complement activation. However, none of the participants in this study had complications associated with infection, malignancy, impaired circulation, tissue ischaemia, or thrombosis at the time of blood collection and all were negative for C-reactive protein. The hypothesis is further suggested by the data that non-SLE controls who had a history of thrombosis did not show hypocomplementaemia. No patients were receiving heparin or its derivatives, which are known to modify complement activation. Additionally, most of the patients with APS were receiving one or more antiplatelet agents and blood of all patients with primary APS was not examined at the acute stage of thrombosis. In SLE or other immune complex-mediated diseases, immune complex formation can promote activation of the classical pathway. We showed in this study that raised immune complex levels were frequently found in patients with primary APS, suggesting the circulating immune complex potentiates the triggering of complement activation, ultimately leading to thrombotic events. Those immune complexes may include an antigen–antiphospholipid antibody complex in patients with primary APS, as previously described.[36] We found that LA activity was correlated with hypocomplementaemia, and LA from APS plasma may reflect the sum of multiple aPL involved in the pathophysiology of APS. Therefore, our results partially support the hypothesis that an aPL–autoantigen complex drives complement activation.

The prevalence of aPS/PT in patients with hypocomplementaemia was higher than that in patients with a normal CH_{50} level. IgG1 has been proposed as a dominant subclass in aCL in patients with thrombotic events,[37] but we[7] and others[38] have found that IgG2 is the dominant IgG subclass of aCL or anti-β_2GPI in APS. Since IgG2 is less effective in activating complement than other subclasses, aPS/PT might be more potent in activating complement than aCL or anti-β_2GPI. Additional mechanisms may contribute to complement activation in patients with primary APS. In SLE, reduced complement receptor type 1 levels on erythrocytes and impaired CD55 and CD59 expression (downregulators of the complement system) have been proposed as additional mechanisms of complement activation.[39] Equivalent data are unavailable in primary APS, but since many patients with primary APS evolve towards SLE, there may be common mechanisms of complement activation in SLE and APS.

We have previously reported that raised plasma TNFα levels in APS are not associated with HLA haplotypes including class

III.[40] In this study, some patients with hypocomplementaemia showed raised TNFα. Although the relationship of hypocomplementaemia and plasma TNF levels shown in this study was borderline, as TNFα is produced by activated monocytes, complement activation might accelerate prothrombotic reactions in APS, further supporting a role for complement activation in some manifestations in patients with APS. In the animal APS pregnancy model, TNFα has been proposed as a candidate therapeutic target,[41] and our data in patients with APS partly support this idea. Recognition of cross-talk between complement activation and prothrombotic status highlights an important role for the complement system in the physiopathology of primary APS, since complement activation may participate in coagulation processes and contribute to tissue damage. Taken together, these results suggest that the complement system could be a potential therapeutic target in patients with APS.

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STAT4 single nucleotide polymorphism, rs7574865 G/T, as a risk for antiphospholipid syndrome

Genetic factors are hypothesised to play a role in the susceptibility to antiphospholipid syndrome (APS) based on several family studies in patients with anti-phospholipid antibodies (aPL) and/or clinical manifestations of APS. APS can occur alone (primary APS) or in conjunction with systemic lupus erythematosus (SLE). Therefore, APS and SLE may, in part, share a common mechanism for disease onset or progression. Likewise SLE, human leukocyte antigen (HLA) associations have been reported in APS.[1] In addition, polymorphisms of target antigens for aPL and thrombotic genetic risk factors have been described.[1] Despite the numerous studies on the immunogenetic predisposition to APS, only a few genes such as HLA region have been identified.

Signal transducer and activator of transcription 4 (STAT4) is a transcription factor expressed on myeloid cells, T cells, dendritic cells, monocytes and so forth. Since the STAT4 pathway plays crucial roles in the Th1 and Th17 lineages,[2 3] alterations in the STAT4 pathway may lead to autoimmune and/or chronic inflammatory disorders. The STAT4 gene is located in the chromosome 2q that has been considered to be associated with SLE and RA.[4 5] Recently, Remmers *et al*[6] demonstrated that the STAT4 haplotype tagged by rs7574865 G/T single nucleotide polymorphism (SNP) was strongly associated with rheumatoid arthritis (RA) and SLE in the North American population. Moreover, recent genome wide association study confirmed not only HLA but also STAT4 associations in Caucasian patients with SLE.[7] STAT4 association was also found in Korean patients with RA[8] and Caucasian patients with primary Sjögren syndrome (SS).[9] In this study, we investigated rs7574865, the most strongly associated SNP of STAT4, in Japanese patients with APS and compared them with healthy controls.

We enrolled 74 patients with APS (37 with primary APS and 37 with secondary APS complicated with SLE) and 414 ethnically matched healthy controls. Written informed consent was obtained from each participant. All patients with APS met the criteria for the classification of APS.[10] In total, 70 patients met thrombotic criterion and 19 patients met obstetrical criterion. Genomic DNA samples were extracted from peripheral blood. Genotyping of rs7574865 was performed using TaqMan Genotyping Assay kit. Genotype and allele frequencies in each group were compared using the χ^2 test and the related risk for having APS was approximated by the odds ratios.

Genotypes of rs7574865 did not show any significant deviation from Hardy–Weinberg equilibrium in healthy control and APS groups. The T allele frequency of rs7574865 in APS (42.6%) was significantly elevated compared with that in healthy controls (31.6%). When analysed only in patients with primary APS, the T allele frequency of rs7574865 (48.6%) was higher still (table 1). This association was still observed after stratification by clinical manifestations of APS.

Our results show, for the first time, the positive correlation between the T allele of STAT4 rs7574865 and APS. Moreover, the correlation was even enhanced when focusing on primary APS, indicating that this SNP is also associated with APS itself. Our data suggest that the STAT4 SNP plays a crucial role, independent of ethnicity, in the pathogenesis of autoimmune disorders, including RA, SLE, SS and APS.

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Table 1 The allele frequencies of STAT4 SNP, rs7574865 G/T, in controls and patients with APS and primary APS

	T allele	G allele	p Value	OR (95% CI)
Controls (n = 414)	262 (31.6%)	566 (68.4%)	–	–
APS (n = 74)	63 (42.6%)	85 (57.4%)	<0.001	1.60 (1.12 to 2.29)
Primary APS (n = 37)	36 (48.6%)	38 (51.4%)	0.003	2.05 (1.27 to 3.30)
Thrombosis (n = 70)	58 (41.4%)	82 (58.6%)	0.023	1.53 (1.06 to 2.21)
Obstetrical complication (n = 19)	19 (50.0%)	19 (50.0%)	0.018	2.16 (1.12 to 4.15)

The p value and OR (95% CI) for each group were determined in comparison with controls.
APS, antiphospholipid syndrome; OR, odds ratio; SNP, single nucleotide polymorphism; STAT4, signal transducer and activator of transcription 4 (STAT4).

