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Novel lymphocyte-independent mechanisms to initiate inflammatory arthritis via bone marrow-derived cells of *Ali18* mutant mice

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Objective. In a large-scale ENU (*N*-ethyl-*N*-nitrosourea) mouse mutagenesis programme, we previously have identified and characterized a novel mutation *Ali18* that causes inflammatory arthritis like lesions in peripheral joints. In this study, we analysed the immune system of *Ali18* mice to understand mechanisms underlying the spontaneous inflammation.

Methods. Humoral and cellular components of the immune system were phenotyped by ELISA and flow cytometry. The contribution of the immune system for phenotype expression was analysed in disease transfer experiments. The involvement of the adaptive immune system was investigated in *Ali18;Rag1* double mutants and the influence of environmental factors was analysed in *Ali18* mice reared under germ-free conditions.

Results. Bone marrow cells from *Ali18* mice were able to transfer the disease phenotype to naïve wild-type recipients suggesting that cellular components of the reconstituted immune system were sufficient to induce arthritis. *Ali18* mice revealed abnormal leucocyte populations including lymphocytes and granulocytes, as well as increased plasma IL-5 and IgE levels. *Ali18;Rag1* double homozygous mutants, which lack mature lymphocytes, still developed arthritis, suggesting that the phenotype is independent of the adaptive immune system. In addition, the arthritis phenotype appeared to be independent from environmental conditions as demonstrated in mice reared under germ-free conditions.

Conclusions. The *Ali18* mutation induces inflammatory arthritis through bone marrow-derived cells. However, non-pro-inflammatory cytokine cascades and mature lymphocyte independent-mechanisms are crucial for initiation and progression of the phenotype. *Ali18* mice may thus represent a model to study mechanisms involved in seronegative arthritis induced by cells of the innate immune system.

Key words: Psoriatic arthritis, Animal model, Inflammatory arthritis, ENU, Mouse mutant.

Introduction

Autoimmune diseases cause unstrained inflammation and tissue destruction by individual's immune system [1, 2]. Approximately 3% of the human population suffers from a recognized autoimmune disease [3, 4]. Despite the huge impact on the society, to date only symptomatic therapies, which suppress the entire immune response or interfere with inflammatory cytokine networks, are available for the patients [5]. One of the reasons for this is the complex aetiology of these diseases. Because the traditional self–non-self discrimination model does not sufficiently explain the underlying mechanisms, concepts of autoimmunity are still controversial in modern immunology (see details in discussion of Matzinger [6, 7]). This hampers the application of potent reductive molecular biology approaches against autoimmune diseases. Alternatively, linkage analysis of disease pedigrees with subsequent positional cloning is a very powerful tool to dissect complex disease pathways. However, traditional linkage studies in humans have not been successful for autoimmune diseases with a few exceptions so far [8, 9]. This strongly suggests that most of the autoimmune diseases are of multifactorial bases, which consist of complex combinations of environmental and genetic factors.

Animal models for human diseases have provided essential tools for dissecting complexity of disease pathways; the use of inbred strains and standardized controlled laboratory conditions offers more precise studies of interactions among genetic and environmental factors. In addition to these advantages, forward genetic (phenotype-driven) and reverse genetic (gene-driven) approaches are available in mice [10]. Indeed, many genetically engineered mice, such as knockout and transgenic mice, show unrestrained inflammation with systemic and tissue-specific manifestations [2], and in some cases phenotype-driven approaches have uncovered gene functions where a gene-driven approach has failed. For instance, a spontaneous point mutation in one of the SH domains of the T cell receptor-associated kinase 70 (ZAP70) causes an inflammatory arthritis phenotype and autoantibody overproduction [11], and another ENU (*N*-ethyl-*N*-nitrosourea)-induced allele shows increased IgE plasma levels [12]. These phenotypes were not observed in ZAP70 null mutants [13]. Another example, an ENU-induced mutation in a catalytic domain of the Plcg2, causes swollen paws and autoantibody production [14], and these phenotypes also do not appear in the Plcg2 knockout mice [15]. Thus, a selection of different alleles of a gene, such as hypomorph and hypermorph, uncovers hidden phenotypes that do not appear in null allele-like knockout mice. These variations are obviously valuable to understand the underlying molecular pathway of inflammation and to create new models for the disease.

In the Munich large-scale screen of ENU-induced mutant mice [16–19], we have isolated and characterized the *Ali18* mutant mice that show defects resembling human inflammatory arthritis, dermatitis and osteoporosis [20]. However, the mechanisms and pathways causing the phenotypes of *Ali18* mice are still unknown. Here we present a detailed analysis of the immune system of *Ali18* mutant mice. Bone marrow cells from *Ali18* mice were able to transfer the disease phenotype to naïve wild-type recipients suggesting an autoimmune phenomenon in which bone marrow-derived cells initiated the inflammatory response. *Ali18* mice exhibited abnormalities in peripheral blood and spleen leucocyte

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populations including lymphocytes and granulocytes, as well as increased plasma IL-5 and IgE levels. Interestingly, absence of mature B and T cells in *Ali18;Rag1* double mutant mice did not prevent the swelling phenotype suggesting that cells of the innate rather than adaptive immune system play a central role in the initiation and maintenance of the inflammatory arthritis in *Ali18* mice.

Materials and methods

Mice

The *Ali18* mutation in the C3HeB/FeJ genome was identified in the Munich mouse ENU mutagenesis programme as previously described (Fig. 1A–D and [20]). For maintenance of the strain, *Ali18*/+ mice have been backcrossed to wild-type C3HeB/FeJ mice (Jackson Laboratory, Bar Harbor, ME, USA), and *Ali18*/*Ali18* mice were maintained by intercrossing of homozygous animals. Severity of swelling of paws of the mice was assessed using a semiquantitative score as described previously [20]. Briefly, the severity of arthritis was scored on a scale of 0–3 for each paw, where 0 = no swelling, 1 = slight swelling and/or redness of footpads, 2 = obvious swelling and 3 = severe swelling with deformity of digits. For germ-free experiments, *Ali18*/*Ali18* fetuses were imported via Caesarean section into a germ-free facility (Microbiology and Tumorbiology Centre, Karolinska Institutet, Stockholm, Sweden) according to the procedure of the facility [21]. *Rag1*^{tm1Mom} mice [22] on the C57BL/6J background were purchased from the Jackson Laboratory. The genotype of the RAG-1 locus was determined by PCR reaction with the primers, oIMR0189 (TGG ATG TGG AAT GTG TGC GAG), oIMR1746 (GAG GTT CCG CTA CGA CTC TG) and oIMR3104 (CCG ACA AGT TTT TCA TCG T). For the genotyping of *Ali18*;*Rag1*^{tm1Mom} compound mutant mice, we used the above primers for the RAG-1 locus and the non-recombinant markers, D4Mit204 and D4Mit71 [23], for the *Ali18* locus. The study was conducted under federal and institutional guidelines for the use and care of laboratory animals, and was approved by the Government of the District of Upper Bavaria in Germany.

