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Avenues to autoimmune arthritis triggered by diverse remote inflammatory challenges

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ABSTRACT

Environmental factors contribute to development of autoimmune diseases. For instance, human autoimmune arthritis can associate with intestinal inflammation, cigarette smoking, periodontal disease, and various infections. The cellular and, molecular pathways whereby such remote challenges might precipitate arthritis or flares remain unclear. Here, we used a transfer model of self-reactive arthritis-inducing $CD4^+$ cells from KRNtg mice that, upon transfer, induce a very mild form of autoinflammatory arthritis in recipient animals. This model enabled us to identify external factors that greatly aggravated disease. We show that several distinct challenges precipitated full-blown arthritis, including intestinal inflammation through DSS-induced colitis, and bronchial stress through *Influenza* infection. Both triggers induced strong IL-17 expression primarily in self-reactive $CD4^+$ cells in lymph nodes draining the site of inflammation. Moreover, treatment of mice with IL-1β greatly exacerbated arthritis, while transfer of KRNtg $CD4^+$ cells lacking IL-1R significantly reduced disease and IL-17 expression. Thus, IL-1β enhances the autoaggressive potential of self-reactive $CD4^+$ cells, through increased Th17 differentiation, and this influences inflammatory events in the joints. We propose that diverse challenges that cause remote inflammation (lung infection or colitis, etc.) result in IL-1β-driven Th17 differentiation, and this precipitates arthritis in genetically susceptible individuals. Thus the etiology of autoimmune inflammatory arthritis likely relates to diverse triggers that converge to a common pathway involving IL-1β production and Th17 cell distribution.

1. Introduction

Autoimmune diseases represent a heterologous family of chronic, debilitating diseases with a wide spectrum of clinical symptoms. Depending on the type, they attack different types of tissue, e.g. pancreatic cells in type-1 diabetes, the thyroid glands in Hashimoto's disease or the intestine in inflammatory bowel diseases. Autoimmune inflammatory arthritis typically affects synovial tissue in the joints and leads to destruction of articular cartilage. It comprises a heterogeneous grouping of different arthritis types, including rheumatoid arthritis (RA), arthritis associated with connective tissue diseases or vasculitis, and the family of spondyloarthritis which includes sub-types such as ankylosing spondylitis, psoriatic arthritis, reactive arthritis or enteropathic arthritis

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associated with inflammatory bowel disease.

Generally, autoimmune diseases result through breakdown of immune tolerance and development of effector T cells, or autoantibody-producing B cells. Tolerance breakdown and progression to autoimmune disease is multifactorial and usually involves a complex interplay between both genetic and external environmental factors [1]. Accordingly, many autoimmune diseases are associated with HLA types, such as HLA-B27, that is classically involved in spondyloarthritis [2]. Regarding the pathogenesis of RA, genetic studies have established the importance of HLA-DR1 as well as other genes such as *PTPN22* and *TRAF1-C5*, as well as *Ccr6* which encodes a Th17 expressed chemokine receptor [2–8]. These genetic associations fit with the general notion that autoimmune inflammatory arthritis is a T cell- and antibody-driven disease, that also involves inflammatory cytokines such as TNF, IFNγ, IL-17 and IL-1β [3,9].

Less attention has been paid to defining the environmental factors that precipitate disease, or flares. For RA, strong links have been reported for bronchial stress (e.g. smoking), and infections, including periodontal disease [3]. Microbial pathogens have also been suspected as triggers for various other autoimmune diseases [10]. Mechanisms that have been suggested include molecular mimicry, epitope spreading, bystander activation and epigenetic changes [11,12].

In the case of spondyloarthritis, clinical arthritis frequently coincides with intestinal inflammation [13,14]. Intriguingly, arthritis is one of the most common extra-intestinal complications in inflammatory bowel disease (IBD) [14]. Furthermore, arthritis often occurs before gastrointestinal symptoms, and articular disease coincides with flare-ups of intestinal disease [14]. An association between intestinal inflammation and arthritic complications is also a frequent occurrence in patients suffering from microscopic colitis (MC) [15].