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Influenza infection in suckling mice expands an NKT cell subset that protects against airway hyperreactivity

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Infection with influenza A virus represents a major public health threat worldwide, particularly in patients with asthma. However, immunity induced by influenza A virus may have beneficial effects, particularly in young children, that might protect against the later development of asthma, as suggested by the hygiene hypothesis. Herein, we show that infection of suckling mice with influenza A virus protected the mice as adults against allergen-induced airway hyperreactivity (AHR), a cardinal feature of asthma. The protective effect was associated with the preferential expansion of $CD4^-CD8^-$, but not $CD4^+$, NKT cells and required T-bet and TLR7. Adoptive transfer of this cell population into allergen-sensitized adult mice suppressed the development of allergen-induced AHR, an effect associated with expansion of the allergen-specific forkhead box $p3^+$ ($Foxp3^+$) Treg cell population. Influenza-induced protection was mimicked by treating suckling mice with a glycolipid derived from *Helicobacter pylori* (a bacterium associated with protection against asthma) that activated NKT cells in a CD1d-restricted fashion. These findings suggest what we believe to be a novel pathway that can regulate AHR, and a new therapeutic strategy (treatment with glycolipid activators of this NKT cell population) for asthma.

Introduction

Bronchial asthma, a complex and heterogeneous trait, is a major public health problem, affecting nearly 10% of the general population and disproportionately affecting children. Moreover, the prevalence of asthma has increased dramatically over the past 3 decades, an increase thought to be due to changes in our environment. These environmental changes include reductions in the incidence of infectious diseases that may exert protective effects against asthma, as suggested by the hygiene hypothesis (1). While the infectious agents responsible for this relationship, and the precise mechanisms by which infectious microorganisms might protect against asthma, are very poorly understood, epidemiological studies suggest that infection with bacteria (e.g., *Helicobacter pylori* [refs. 2, 3], endotoxin [ref. 4], or *Acinetobacter lwoffii* [ref. 5]) or viruses (e.g., hepatitis A virus [refs. 6, 7]) might reduce the likelihood of developing asthma.

The role of viral infection in modulating the development of asthma is particularly complex because many different viruses affect the respiratory tract, some appearing to enhance and some to protect against the development of asthma. For example, infection with human rhinovirus in children before 3 years of age increases the later risk of developing asthma (8), while other respiratory viral infections appear to protect against the later development of asthma (9–14). However, in older individuals with established asthma, respiratory viral infection, particularly with influenza A virus, almost always triggers acute symptoms of asthma (15–17). These discrepancies may be due to the timing of the infection, since infection in very young children may profoundly alter the developing innate immune system in such a way as to protect against the later development of asthma, or to the specific immunological cell types activated by a given infectious agent.

To improve our understanding of the role of respiratory viral infection in children in the development of asthma, we studied a mouse model of asthma in which suckling mice were infected with the influenza A virus (H3N1), and were subsequently studied as adults for susceptibility to allergen-induced airway hyperreactivity (AHR), a cardinal feature of asthma. We found that H3N1 infection in suckling mice protected the mice as adults against allergen-induced AHR. The protective effect was associated with the preferential expansion of a subpopulation of suppressive double-negative (DN) NKT cells and was mimicked by treatment of suckling mice with several specific glycolipids, including one derived from *H. pylori*.

Results

Infection of suckling mice with H3N1 protects against AHR. We infected suckling pups (2 weeks old) or adult mice (8 weeks old) with the influenza A/Mem71 (H3N1) virus, and 6 weeks later the mice were examined for susceptibility to OVA-induced AHR (Figure 1A). H3N1 infection

Authorship note: Michio Shimamura, Petr Illarionov, and Dale T. Umetsu contributed equally to this work.

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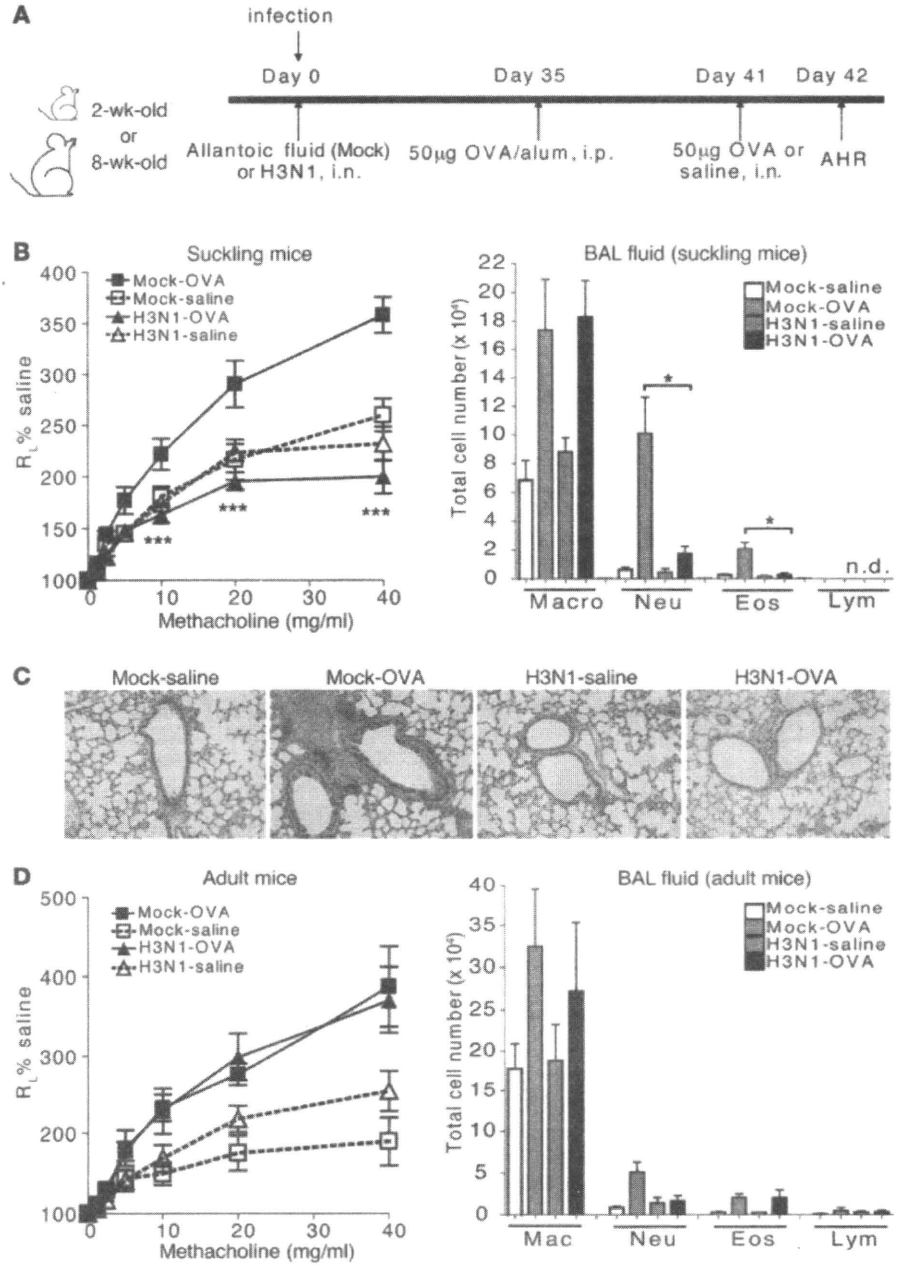