Disease transfer experiments

For generation of bone marrow chimeras, to avoid contamination and complications, we performed a transplant from one donor to one recipient. Donor bone marrow cells collected from 12-week-old *Ali18*/*Ali18* or wild-type mice were purified by Ficoll gradient, and 1×10^7 cells were then intravenously injected into each lethally irradiated (11 Gy) recipient. All lethally irradiated mice were housed in ventilated cages during the experiments. The recipient mice were inspected every other week after 6 weeks from the date of bone marrow transfer experiments. The experiments were performed in accordance with German laws after appropriate permission was obtained from the government (209-2531-39/02).

Cytokine measurement

Peripheral cytokine levels were measured on a Bioplex reader (BioRad) using an 8-plex assay kit (BioRad) allowing quantification of the following cytokines in a single sample: IL-1β, IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN-γ and TNF-α. All procedures were carried out according to the manufacturer's specifications.

Flow cytometric analysis

A detailed flow cytometry procedure is described in [24]. Briefly, single-cell suspensions from peripheral blood, spleens and bone marrow were prepared with ethidium monoazide (Molecular Probes), which stains dead cells, and Fc blocking antibody (Clone: 2.4G2, Pharmingen). Erythrocytes were lysed by osmotic shock before the staining procedure. Cells were then stained with fluorescence-conjugated monoclonal antibodies (Pharmingen) and were analysed by a FACSCalibur (BD Bioscience). Data analysis was carried out using FlowJo program (Tree Star Inc.) or CellQuest Pro (BD Bioscience).

ELISA

Levels of circulating immunoglobulin (Ig), autoantibody against single- and double-strand DNA (anti-DNA) and RF were measured by standard ELISA procedures as previously described [24, 25]. For measurement of autoantibody production, test sera were diluted 1:100 for anti-DNA or 1:200 for RF and pooled serum from MRL-lpr mice (Jackson Laboratory) with appropriate dilutions were used as positive controls.

Statistics

Comparisons of the mean values were performed by the paired *t*-test using the JMP software (www.jmp.com). *P*-values <0.05 were considered statistically significant. Data are expressed as the mean ± S.E.M.

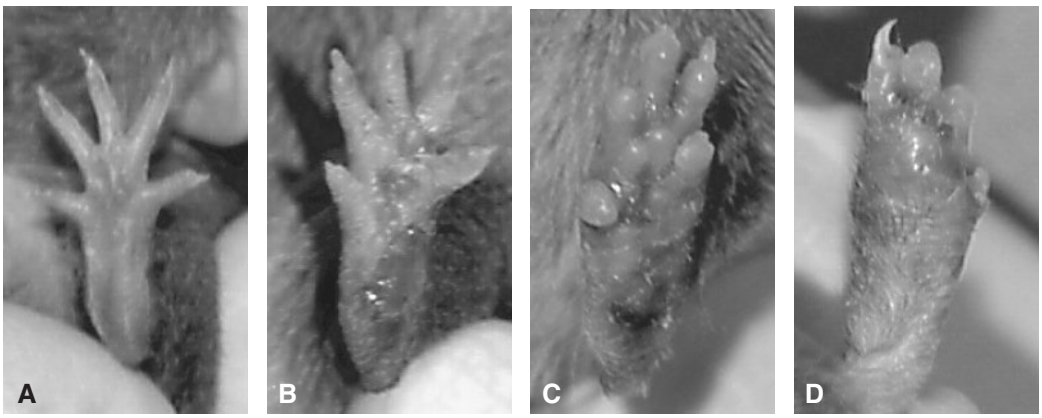
Results

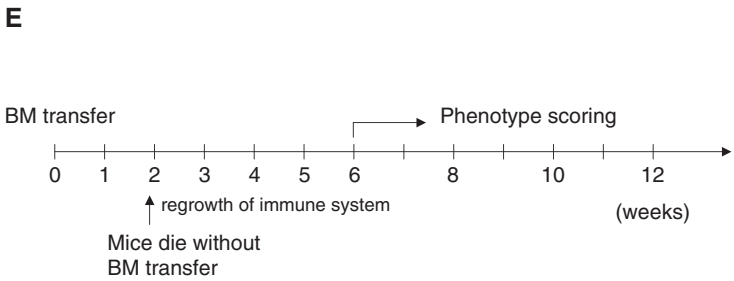
The *Ali18* line was identified in the dysmorphology screen in the Munich ENU mutagenesis project, and the offspring showed swollen paws in a semi-dominant manner (Fig. 1B–D and [20]). Histological analysis of the affected paws of *Ali18* mice revealed inflammatory lesions with a mixed cellular infiltrate including lymphocytes and granulocytes in affected area ([20] and data not shown). From these indicative results, we assumed that the inflammatory lesions in the paws of *Ali18* mice are caused by abnormalities in the immune system. Therefore, we employed various immunological techniques to elucidate contribution of haematopoietic cells to initiation and progression of the phenotype. Due to the low penetrance and late onset of the phenotypes in heterozygous animals [20], comparative analysis was performed using *Ali18* homozygous (*Ali18*/*Ali18*) animals, which display an early disease onset, and wild-type (+/+) animals on C3HeB/FeJ background at 12 weeks of age.

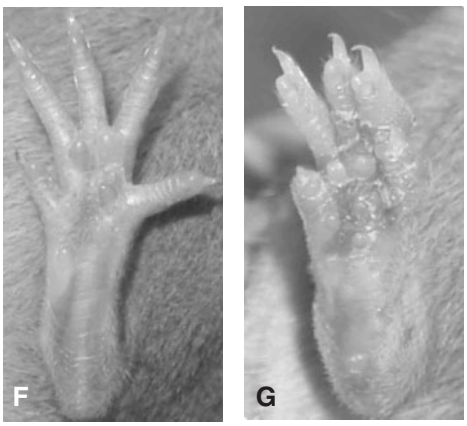
Disease transfer via bone marrow transplantation

To analyse whether the arthritis phenotype is dependent on the immune system of *Ali18* mice, we employed bone marrow transplantation. In a pilot experiment, we selected wild-type C3HeB/FeJ mice as recipients and F1 mice from C3HeB/FeJ and C57BL/6J parents as donors. Because the cells of the F1 animals express both $H2K^k$ and $H2K^b$ antigen, $H2K^b$-positive cells can be distinguished as donor-derived cells from recipient cells (only $H2K^k$ positive) without rejection. Four groups, each containing four animals, were exposed to 8, 9, 10 and 11 Gy of X-ray, respectively. Between 6 and 10 h after irradiation, purified donor bone marrow cells or phosphate-buffered saline (PBS) were intravenously injected into each of the two recipient mice. All PBS-injected recipients died by 2 weeks after irradiation (Fig. 1E), but all bone marrow cell-injected recipients survived. In addition, we detected >90% of $H2K^b$-positive haematopoietic cells in surviving bone marrow-transferred animals by using flow cytometry.