Despite the above associations between arthritis and either colitis, or bronchial stress/infections, there are no clear molecular pathways that could substantiate the notion that environmental challenges at remote sites trigger arthritic inflammation. Additionally, it is unclear if diverse external or environmental factors induce common or different pathways. The resolution of these questions was the focus of this study, which utilized a refined version of the well-established KRN model of autoimmune arthritis [16,17]. This refined model results in very mild arthritic symptoms, which allowed us to study the role of external factors in remote tissues, including colitis and lung infection, and the molecular mechanisms by which they triggered full-blown arthritis.

2. Materials and methods

2.1. Mice

KRNtg (KRN TCR transgenic C57BL/6) mice were obtained from D. Mathis and C. Benoist, B6.H-$2^{g7/g7}$ mice were purchased from The Jackson Laboratory, NOD/ShiLtJArc and CD45.1 congenic C57BL/6 (B6.SJL/ptprc^a) mice were obtained from the Animal Resources Centre (Perth, Australia), IL-1r$^{-/-}$ mice and Caspase-1$^{-/-}$ mice were bred by Seth Masters, NLRP3$^{-/-}$ mice were obtained from Ashley Mansell. IL-1r$^{-/-}$.KRNtg mice were generated by crossing IL-1r$^{-/-}$ with KRNtg mice. Crossing KRNtg with NOD/Lt mice generates either KRNtg-expressing arthritic K/BxN (KRNtgxNOD)F1 mice or KRNtg-negative and healthy littermates (BxN). Crossing B6.SJL/ptprc^a with NOD mice generates BxN.45.1 mice (B6.SJL/ptprc^a x NOD)F1. Crossing B6.H-$2^{g7/g7}$ with Caspase-1$^{-/-}$ or NLRP3$^{-/-}$ mice generates Caspase-1$^{-/-}$.$A^{g7+/-}$ or NLRP3$^{-/-}$.$A^{g7+/-}$ mice, crossing B6.H-$2^{g7/g7}$ with C57BL/6 generates WT $A^{g7+/-}$ mice. When indicated some of the strains were further crossed with B6.SJL/ptprc^a mice to generate CD45.1 or CD45.1.2 congenic mice. Genotypes were assessed by genomic PCR or FACS. Both male and female mice were used for experiments at an age of 8–12 weeks. To the greatest possible extent, groups consisted of equal numbers of male and female animals. The work described was carried out in accordance with the EU directive 2010/63/EU for animal experiments. Experiments were approved by the Garvan-St. Vincent's and the Monash Animal Ethics Committees.

2.2. Adoptive transfer experiments and treatment of mice

Cell suspensions were prepared from pooled spleens and lymph nodes (LN) and ≈2.5 × 10^6 cells (≈2.5 × 10^5 CD4$^+$) were injected i.v. into recipient mice which received 2.5 Gy irradiation prior to transfer of KRNtg cells, to enhance engraftment of transferred cells [18].

DSS feeding: for induction of colitis, mice were given 3% DSS (dextran sulphate sodium) in drinking water ad libitum over a period of 7 days (day −8 till day −1 before transfer of KRNtg cells at day 0). Control mice were given autoclaved tap water.

Influenza infection: For induction of respiratory *Influenza* infection, mice were infected i.n. with 1 × 10^5 PFU of ×31 in 50 μl PBS or mock-infected with 50 μl PBS alone.

Intra-tracheal (I.t.) LPS Instillation: Mice were anesthetized with i.p. ketamine/xylazine (80–100 mg/kg and 10 mg/kg respectively) before exposure of the trachea. LPS (3.75 μg/g in 30–50 μl saline) was then placed in the top of the catheter and i.t. breathed in naturally. I.t. instillation of PBS was used as control.