Figure 1
Infection of suckling mice with H3N1 protects the mice against AHR when adults. (**A**) Schematic showing the protocol for OVA-induced AHR. Two-week-old (suckling) or 8 week-old (adult) mice were treated with influenza A virus (H3N1) or control AF (mock infection) and assessed 6 weeks later as adults for AHR. (**B**) BALB/c mice ($n = 8$ per group) treated with H3N1 or AF at 2 weeks of age were assessed 42 days after infection for OVA-induced AHR. Changes in lung resistance (R_L) were measured in anesthetized, tracheotomized, intubated, and mechanically ventilated mice (left panel). ***$P < 0.001$ compared with mock-infected group. Cells in BAL were collected and analyzed 24 hours after the final OVA challenge (right panel). *$P < 0.05$ compared with mock-infected group. (**C**) Representative lung sections stained with H&E (original magnification, ×10) from mock- or H3N1-infected mice treated with saline or challenged with OVA. (**D**) Eight-week-old BALB/c mice ($n = 5$ per group) were infected with H3N1 or AF. Six weeks after infection, the mice were assessed for OVA-induced AHR by measuring lung resistance (left panel). Cells in BAL were collected and analyzed 24 hours after the final OVA challenge (right panel). Data are representative of 3 independent experiments.

in 2-week-old mice protected the mice as adults (at 8 weeks of age) against OVA-induced AHR (Figure 1B) and airway inflammation (Figure 1, B and C). In contrast, severe OVA-induced AHR and airway inflammation developed in the mock-infected mice at 8 weeks of age. Whereas infection in 2-week-old suckling mice conferred protection, infection in 8-week-old adult mice with H3N1 did not protect against subsequent OVA-induced AHR or airway inflammation (Figure 1D).

Adoptive transfer of NKT cells cannot reconstitute OVA-induced AHR in Jα18$^{-/-}$ mice. Infection with a different influenza virus strain (H3N2) enhanced the ability of respiratory tolerance to prevent OVA-induced AHR (11), consistent with the idea that influenza infection is complex and can affect multiple compartments of the immune system. Because infection with the influenza A virus has been shown to directly activate NKT cells (18), which play a very important role in asthma (19), we asked whether infection with the H3N1 virus affected the function of NKT cells. We therefore purified NKT cells from mice infected with H3N1 as sucklings (42 days after infection) and adoptively transferred these cells (92%–97% purity; Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI44845DS1) into adult OVA-sensitized, NKT cell–deficient recipients (Jα18$^{-/-}$ mice) (Figure 2A). After receiving the H3N1-exposed NKT cells, the Jα18$^{-/-}$ mice, which have CD1d-restricted non-invariant (but not invariant) TCR NKT cells, and which cannot develop