Next, we used donor bone marrow cells from +/+ and *Ali18*/*Ali18* mice on C3HeB/FeJ genetic background by the same protocol described above with 11 Gy irradiation. The survival rates of recipients with +/+ and *Ali18*/*Ali18* bone marrow cells were 80% (4 out of 5) and 100% (6 out of 6), respectively. Between 6 and 12 weeks after bone marrow transfer, we scored strength of swelling on paws of the recipient mice every other week (Fig. 1E). At 6 weeks after bone marrow transfer, 33.3% (2 out of 6) of the wild-type animals that were transferred with







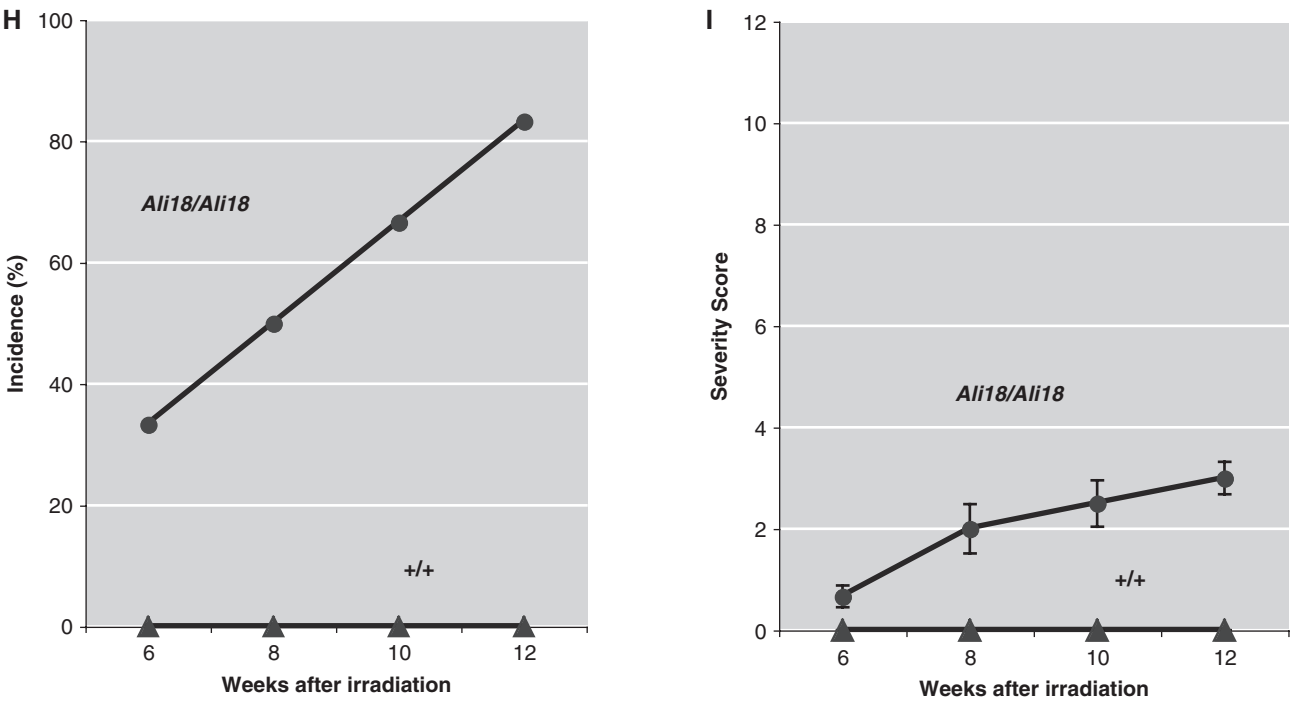


FIG. 1. Macroscopic view of hind paws of *Ali18* mice and disease transfer experiments. (**A**) Bottom of a hind paw of a wild-type C3HeB/FeJ mouse, (**B**) bottom of a hind paw of an *Ali18*/+ mouse, (**C**) bottom and (**D**) dorsum of a hind paw of an *Ali18*/*Ali18* mouse are shown. Mild swelling and bleeding from skin were observed in the heterozygous mouse (**B**). Severe swelling causes defects of nails and finger tips in the homozygous animals (**C** and **D**). Deformities of fingers are also remarkable in the homozygote (**D**). (**E**) Time schedule of the bone marrow transfer experiments. The week when bone marrow cells are transferred to lethally irradiated recipients is set as 0 week in the figure. Development of arthritis was inspected every other week after 6 weeks from bone marrow transfer. (**F** and **G**) Morphological appearance of footpads after bone marrow transfer. +/+ (F) and *Ali18/Ali18* (G) bone marrow cells were used as a donor, respectively. (**H**) Incidence of arthritis after bone marrow transfer. Frequency of arthritis mice (over score 1) is shown. (**I**) Arthritic severity score of the recipient mice. The average (dots) ± S.E.M. (axial bar) are shown. *Ali18/Ali18* mice: $n=6$; +/+ mice: $n=4$.

Ali18/*Ali18* bone marrow cells exhibited swollen paws (Fig. 1G). No swelling was observed in control animals that had received wild-type bone marrow cells as expected (Fig. 1F). The incidence of the arthritis phenotype reached a maximum of 83.3% (5 out of 6) until 12 weeks after bone marrow transfer (Fig. 1H). The total numbers for clinical scoring were lower than that observed previously in *Ali18*/*Ali18* animals (Fig. 1I, [16]). All of six control wild-type recipients with wild-type donor cells showed no swollen paw phenotypes (Fig. 1H and I).

Furthermore, we tried to transfer wild-type bone marrow cells into irradiated *Ali18*/*Ali18* recipients. As controls, we injected wild-type and *Ali18*/*Ali18* donor cells into wild-type recipients separately. The survival rate of the animals after bone marrow transfer was low. Numbers of surviving animals were 2 out of 14 *Ali18*/*Ali18* recipients with wild-type donor cells, 2 out of 15 wild-type recipient with wild-type donor cells, 2 out of 5 wild-type recipients with *Ali18*/*Ali18* donor cells. Despite these low survival rates, the two surviving *Ali18*/*Ali18* recipients with wild-type bone marrow cells showed repressed swollen paws, clinical score 0, as well as two surviving wild-type recipients with wild-type bone marrow cells (data not shown). The two control wild-type recipients with *Ali18*/*Ali18* donor cells showed swelling on paws, clinical score 5 and 2 (data not shown).