I.t. Infection with Klebsiella pneumoniae: Mice were anesthetized with i.p. ketamine/xylazine (80–100 mg/kg and 10 mg/kg respectively) before exposure of the trachea. *Klebsiella pneumoniae* (ATCC strain 27736, 10^7 PFU) in 30–50 μl PBS was then placed in the top of the catheter and i.t. breathed in naturally. I.t. instillation of PBS was used as control.

*Injection of recombinant mouse IL-1β (*Peprotech*) or IL-17* (Peprotech): 100 ng of recombinant mIL-17 or mIL-1β in 100 μl PBS were applied subcutaneously in the flank, PBS was used as a control. To examine arthritis development, recombinant mIL-1β or mIL-17 were applied on days 0, 1, 2, 4 and 6. To determine IL-17 expression, recombinant mIL-1β was applied on day 0, 1, 2 and 3 before harvest of draining LN at day 3.5.

2.3. Arthritis scoring

Measurement of arthritis development was done as previously described [17,19]. Briefly, clinical severity of arthritis was assessed every 1–2 days for all 4 paws on a scale from 0 to 3 and indicated as cumulative score. 0: normal; 1: erythema, swelling limited to individual digits or mild ankle swelling insufficient to reverse the normal V shape of the foot; 2: swelling sufficient to make the ankle and midfoot approximate in thickness to the forefoot; 3: reversal of the normal V shape of the foot, swelling of the entire paw including multiple digits. Arthritis scores were evaluated by 2 independent observers.

2.4. Flow cytometry

Cells were collected from crushed spleen, colon-draining mesenteric, lung-draining mediastinal or flank-draining inguinal LN. Intra-cellular cytokine expression was detected after re-stimulation of cells with 50 ng/ml PMA and 1 μg/ml ionomycin (Sigma-Aldrich) in the presence of brefeldin A and monensin (ebiosciences). Cells were analysed with BD LSRII and FACSCantoII. The following antibodies were used: anti-CD4 PE-Cy7 (Biolegend, #100422), anti-CD45.2 V450 (BD, #560697), anti-CD45.1 APC-

efluor 780 (ebiosciences, #47-0453-82), anti-IL-17 PE (BD, #559502), anti-CD44 FITC (BD, #553133), anti-CCR6 PE (R&D #FAB590P).

2.5. Anti-GPI IgG1 ELISA

Serum samples were plated at an initial dilution of 1:10 and diluted serially 1:2 in 384-well high-binding multiwall plates (Corning) that were coated over night with GPI (5 μg/ml, Sigma). Goat-anti-mouse IgG1-HRP was used as secondary antibody. TMB substrate solution (BD) was used for detection.

2.6. Real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and the QuantiTect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis according to the manufacturer's instructions. Quantitative real-time PCR was performed using an ABI Prism 7900HT Real-Time PCR system (Applied Biosystems). For each sample, mRNA abundance was normalized to the amount of the housekeeping gene *Gapdh* and results expressed as arbitrary units.

2.7. IL-1β protein measurement

Samples (lung and colon tissue) were homogenized, proteins extracted and IL-1β concentrations determined according to established protocols (Mouse IL-1β Singleplex Bead Kit, Life Technologies, #LMC0011).

2.8. Statistics

Statistical significance was determined by calculating *p-values* using an unpaired *t-test* on Instat software (GraphPad Software, San Diego, CA). All data are means ± SD and representative of at least two independent experiments. The *p-values* $\leq$0.05 were considered significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

3. Results

3.1. DSS-induced colitis and respiratory Influenza viral infection significantly exacerbate inflammatory arthritis in a KRNtg CD4$^+$ T cell transfer model