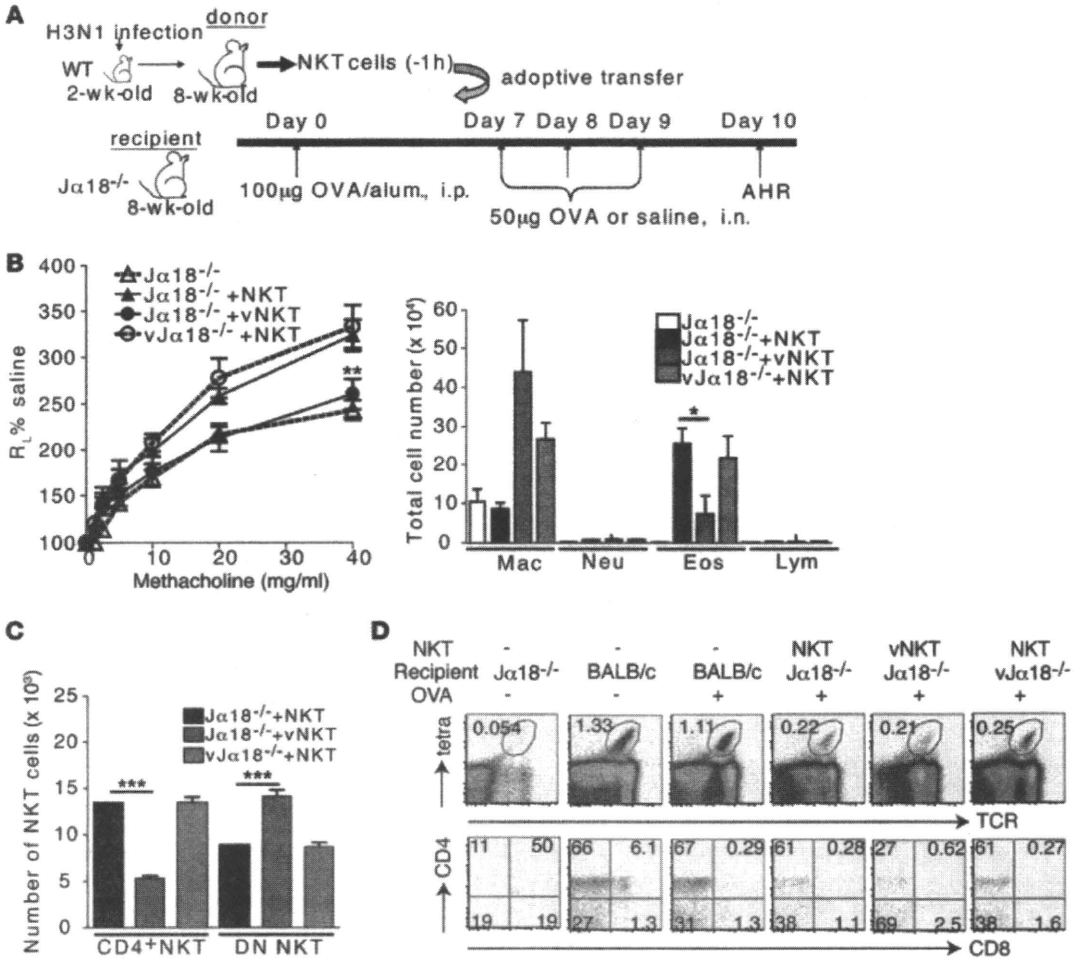

Figure 2

Adoptive transfer of H3N1-exposed NKT cells fails to reconstitute OVA-induced AHR. (**A**) Schematic showing the protocol for adoptive transfer of NKT cells to OVA-immunized *Jα18*$^{-/-}$ recipients. The donor mice were infected with H3N1 or mock infected at 2 weeks of age. Six weeks after infection, NKT cells were purified and adoptively transferred into OVA-sensitized *Jα18*$^{-/-}$ mice, which were then challenged with OVA and assessed for AHR. (**B**) Adoptive transfer of H3N1-exposed NKT cells (vNKT) to *Jα18*$^{-/-}$ mice failed to reconstitute OVA-induced AHR (measured as lung resistance in response to methacholine challenge) (left panel). Adoptive transfer of NKT cells from mock-infected mice (NKT) fully reconstituted AHR. H3N1 infection at 2 weeks of age of *Jα18*$^{-/-}$ mice (v*Jα18*$^{-/-}$) and reconstitution at 8 weeks of age with NKT cells from mock-infected mice did not protect against AHR (n = 8–10 per group). BAL fluid was collected and analyzed (right panel). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with *Jα18*$^{-/-}$ + NKT group. (**C** and **D**) Lung cells were isolated from the recipients after measurement of AHR, and the absolute numbers (**C**) and percentages (**D**) of lung CD4^{+} or CD4^{-}CD8^{-} (DN) NKT subsets were assessed by FACS. Upper panels show dot plots for NKT cells in lung leukocytes. After gating on the NKT cells, the cells were analyzed for CD4 and CD8 (lower panels). $^{***}P < 0.001$ compared with WT NKT group. Data are representative of 3 independent experiments.

allergen-induced AHR unless reconstituted with functional invariant TCR NKT cells (20–22), failed to develop OVA-induced AHR (Figure 2B). In contrast, transfer of NKT cells from mock-infected mice to *Jα18*$^{-/-}$ mice fully reconstituted AHR. Moreover, H3N1 infection in 2-week-old *Jα18*$^{-/-}$ suckling mice (*vJα18*$^{-/-}$ mice) and later reconstitution (at 8 weeks of age) with NKT cells from mock-infected mice did not prevent OVA-induced AHR (Figure 2B), indicating that early exposure of all of the non-NKT cells in *Jα18*$^{-/-}$ mice (e.g., conventional CD4^{+} and CD8^{+} T cells) to H3N1 was not effective in preventing AHR. Finally, in the lungs of mice receiving the H3N1 virus–exposed NKT cells (42 days after infection), significantly more CD4^{-}CD8^{-} (DN) NKT cells and significantly fewer CD4^{+} NKT cells were present (Figure 2, C and D), suggesting that H3N1 infection of 2-week-old suckling mice reduced the inflammatory function of the NKT cells, possibly by altering the CD4^{+} versus DN NKT cell subset proportions.

H3N1 infection accelerates the expansion of pulmonary NKT cells in suckling mice. In 2-week-old naive suckling mice, few NKT cells were present in the lungs, although this number increased normally to adult levels over a 6-week period (Figure 3A). Importantly, H3N1 infection but not mock infection in suckling mice greatly accelerated the expansion of the pulmonary NKT cell numbers (Figure 3B).

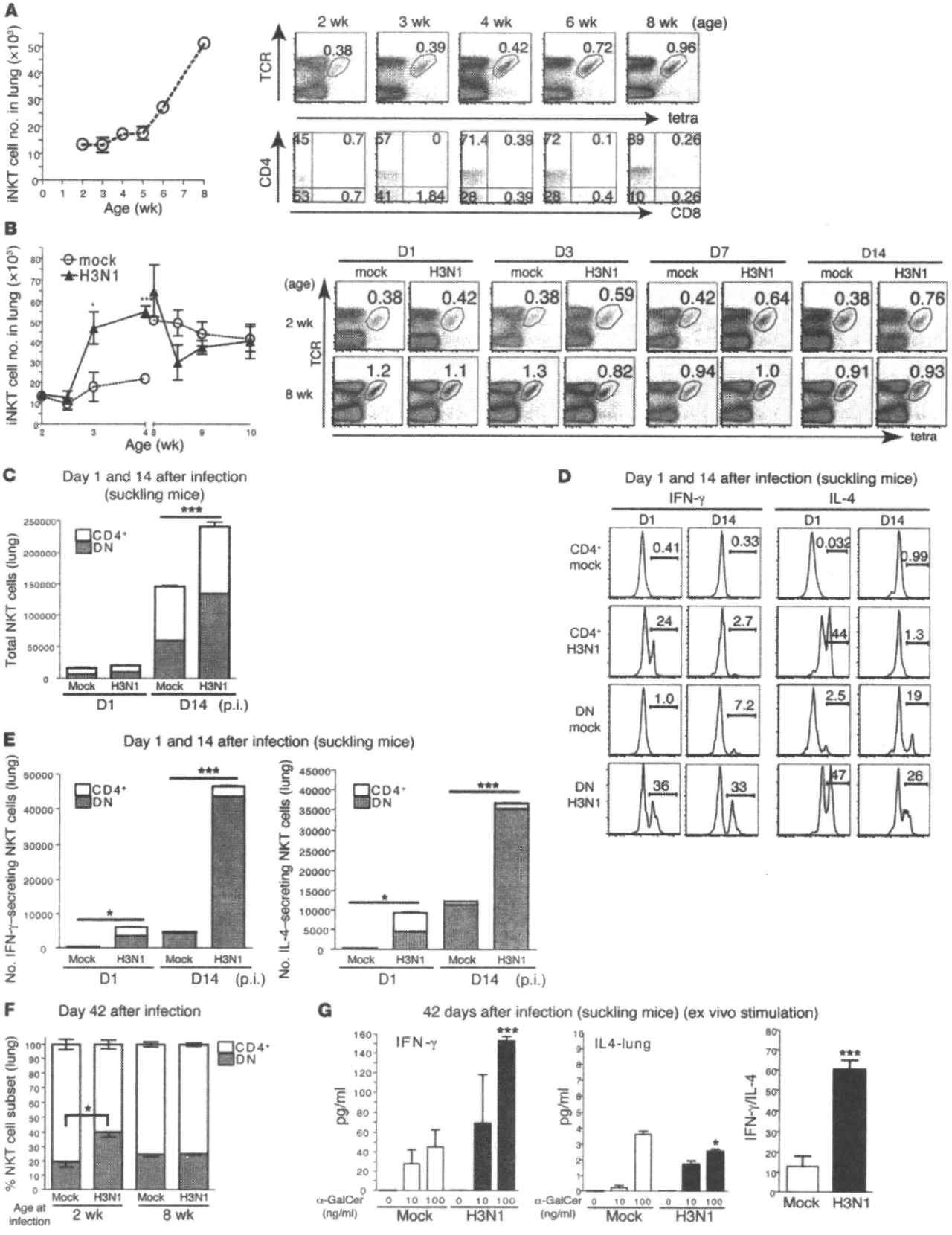
A
iNKT cell no. in lung (×10³)
Age (wk)
2 wk
3 wk
4 wk
6 wk
8 wk (age)
TCR
tetra
CD4
CD8
0.38
0.39
0.42
0.72
0.96
B
mock
H3N1
D1
D3
D7
D14
(age)
2 wk
8 wk
0.38
0.42
0.38
0.59
0.42
0.64
0.38
0.76
1.2
1.1
1.3
0.82
0.94
1.0
0.91
0.93
C
Day 1 and 14 after infection (suckling mice)
Total NKT cells (lung)
CD4⁺
DN
Mock
H3N1
D1
D14 (p.i.)
D
Day 1 and 14 after infection (suckling mice)
IFN-γ
IL-4
CD4⁺ mock
CD4⁺ H3N1
DN mock
DN H3N1
0.41
0.33
0.032
0.99
24
2.7
44
1.3
1.0
7.2
2.5
19
36
33
47
26
E
Day 1 and 14 after infection (suckling mice)
No. IFN-γ–secreting NKT cells (lung)
No. IL-4–secreting NKT cells (lung)
F
Day 42 after infection
% NKT cell subset (lung)
Age at infection
2 wk
8 wk
G
42 days after infection (suckling mice) (ex vivo stimulation)
IFN-γ
IL4-lung
pg/ml
α-GalCer (ng/ml)
IFN-γ/IL-4