Plasma cytokine levels and granulocyte populations in Ali18 mice

From disease transfer experiments it is obvious that the reconstituted immune system of *Ali18* mutant mice is sufficient to cause swollen paws. Next, we questioned whether humoral factors influence the phenotype or not. Because of the importance of pro-inflammatory cytokines in the induction and maintenance of chronic inflammatory diseases, we measured the levels of a variety of different cytokines in plasma samples from *Ali18*/*Ali18* and +/+ control animals by Bioplex (Table 1). Our data revealed significantly increased levels of IL-5 in both sexes (male, $P < 0.005$; female, $P < 0.05$) and of IL-1β in females ($P < 0.005$) in *Ali18*/*Ali18* plasma. The magnitude of up-regulation of IL-5 in *Ali18*/*Ali18* mice was 6 and 11 times higher in males and females, respectively. The magnitude of IL-1β up-regulation in females was approximately 2-fold. Other pro-inflammatory cytokines, such as TNF-α or IFN-γ or anti-inflammatory cytokines such as IL-10, were found to be normal or below reliable levels for comparison in *Ali18*/*Ali18* animals (Table 1 and data not shown).

IL-5 controls the production and functions of granulocytes, such as eosinophils and basophils. Thus, elevated IL-5 serum levels in *Ali18* mutant might be correlated with changes within the granulocyte compartment. Therefore, we analysed granulocyte populations in splenocytes and peripheral blood by flow cytometry. In *Ali18*/*Ali18* mice, proportions of Gr-1$^+$ CD11b$^+$ granulocytes were significantly higher in the spleen (Fig. 2A and B) and in blood (Fig. 2C). When analysing additional surface markers to distinguish subpopulations of granulocytes, we detected significantly increased numbers of Gr-1$^+$CD11b$^+$CCR3$^+$ eosinophils (Fig. 2D), an observation that was very much in line with the increased levels of IL-5 in *Ali18*/*Ali18* mice.

TABLE 1. Plasma cytokine levels in *Ali18* mice

	Level (pg/ml)			
	Male		Female	
Cytokine	*Ali18/Ali18* ($n=10$)	Wild-type ($n=8$)	*Ali18/Ali18* ($n=5$)	wild-type ($n=5$)
IL-1β	2.13 ± 0.56	6.25 ± 0.83	$6.09 \pm 2.12^{**}$	3.36 ± 1.36
IL-5	$26.63 \pm 5.93^{**}$	4.00 ± 1.68	$68.85 \pm 17.57^{*}$	5.85 ± 3.76
TNF-α	4.34 ± 0.44	11.86 ± 4.95	21.48 ± 6.95	6.28 ± 1.95

The concentrations of IL-2, IL-4, IL-10, GM-CSF and IFN-γ in wild-type and *Ali18/Ali18* mice were below reliable levels for exact comparison. The average $\pm$ S.E.M. is shown. Statistical significance was calculated by the paired *t*-test. $^{*}P < 0.05$; $^{**}P < 0.005$.

Plasma Ig levels and lymphocyte populations in Ali18 *mice*

We also analysed levels of different Ig isotypes in plasma of *Ali18*/*Ali18* mice, since increased Ig levels are often observed in human inflammatory arthritis patients. The ELISA assays demonstrated that IgG1, IgG2a, IgG3 and IgA plasma levels in *Ali18*/*Ali18* mice were normal (Fig. 3A, B, D and E), while the levels of IgG2b and IgE were significantly increased, $P < 0.0005$ and $P < 0.0001$, respectively (Fig. 3C and F). We also investigated autoantibody concentrations in the plasma of *Ali18*/*Ali18* and wild-type mice. Low plasma dilution allowed the detection of anti-DNA (double and single stranded) and rheumatoid factor (RF) even in wild-type animals. *Ali18* mutants displayed about 2- and 1.5-fold higher reactivity for anti-DNA and RF, respectively ($P < 0.05$, Fig. 3G and H). However, in comparison with *MRL*/*lpr* mice, which develop an autoimmune disease similar to systemic lupus erythematosus [26], titres of anti-DNA antibody and RF were about 20- and 4-fold lower, respectively, in *Ali18*/*Ali18* mice.

We next analysed peripheral blood lymphocyte populations by flow cytometry. In the T-cell population of *Ali18*/*Ali18* mice, we detected a relative decrease in the frequency of CD4$^+$ T cells ($P < 0.0001$) and of CD8$^+$ T cells ($P < 0.0001$) (Fig. 3I and J). In contrast to T cells, B220$^+$ B cell populations in peripheral blood were not significantly changed in *Ali18* mice (Fig. 3K). Because we observed a tendency of spleens and lymph nodes in paws to enlarge macroscopically in *Ali18* mice (data not shown), a total cell number of the splenocytes and lymph node cells was counted. The number of splenocytes was increased moderately in *Ali18*/*Ali18* mice at 16–18 weeks of age (Fig. 3L), and a significant increase (approximate doubling number) was observed in the lymph node cells of *Ali18*/*Ali18* mice at same age (Fig. 5M).

Phenotypes of Ali18;Rag1 compound mutant mice

Since we had observed abnormalities involving lymphocytes and Ig levels (i.e. decreased T cells and increased levels of IgE), we conducted further studies to analyse whether lymphocytes might be involved in the development of the inflammatory phenotype. We set a mating scheme to produce *Ali18;Rag1*^{tm1Mom} compound mutant mice. RAG-1, the recombination activating gene, activates V(D)J recombination during lymphocyte differentiation; homozygous RAG-1-deficient mice (*Rag1*^{tm1Mom}, [22]) lack mature B and T cells because of inability to perform V(D)J recombination [27]. We first crossed *Ali18*/*Ali18* animals on C3HeB/FeJ background to *Rag1*$^{tm1Mom/tm1Mom}$ animals on C57BL/6 background. Subsequently, the F1 compound heterozygous animals were intercrossed to obtain double homozygous animals. The F2 animals were genotyped by PCR using primers for D4Mit204 and D4Mit71, non-recombinant polymorphic markers in the critical region of the *Ali18* locus, and primers for either wild-type or knockout alleles of the RAG-1 locus (see Materials and methods section). We confirmed the RAG-1 phenotypes of representative animals by flow cytometric analysis using CD8, B220 and Gr-1, and by ELISA analysis of serum IgE levels. As expected, F2 RAG-1 knockout homozygous animals lacked mature B and T cells (Fig. 4A) and IgE (data not shown). A total of 658 F2 animals were inspected, and the macroscopical score of swollen paws after 6 months of age was counted. Although modifier effects from C57BL/6 background hinder the *Ali18* swollen limb phenotype, only homozygous and heterozygous animals, which were confirmed by the non-recombinant markers for the *Ali18* locus, exhibited the swollen limb phenotype (data not shown). We then selected only the *Ali18* homozygous animals, and compared the RAG-1 genotype with IgE levels and arthritis frequency. As expected,