Figure 3
H3N1 infection in 2-week-old mice alters the phenotype of the NKT cells. (**A**) Lung cells were isolated over a 6-week period and analyzed for NKT cells. Left: Absolute numbers of lung NKT cells. Right: Percentage of NKTs (top) in lung leukocytes. NKT cells were analyzed for CD4 and CD8 (bottom). (**B**) Left: BALB/c mice (n = 3/group) were infected with H3N1 or AF at 2 or 8 weeks of age, and lung NKT cells were assessed over 2 weeks. Right: Percentage of NKT cells in lungs of 2-week-old and 8-week-old mice. (**C**) Two-week-old BALB/c mice were mock infected or infected with H3N1, and pulmonary $CD4^+$ NKT and DN NKT cell numbers were assessed on days 1 and 14 after infection. (**D** and **E**) NKT cells from **C** were assessed for CD4, IFN-γ, and IL-4 expression (**D**) and absolute numbers quantified (**E**). (**F**) BALB/c mice (n = 4–5/group) were infected with H3N1 or mock infected at 2 or 8 weeks of age, and lung samples were taken 42 days later to assess NKT cell subsets. One of 2 independent experiments is shown. (**G**) Two-week-old BALB/c mice were infected with H3N1 or mock infected. After 42 days, lung cells were harvested and stimulated ex vivo with vehicle or α-GalCer for 96 hours. IFN-γ and IL-4 in supernatants from triplicate wells were determined by ELISA and the IFN-γ/IL-4 ratio calculated. $^*P < 0.05$, $^{***}P < 0.001$ compared with mock infection.

In contrast, H3N1 infection in adult mice had little effect on pulmonary NKT cell numbers. In fact, H3N1 infection in the adult mice transiently reduced the number of NKT cells, possibly due to activation-induced TCR downregulation (Figure 3B). In 2-week-old suckling naive mice, approximately 50% of the pulmonary NKT cells were $CD4^+$, and over time this fraction increased such that in 8-week-old adult naive mice, 89% of the pulmonary NKT cells were $CD4^+$ (dot plots in Figure 3A). However, H3N1 infection of suckling mice preferentially increased the number of DN NKT cells by day 14 after infection (Figure 3C). Both $CD4^+$ and DN NKT cells from the suckling mice secreted IFN-γ on day 1 of infection, but 14 days after infection only DN but not $CD4^+$ pulmonary NKT cells continued to secrete IFN-γ (and IL-4), as assessed with intracellular staining without in vitro restimulation (Figure 3D). Thus, 14 days after infection the great majority of cytokine-secreting cells in the lungs were DN NKT cells (Figure 3E).

Analysis of the mice 42 days after H3N1 infection showed that the proportion of DN versus $CD4^+$ NKT cells in the lungs doubled, whereas 42 days after H3N1 infection in 8-week-old mice, there was no effect on the proportion of DN NKT cells in the lungs (Figure 3F). Assessment of the cytokine profile of NKT cells 42 days following infection after ex vivo stimulation with α-galactosylceramide (α-GalCer, which specifically activates NKT cells) demonstrated increased IFN-γ but not IL-4 production by the H3N1-exposed NKT cells (Figure 3G), resulting in a greatly increased IFN-γ/IL-4 ratio (Figure 3G). These results suggested that H3N1 infection in suckling mice preferentially expanded a unique NKT cell population in the lungs that, by day 42, preferentially produced IFN-γ but not IL-4 and was associated with a reduced expression of CD4.

Adoptive transfer of H3N1-exposed NKT cells suppresses AHR and induces Treg cells. While the H3N1-exposed NKT cells (vNKT) could not induce AHR when transferred into $J\alpha18^{-/-}$ mice (Figure 2), they were not anergic, but instead potently suppressed OVA-induced AHR (Figure 4, A and B) and inflammation (Figure 4C), as assessed by adoptive transfer 42 days after infection into adult WT OVA-sensitized mice. In contrast, NKT cells from mock-infected mice (WT NKT) (Figure 4, B and C) or from adult mice infected with H3N1 (data not shown) did not suppress OVA-induced AHR. The proportion of DN NKT cells in the lungs of mice receiving the H3N1 virus–exposed NKT cells was increased (Figure 4D), consistent with the idea that H3N1 infection in suckling mice preferentially expands a subpopulation of DN NKT cells.

To more clearly demonstrate that the DN NKT cell subpopulation was responsible for the suppression of AHR, we purified $CD4^+$ and DN NKT cell subpopulations from the spleens of mice (purity 96%–99%) (Supplemental Figure 1C), which had been infected with H3N1, and adoptively transferred these cells into OVA-sensitized mice. Figure 4E shows that the DN but not the $CD4^+$ NKT cell population suppressed AHR that developed on challenge of the mice with OVA, confirming that the H3N1-exposed DN NKT cell population was responsible for this effect.

The suppression of AHR by the transferred H3N1-exposed NKT cells was associated with a 50% increase in the number of natural $Foxp3^+$ Treg cells and with a 300% increase in the number of adaptive OVA-specific $Foxp3^+$ Treg cells in the lungs (assessed by transferring DO11.10 Tg OVA-specific $Foxp3^-$ T cells from DO11.10 Tg × $Rag^{-/-}$ mice), compared with when NKT cells from mock-infected mice were transferred (Figure 4F). Furthermore, the inhibitory effect of the NKT cells exposed to H3N1 was reversed by treatment of the recipient mice with an anti-CD25 mAb (Figure 4G). These results together indicated that H3N1-exposed NKT cells could suppress the development of experimental asthma, and that natural and adaptive Treg cells might mediate the suppressive effects of the NKT cell population.

We found a similar suppressive NKT cell population in Vα14 TCR Tg mice. Adult Vα14 TCR transgenic mice have a 5- to 10-fold increase in the number of NKT cells in the spleen, of which the majority (53%) are DN NKT cells (Supplemental Figure 1B), whereas in WT BALB/c mice, only 11% of the splenic NKT cells are DN (Supplemental Figure 1B). Adoptive transfer of NKT cells purified from Vα14 TCR Tg mice into adult WT OVA-sensitized BALB/c mice greatly suppressed the development of OVA-induced AHR and airway inflammation (Figure 4, H–J). Transfer of Vα14 TCR Tg NKT cells was also associated with a 50% increase in the number of natural $Foxp3^+$ Treg cells and in a 300% increase in the number of adaptive OVA-specific $Foxp3^+$ Treg cells (assessed by transfer of DO11.10 Tg OVA-specific cells), compared with transfer of naive (WT) NKT cells (Figure 4F). These results suggest that NKT cells in Vα14 Tg mice were similar to NKT cells from suckling mice exposed to H3N1, in that they had suppressive activity for allergen-induced AHR.

The protective effect of H3N1 infection depends on TLR7 and T-bet. Since influenza A virus is a single-stranded RNA (ssRNA) virus, and since T-bet participates in IFN-γ production and in NKT cell maturation (23), we infected 2-week-old $Tlr7^{-/-}Tbet^{-/-}$ mice and control WT BALB/c mice with the H3N1 virus. Six weeks later, the mice were examined for OVA-induced AHR (protocol shown in Figure 5A). Whereas H3N1 infection in suckling WT mice protected against subsequent OVA-induced AHR and airway inflammation (Figure 5, B and C), H3N1 infection in suckling $Tlr7^{-/-}$ or suckling $Tbet^{-/-}$ mice failed to protect against, and even exacerbated, OVA-induced AHR and airway inflammation. Furthermore, the ratio of IFN-γ production to IL-4 production in NKT cells from $Tlr7^{-/-}$ mice was reduced (Supplemental Figure 2D), while IFN-γ was reduced and IL-13 and IL-17 production increased in NKT cells in $Tbet^{-/-}$ mice compared with WT mice (Supplemental Figure 2, A and E). (Note that $Tbet^{-/-}$ mice have reduced numbers of NKT cells, particularly in the liver [ref. 23] but have significant numbers of pulmonary NKT cells compared with WT mice [ref. 24]).

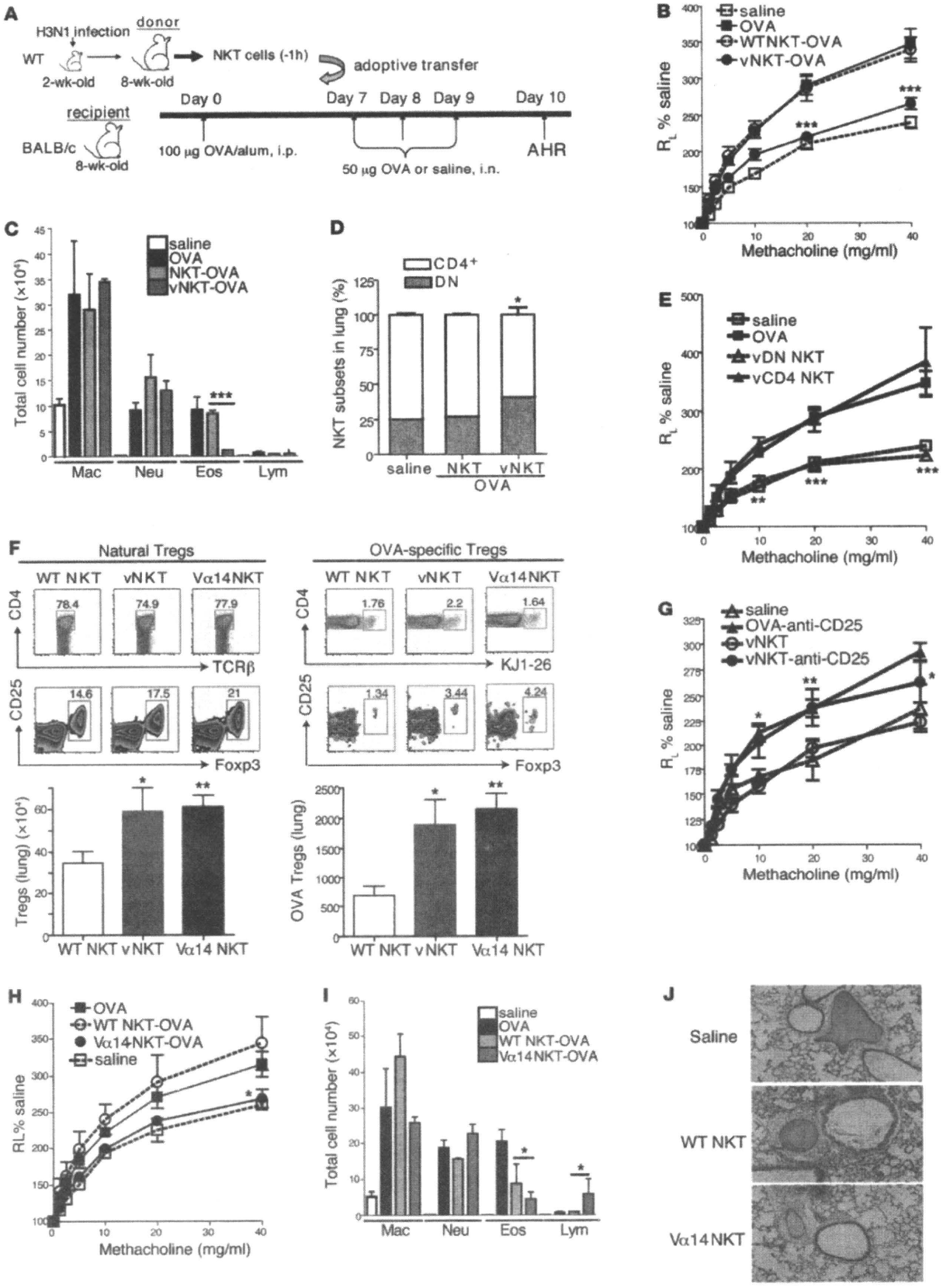
A
H3N1 infection
donor
WT
2-wk-old
8-wk-old
NKT cells (-1h)
adoptive transfer
recipient
BALB/c
8-wk-old
Day 0
Day 7 Day 8 Day 9
Day 10
100 μg OVA/alum, i.p.
50 μg OVA or saline, i.n.
AHR
B
saline
OVA
WTNKT-OVA
vNKT-OVA
R_L % saline
Methacholine (mg/ml)
C
saline
OVA
NKT-OVA
vNKT-OVA
Total cell number (×10⁴)
Mac
Neu
Eos
Lym
D
CD4⁺
DN
NKT subsets in lung (%)
saline
NKT
vNKT
OVA
E
saline
OVA
vDN NKT
vCD4 NKT
R_L % saline
Methacholine (mg/ml)
F
Natural Tregs
OVA-specific Tregs
WT NKT
vNKT
Vα14NKT
CD4
TCRβ
KJ1-26
CD25
Foxp3
78.4
74.9
77.9
14.6
17.5
21
1.76
2.2
1.64
1.34
3.44
4.24
Tregs (lung) (×10⁴)
OVA Tregs (lung)
WT NKT vNKT Vα14 NKT
G
saline
OVA-anti-CD25
vNKT
vNKT-anti-CD25
R_L % saline
Methacholine (mg/ml)
H
OVA
WT NKT-OVA
Vα14NKT-OVA
saline
RL% saline
Methacholine (mg/ml)
I
saline
OVA
WT NKT-OVA
Vα14NKT-OVA
Total cell number (×10⁴)
Mac
Neu
Eos
Lym
J
Saline
WT NKT
Vα14NKT