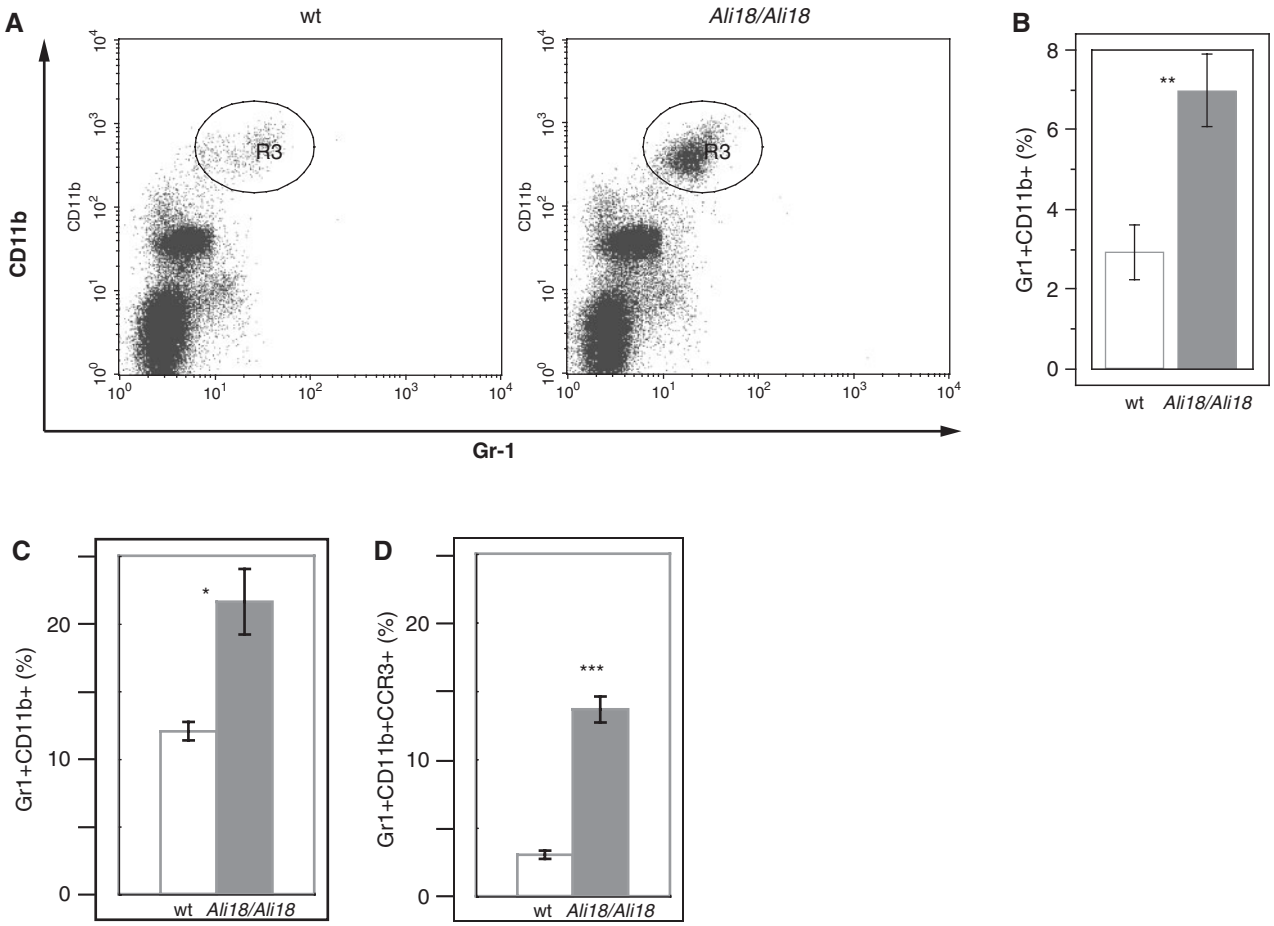


FIG. 2. Flow cytometric analysis of granulocyte populations in *Ali18* mice. (**A** and **B**) Splenocytes from 16- to 18-week-old wild-type ($n=7$) and *Ali18/Ali18* ($n=8$) animals were stained with anti-Gr-1, IgD, B220 and CD11b monoclonal antibodies to detect granulocyte subsets. (**A**) Gr-1$^+$ CD11b$^+$ cells are indicated by circles. (**B**) The average percent of Gr-1$^+$CD11b$^+$ cells ± S.E.M. is shown. (**C** and **D**) Peripheral blood cells from 12-week-old male wild-type ($n=10$) and *Ali18/Ali18* ($n=9$) mice were stained for Gr-1, CD11b and CCR3 to detect granulocyte subsets. The average percent of Gr-1$^+$CD11b$^+$ cells (**C**) and Gr-1$^+$CD11b$^+$CCR3$^+$ eosinophils (**D**) is shown. Statistical significance was calculated by the paired *t*-test. $^*P<0.01$; $^{**}P<0.005$; $^{***}P<0.001$.

all *Ali18/Ali18;Rag1*$^{tm1Mom/tm1Mom}$ animals had no IgE in plasma because of a lack of RAG-1 function (data not shown). However, these animals exhibited the swollen paw phenotype. In addition, the frequency of arthritis development was not significantly different among *Ali18/Ali18;Rag1*$^{tm1Mom/tm1Mom}$, *Ali18/Ali18;Rag1*$^{tm1Mom/+}$ and *Ali18/Ali18;Rag1*$^{+/+}$ animals (Fig. 4B).

Environmental influences on the Ali18 phenotype

Inflammatory phenotypes are often aggravated by exposure to infection with micro-organisms from the environment, and the clinical or subclinical bacterial infections can act as triggers for inflammatory dermatitis and/or arthritis. Therefore, we initially hypothesized that the strength of the inflammatory reaction, which leads to swollen paws of *Ali18* mice, may be influenced by commensal microbial colonization. Alterations in the innate or adaptive immune system could lead to increased susceptibility to pro-inflammatory microbial signals. Although we did not observe any difference in the severity of arthritis nor IgE up-regulation between specific pathogen-free (SPF) and conventional mouse housing (K.A. and T.J., unpublished data), it still was conceivable that some commensal micro-organisms in the SPF facility influenced the *Ali18* arthritis phenotype. To test this hypothesis, we imported *Ali18/Ali18* mice into a germ-free facility and analysed the morphological and immunological phenotypes. We compared severity scores and blood samples of animals at the same age (12 weeks) from both germ-free (*Ali18/Ali18* mice) and SPF (*Ali18/Ali18* and wild-type mice) conditions. The blood samples were taken at the same day from different facilities, and were analysed in the same series of experiments. Interestingly, the severity scores of arthritis were comparable in both environments (Fig. 5A). Increased IgE levels were also detected in germ-free *Ali18/Ali18* mice, which were higher than the wild-type values found in the SPF facility (Fig. 5B).