Figure 4
H3N1-exposed NKT cells suppress AHR and increase OVA-specific Tregs. (**A**) Protocol for adoptive transfer of NKT cells. (**B** and **C**) Lung resistance was measured in recipient mice (**B**; n = 15/group) and BAL cells collected (**C**). (**D**) Relative numbers of $CD4^+$ versus DN NKT cells in recipients' lungs were assessed (**E**) H3N1-exposed $CD4^-CD8^-$NKT (vDN NKT) or $CD4^+$NKT (vCD4 NKT) cells were purified and transferred as in **A**. Lung resistance was measured in recipient mice (n = 5/group). (**F**) Eight-week-old WT BALB/c mice received 5×10^4 DO11.10 *Rag*$^{-/-}$ T cells and were sensitized with OVA/alum. Seven days later, NKT cells from WT BALB/c, Vα14tg, or H3N1-infected mice were adoptively transferred into OVA-sensitized mice. After OVA challenge, the numbers of natural Tregs ($CD4^+C25^+Foxp3^+$) and adaptive OVA antigen–specific Tregs ($CD4^+$ $CD25^+$ $Foxp3^+KJ1\text{-}26^+$) were determined. Absolute cell numbers were calculated (n = 5/group). (**G**) Eight-week-old WT BALB/c recipients were depleted of Tregs through injections of anti-CD25 mAb (clone PC61; 0.5 mg) and assessed as in **A** (n = 5/group). (**H** and **I**) NKT cells from WT or Vα14 Tg were transferred to OVA-sensitized BALB/c mice (n = 4–6/group), which were assessed as in **A** (**H**), and BAL cells were analyzed (**I**). (**J**) Representative lung sections from recipients described in **H** were H&E stained (original magnification, ×10). Data represent 2–3 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus WT NKT-OVA (**B–D**), OVA (**E**), WT NKT (**F**, **H**, and **I**), and OVA-vNKT (**G**).

As noted above (Figure 3F), protection against AHR was associated with an increase in the number of DN NKT cells following H3N1 infection in WT mice, which did not occur in *Tlr7*$^{-/-}$ or *Tbet*$^{-/-}$ mice (Figure 5D). Moreover, adoptive transfer of NKT cells purified 6 weeks after H3N1 infection of WT, but not *Tlr7*$^{-/-}$ or *Tbet*$^{-/-}$ mice, into OVA-sensitized WT BALB/c mice suppressed OVA-induced AHR and airway inflammation (Figure 5, E and F). Taken together, these results indicate that protection by H3N1-exposed NKT cells against AHR depends on TLR7 and T-bet.

Induction of protection with α-C-GalCer and a glycolipid from H. pylori. Since NKT cells appeared to mediate the effects of H3N1 infection, we examined a panel of glycolipids that specifically activate NKT cells for the capacity to replicate the beneficial effects of H3N1 infection. We first examined the effects of α-C-GalCer, a synthetic C-glycoside analog of α-GalCer that preferentially induces IFN-γ but not IL-4 synthesis (25–27). Treatment of suckling mice with α-C-GalCer (5 μg), but not α-GalCer, which induces production of both IFN-γ and IL-4, protected the mice as adults (42 days later) from the development of OVA-induced AHR (Figure 6A). The protective effect was dependent on T-bet, since *Tbet*$^{-/-}$ mice were not protected by treatment with α-C-GalCer (Figure 6B). Moreover, adoptive transfer of NKT cells exposed to α-C-GalCer protected recipients against the development of AHR and airway inflammation (Figure 6C).

We also found a second glycolipid, PI57, a cholesterol-derived lipid from *H. pylori* (28), that could protect against the development of AHR (Figure 6D). *H. pylori*, a bacteria that colonizes the stomach (29) and is associated with protection against asthma (2, 3), produces cholesteryl α-glucosides (30), including cholesteryl 6-O-acyl α-glucoside (AGlc-Chol) (Supplemental Figure 4), which was chemically synthesized (PI57) (Figure 6D). PI57, when administered i.p. to 2-week-old mice, increased the total number of NKT cells, particularly the number of DN NKT cells, found in the lung 2 weeks later (Figure 6, E and F). In contrast, treatment with α-GalCer increased both $CD4^+$ and DN NKT cells in the lungs. Importantly, treatment of 2-week-old mice with PI57 (50 or 100 μg) (Figure 6G) protected the mice from the development of OVA-induced AHR, induced 6 weeks after the glycolipid treatment. On the other hand, treatment of 2-week-old mice with PBS30, a lipid present in the cell walls of *Sphingomonas* bacteria (31, 32), failed to protect the mice from OVA-induced AHR (Figure 6H). Moreover, adoptive transfer of NKT cells from PI57-treated, but not vehicle-treated, 2-week-old mice (harvested 6 weeks after treatment) into OVA-sensitized WT mice, suppressed AHR and airway inflammation (Figure 6, I and J). Transfer of NKT cells from α-GalCer–treated mice reduced AHR slightly, but this was not statistically significant (Supplemental Figure 3A). The production of IFN-γ by the NKT cells was important, since the protective effect of PI57, like that of H3N1 and α-C-GalCer, was dependent on T-bet, since PI57 treatment of 2-week-old *Tbet*$^{-/-}$ mice did not protect against subsequent OVA-induced AHR (Supplemental Figure 3B). These results together suggest that a subset of NKT cells that can be specifically activated by some but not all glycolipid antigens, and that preferentially produces IFN-γ, mediates the protective effects of H3N1 infection.