Discussion

In this article, we focused on various immunological parameters in *Ali18* mutant mice. Because the Ali18 mutation and its causal gene have not been identified, interpretation of some of the data is complicated. However, the comparison to other different mouse models for spontaneous inflammation, which have been established by means of genetic manipulation and experimental induction, would give insights into hidden mechanisms of development of arthritis. Interestingly, the abnormality in the immune system of *Ali18* mice is similar to that in some of these models.

In *Ali18/Ali18* mice, significantly increased levels of IgE were detected in peripheral blood (Fig. 2J). In humans, three cases of the NERDS (nodules, eosinophilia, rheumatism, dermatitis and swelling) syndrome were reported [28, 29]. In addition to morphological similarity to the *Ali18* phenotypes, the NERDS patients show elevated serum IgE values. However, roles of IgE up-regulation in NERDS are almost not known. Mice with interleukin 1 receptor antagonist deficiency (IL-1ra−/−), which

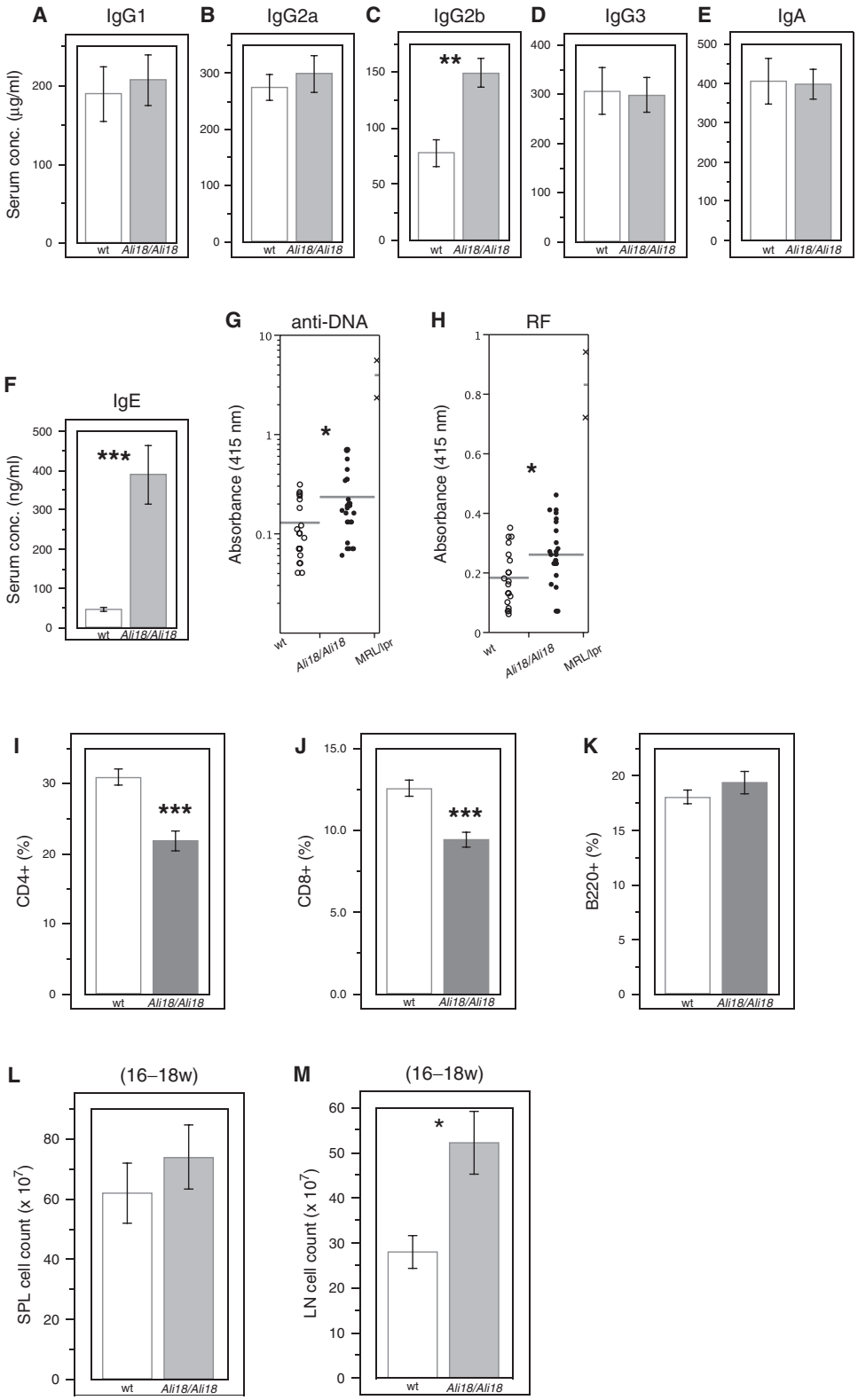


FIG. 3. Plasma Ig levels, autoantibody production and lymphocyte populations in *Ali18* mice. (**A**–**H**) Antibody levels in plasma from arthritis positive and negative littermates from parental mating of (*Ali18*/+ × +/+) and (*Ali18/Ali18* × *Ali18/Ali18*) were determined by ELISA at 12 weeks of age. (**A**–**D**) Average levels of IgG subclasses and (**E** and **F**) levels of total IgA and IgE are shown. S.E.M. is indicated by axial bar. (**G** and **H**) Levels of autoantibody against single- and double-strand DNA (anti-DNA) and IgG (RF) are shown. Open boxes, closed boxes and crosses indicate wild-type, *Ali18* homozygous and MRL/lpr mice, respectively. Average is indicated by horizontal line. (**I**–**M**) Analysis of lymphocyte populations in *Ali18/Ali18* mice. Cell surface analysis of (**I**) $CD4^+$ and (**J**) $CD8^+$ T lymphocytes, (K) $B220^+$ B lymphocytes in peripheral blood from *Ali18/Ali18* and +/+ mice at 12 weeks of age are shown. *Ali18/Ali18* mice, $n=22$ (12 males and 10 females); +/+ mice, $n=11$ (4 males and 7 females) were used. The average percent of positive cells ± S.E.M. of the flow cytometry are indicated. Comparison of total splenocyte number (**L**) and lymph node cell number (**M**). Erythrocytes were depleted by osmotic shock and living cells were counted by trypan blue exclusion. Statistical significance was calculated by the paired *t*-test. $^*P<0.05$; $^{**}P<0.0005$; $^{***}P<0.0001$.

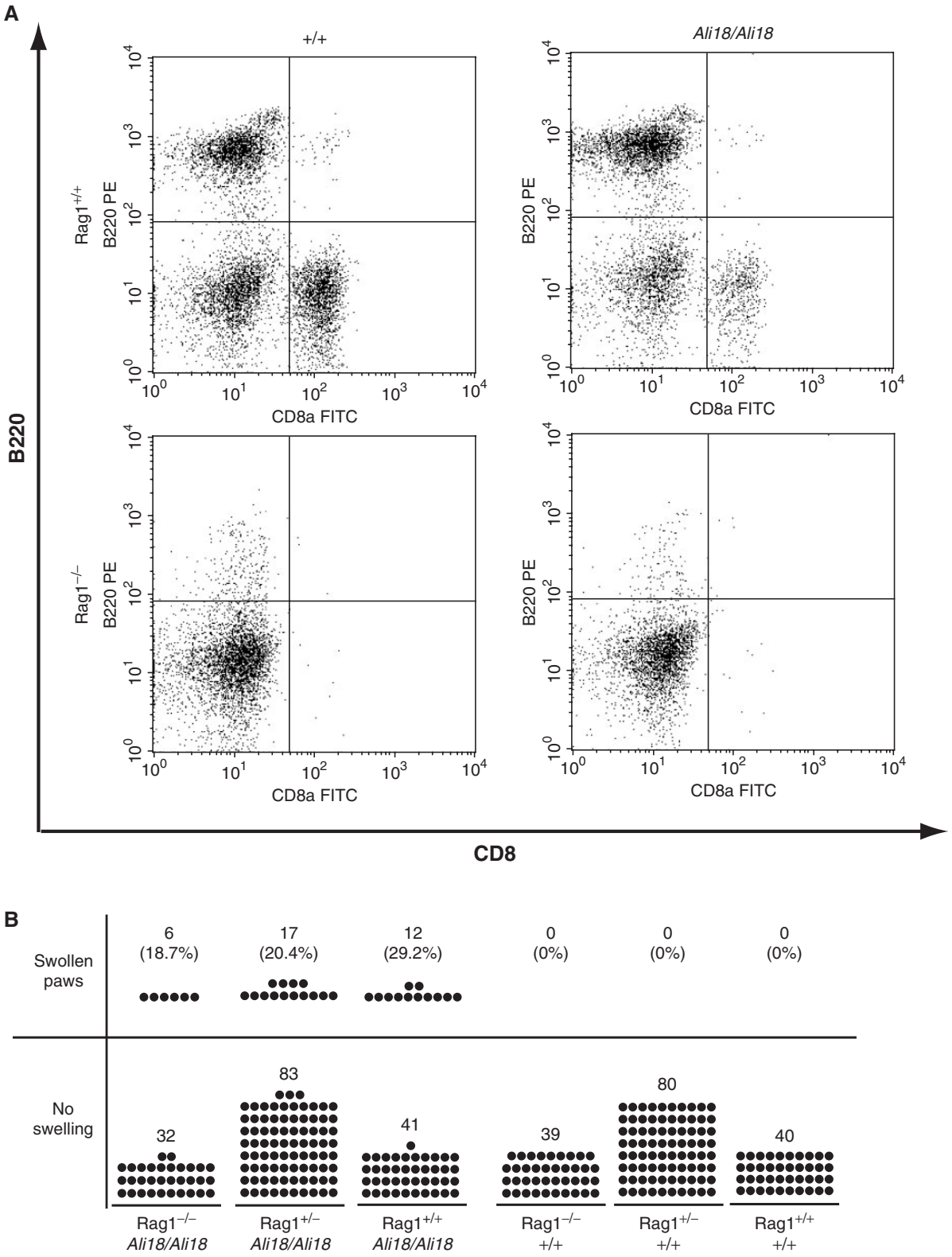


FIG. 4. Lymphocyte-independent phenotypes in *Ali18;Rag1* compound mutant mice. (**A**) Flow cytometric analysis of peripheral blood with B220 and CD8 confirms absence of mature B and T cells in *Rag1*−/− mice. (**B**) Clinical score of *Rag1*;*Ali18* compound mutant mice over age of 6 months. Macroscopically visible swollen footpads were detected in *Ali18;Rag1* compound mutant mice with no significant difference of the frequency.

show swollen paws on a BALB/cA background, exhibit IgE up-regulation in blood [30]. In the peripheral joints of the IL-1ra −/− mice increased levels of mRNA of IL-1β were detected. Although *Ali18*/*Ali18* females only exhibit a slight increase of peripheral IL-1β concentrations, the *Ali18* mutation might trigger the pathway shared with IL-1 signalling leading to IgE up-regulation. In collagen-induced arthritis (CIA), [31] which is an experimentally induced mouse model for rheumatoid arthritis, the IgG2a isotype plays a major pathogenic role in the arthritis development [32]. In *Ali18*/*Ali18* males and females,

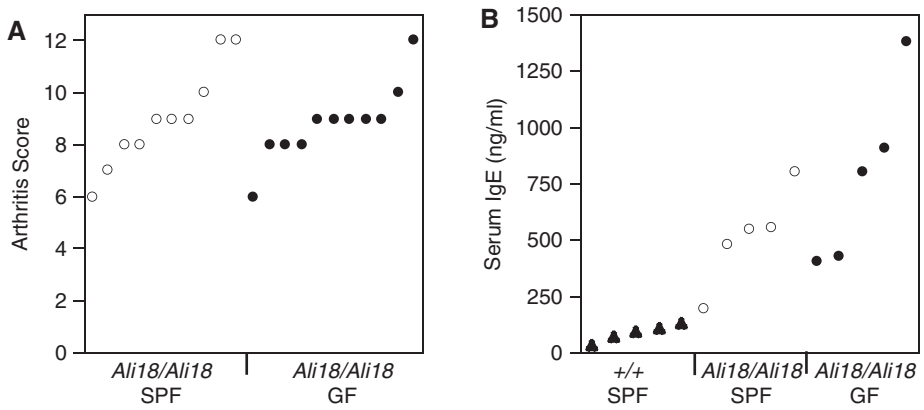


FIG. 5. Comparison of phenotypes of *Ali18/Ali18* mice between germ-free and SPF environment. (**A**) Arthritis severity scores of *Ali18/Ali18* mice at 12 weeks of age in germ-free (closed circles) and SPF environment (open circles). Dots represent total scores of individual mice. (**B**) Levels of IgE in peripheral serum from 12-week-old *Ali18/Ali18* mice in germ-free (closed circles) and SPF (open circles) environment and from 12-week-old +/+ mice (triangle) in SPF environment. All IgE levels were measured in the same set of ELISA experiments.

increased levels of IgG2a were not detected (Fig. 2F). We speculate that the *Ali18* mutation might not regulate a pathway shared with CIA, but it is interesting to note that no IgG2a up-regulation was also detected in peripheral blood of IL-1ra−/− animals [30].