PI57 is a CD1d-dependent NKT cell antigen. To demonstrate that PI57, like α-C-GalCer, can directly activate NKT cells, we showed that PI57, when added to cultures of NKT cell lines plus DCs, induced the production of IFN-γ in a CD1d-restricted manner, since cytokine production was blocked by anti-CD1d mAb (Figure 7A). In addition, PI57 induced higher levels of IFN-γ and less IL-4 in NKT cell lines compared with PBS30 (from *Sphingomonas*) or α-GalCer, and did so in a CD1d-restricted manner, since DCs from *Cd1d*$^{-/-}$ mice failed to support PI57-induced cytokine production (Figure 7B). Furthermore, the PI57 response occurred by direct activation of NKT cells, since PI57 induced cytokine production in NKT cell lines with DCs from *Myd88*$^{-/-}$ or *Trif*$^{-/-}$ mice (Figure 7B), and since 3 different NKT cell hybridomas derived from Vα14 NKT cells but not from $V\alpha14^-$ T cells produced IL-2 in response to immobilized recombinant CD1d previously loaded with PI57 but not with PI56, a control glycolipid (Figure 7C). Moreover, CD1d tetramers loaded with PI57 stained 10%–23% of NKT cells in an NKT cell line (Figure 7D). Of the PI57-CD1d tetramer$^+$ cells, 92% were $CD4^-$ (DN) (data not shown). This strongly suggests that PI57 bound to CD1d was directly recognized by the TCR of a population of NKT cells. Finally, human NKT cells were also activated by PI57, since NKT cells lines (Figure 7E) as well as a $V\alpha24^+$ NKT cell clone (BM2a.3) (Figure 7F) responded to this glycolipid. The response was also directly induced, since plate-bound CD1d loaded with PI57 induced IFN-γ in BM2a.3 cells (Figure 7G). Taken together, these results indicated that both mouse and human NKT cells were directly activated by PI57, an *H. pylori* glycolipid, in a CD1d-restricted manner.

Discussion

Herein, we showed that infection of 2-week-old pups with influenza A virus H3N1 protected against the subsequent development of allergen-induced AHR, whereas infection of adult (8-week-old) mice with H3N1 did not protect against the subsequent development of AHR. The protective effect H3N1 in suckling mice was associated with the maturation and expansion of a specific subset of NKT cells, which suppressed the development of allergen-induced AHR, demonstrated by adoptive transfer of these NKT cells into normal allergen-sensitized adult mice. The protective NKT cell subset required T-bet, as the NKT cells had to be derived from T-bet$^+$ mice; this subset also produced IFN-γ and was present in NKT cell populations enriched for DN ($CD4^-$) NKT cells. Adoptive transfer of the protective NKT cell population was associated

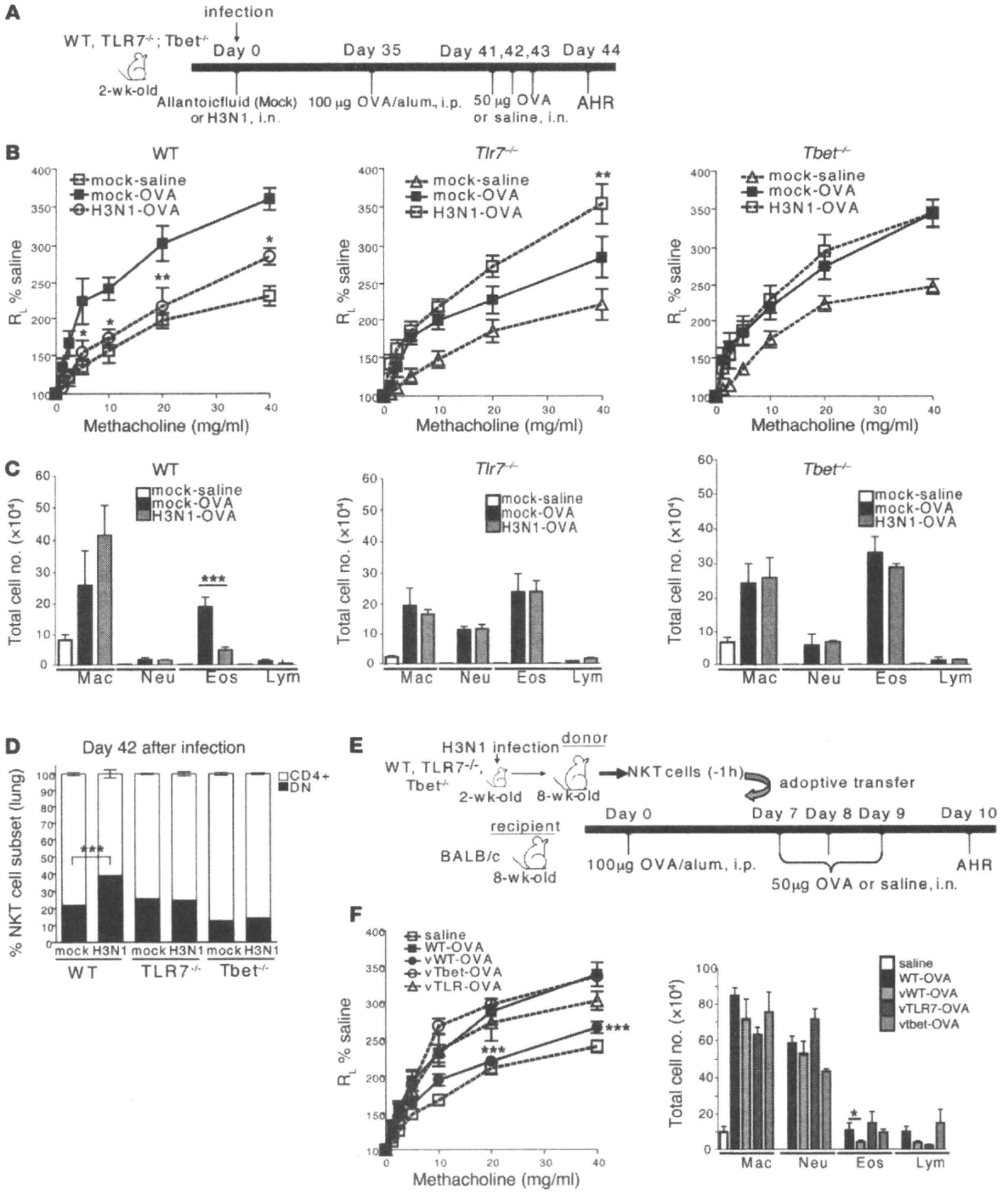

Figure 5

The protective effect of H3N1 infection depends on TLR7 and T-bet. (**A**) Schematic showing the protocol for WT, *Tlr7*$^{-/-}$, or *Tbet*$^{-/-}$ mice infected at 2 weeks of age with H3N1 virus or mock infected and examined for OVA-induced AHR at 8 weeks of age (n = 4–6 per group). (**B**) Lung resistance was measured. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the mock-OVA group. (**C**) BAL cells from **B** were collected. (**D**) WT, *Tlr7*$^{-/-}$, or *Tbet*$^{-/-}$ mice were infected with H3N1 or mock at 2 weeks of age, and lung samples were taken 42 days later to asses for NKT cell subsets. ***P < 0.001 compared with the mock group. (**E**) Schematic showing the adoptive transfer of NKT from virus-infected WT, *Tlr7*$^{-/-}$, or *Tbet*$^{-/-}$ mice to OVA-sensitized BALB/c recipients (n = 4–6 per group). The donor mice were infected with H3N1 or mock-infected at 2 weeks of age. NKT cells were purified from these mice 42 days after infection and transferred to OVA-sensitized BALB/c mice, which were then challenged with OVA to induce AHR. (**F**) Left: After OVA challenge, AHR was measured as described in **D**. Right: Cells in BAL were assessed. ***P < 0.001 compared with the WT-OVA group. Data are representative of 2 independent experiments.