In peripheral blood of *Ali18* mice, increased levels of IL-5 were detected (Table 1). IL-5 plays an important role for induction of eosinophil differentiation [33]. It is possible that increased numbers of eosinophils contribute to the unstrained inflammation of *Ali18* mice. However, IL-5 transgenic mice, which show overproduction of IL-5, show eosinophilia without any gross anatomical changes [34]. IL-5 also plays an important role in B-cell proliferation [35]. In addition, a significant increase of IL-5 mRNA in B-cells of rheumatoid arthritis patients were reported [36]. As shown in Fig. 4, however, *Ali18;Rag1* double mutants, which lack mature B-cells, still develop the arthritis phenotype. This indicates that IL-5 up-regulation alone is not sufficient for the inflammatory phenotype of *Ali18* mice.

Levels of TNF-α in peripheral blood of *Ali18*/*Ali18* mice are comparable with that of wild-type mice (Table 1). TNF-α has many important functions in inflammatory diseases [37]. Especially, transgenic mice expressing human TNF-α (hTNF-α tg) develop polyarthritis [38]. TNF bioreactivity in the serum of these transgenic mice, however, was undetectable [38]. In addition, serum levels of TNF-α in rheumatoid arthritis patients were comparable with that of healthy controls [36]. These results support that levels of TNF-α in peripheral blood do not reflect the disease presentation. Because synovial fibroblast of hTNF-α tg mice make recipient SCID mice arthritic [39], TNF-α up-regulation may function in synovium to lead to arthritis. The levels of TNF-α in synovial fibroblasts of *Ali18* mice could be analysed in future studies.

Our results in Fig. 5B indicated that there was no difference in swelling severity and IgE levels of *Ali18*/*Ali18* mice between germ-free and SPF environment. These results suggest that bacterial flora seems not to influence the development of swelling or the increase of serum IgE level. Since the mice were transferred by Caesarean sections into the germ-free facility, the possibility of exposure to other infectious agents such as viruses or parasites should be extremely low. In addition, the mutant mice showed nearly the same phenotypes in two physically separate facilities (SPF, Helmholtz Zentrum München, Germany; germ-free, Karolinska Institutet, Sweden), suggesting that chemical factors from mouse-holding conditions do not significantly affect the strength of the phenotypes. The results indicate that the *Ali18* mutation triggers the swelling and the IgE up-regulation independently from environmental conditions.

In *Ali18*/*Ali18* peripheral blood, significantly increased levels of Igs (IgG2b, IgE, anti-DNA and RF) were detected (Fig. 3C, F, G and H, respectively). Despite the importance of these factors in autoimmune diseases such as rheumatoid arthritis, *Ali18;Rag1* double mutant mice, which lack mature B lymphocytes resulting in no Ig production, still developed arthritis phenotypes (Fig. 4). This finding suggests that the increased levels of Igs are not primary triggers but a secondary phenomenon in *Ali18* phenotypes. Thus, very distinct from *MRL*/*lpr* mice [26], the *Ali18* mutation appears to induce spontaneous inflammation directly independent of the presence of autoreactive B or T cells. Certain populations of psoriatic arthritis and rheumatoid arthritis patients lack overproduction of autoantibodies [40]. Detailed analysis of *Ali18* mice may contribute to unravel autoimmunity-independent mechanisms to cause inflammatory arthritis.

From disease transfer experiments we learned that bone marrow cells from *Ali18*/*Ali18* mice were able to transmit the inflammatory phenotype to normal wild-type recipient mice. Further, phenotypic analysis of *Ali18*;*Rag1*^{tm1Mom} compound mutant mice showed that mature B and T cells are not required for the phenotype. Other bone marrow-derived cells of the lymphocyte lineage such as NK cells, or cells derived from myeloid progenitors such as granulocytes, macrophages or mast cells may be potential candidates. In this article, we analysed the possible involvement of lymphocytes by crossing *Ali18* mice onto genetically engineered mice; the similar strategies should work for understanding the contribution of other specific cell types to the phenotype. For instance, it is known that *W*/*W*^{v} mutant mice lack mast cells completely [41], and specific monoclonal antibodies can reduce only neutrophils *in vivo* [42]. Using these experimental systems, we can further analyse as to which cell types act as the first trigger of the *Ali18* phenotypes in future studies.

Recently, the mutation of chronic multifocal osteomyelitis (*cmo*), a mouse mutant with swelling on feet and tail, was identified: an amino acid substitution in Pstpip2, also called MAYP (macrophage actin-associated tyrosine-phosphorylated protein), seems to be responsible for the inflammatory phenotypes [43]. In addition, *lupo*, an ENU-induced mouse mutant, turned out to be another amino acid substitution in Pstpip2 [44]. Gross morphological phenotypes of *lupo* are very similar to those of *cmo*. Pstpip2 is selectively expressed in macrophages [45], and macrophage-specific depletion using clodronate liposomes prevented the development of inflammation of hind paws in *lupo* mice [44]. Such liposome-mediated depletion of macrophages [46] could be used to further study the contribution of macrophages in the initiation of spontaneous inflammation in *Ali18* mice. Although many morphological similarities among *cmo*, *lupo* and *Ali18* mice

have been detected, the chromosomal location of *Ali18* on chromosome 4 [20] excludes the possibility of *Ali18* being an allele of *Pstpip2*, which is localized on chromosome 18.

In conclusion, the murine *Ali18* mutation induces spontaneous inflammatory arthritis mediated by bone marrow-derived cells independent of mature lymphocytes. Similar mechanisms may be operative in seronegative arthritis in humans. Dissecting the molecular pathways involved in the manifestation of the *Ali18* phenotype will provide new insights into such mechanisms that may be of relevance for the pathogenesis of inflammatory conditions such as seronegative arthritis in humans.

Rheumatology key messages

- The *Ali18* mutation induces inflammatory arthritis mediated by bone marrow-derived cells independent of mature lymphocytes.
- *Ali18* mice may represent a model for seronegative arthritis induced by the innate immune system.

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