Human inflammatory autoimmune arthritis comprises a heterogeneous grouping of different arthritis types. Although there are many similarities, none of these is exactly represented by one of the numerous murine arthritis models. Given the key role of self-reactive T cells, MHC-class II-association and autoantibody production, a modified version of the well-established KRN mouse model of inflammatory arthritis [16,17] allowed us to examine the impact of remote inflammation on induction or severity of autoimmune arthritis. Disease development in this model relies on KRNtg CD4$^+$ cells that recognize their autoantigen glucose-6 phosphate isomerase (GPI) in the presence of the MHC class II molecule A^{g7}. Primed KRNtg CD4$^+$ cells then drive the expansion of GPI-specific B cells and production of GPI-specific IgG1 that precipitates disease in the joints [20]. Standard K/BxN mice – (KRNtgxNOD)F1 – are characterized by expression of the KRNtg on most CD4$^+$ cells, and this results in an acute model of full blown arthritis by 5 weeks of age (Fig. 1A). Contrary to that, transfer of 2.5×10^5 KRNtg CD4$^+$ cells into A$^{g7+/-}$ BxN.45.1 recipients – which yields a relatively low percentage of autoreactive T cells in blood and therefore more closely resembles human autoimmune arthritis – induced much less aggressive arthritis. This allowed exacerbating influences to be studied (Fig. 1B) [16,17].

In view of the suspected influence of intestinal inflammation or respiratory stress on human arthritis [3], we focused on such influences in our model. To test the impact of colonic injury/inflammation, BxN.45.1 recipients were treated with DSS in drinking water prior to transfer of KRNtg CD4$^+$. Respiratory stress/infection was induced on the day of cell transfer by i.n. treatment with *Influenza* virus, or i.t. with LPS or *Klebsiella pneumoniae* instillation. All inflammatory stimuli markedly increased arthritic symptoms compared to non-treated control mice (Fig. 1B).

Since arthritis exacerbation was most marked following DSS-induced colitis or *Influenza* infection (Fig. 1B), these two models were chosen for further exploration of possible underlying mechanisms. For LPS and *Klebsiella pneumoniae*-treated animals, statistical significance was reached at certain time-points, however variability between individual animals was more pronounced (Fig. 1B). This may be due to the procedure, as i.t. PBS instillation by surgical exposure of the trachea slightly aggravated arthritis while i.n. PBS had no influence (Fig. 1C). Altogether, we developed a model of autoimmune arthritis to mimic the human clinical disease, to further examine the contribution of intestinal inflammation and respiratory stress in arthritis pathogenesis in a susceptible host.

3.2. Colitis and Influenza lung infection induce IL-17 expression in KRNtg CD4$^+$ cells

We reasoned that peripheral, remote immune mechanisms somehow influence disease processes in the joint. As Th17 cells are central players in RA pathogenesis [21,22], we investigated if colitis or *Influenza* infection had induced increased Th17 differentiation. Mice were treated as described above and spleen and draining LNs (mesenteric LN for DSS-induced colitis and mediastinal LN for *Influenza*-infected animals) collected at 3–4 days post transfer of KRNtg CD4$^+$ cells. As determined by CD44 expression, a stringent activation of adoptively transferred KRNtg CD4$^+$ was observed both in spleen and draining LNs and was independent of the applied immunological challenges (Fig. 2A). In contrast, significant IL-17 up-regulation was detected in adoptively transferred self-reactive CD4$^+$ cells in draining LNs of mice treated with DSS or *Influenza* compared to control mice (Fig. 2B, C). Additionally, IL-17 expression in transferred KRNtg CD4$^+$ cells of challenged mice was much less pronounced in spleen compared to draining LNs, and IL-17 expression in endogenous CD4$^+$ cells was only marginal compared to autoreactive KRNtg CD4$^+$ cells (Fig. 2B, C).

The significant up-regulation of IL-17 by autoreactive CD4$^+$ upon challenge with *Influenza* or DSS enhanced their arthritogenic potential. This may occur through a) joint infiltration with aggravation of synovial inflammation [21] or b) enhancement of autoantibody production through IL-17-mediated effects on B cells [22]. The latter seems improbable as anti-GPI IgG1 levels were not boosted upon challenge with DSS or *Influenza* (Fig. 2D). This implies that the disease triggering effects of self-reactive IL-17$^+$ KRNtg CD4$^+$ cells are most probably mediated through direct inflammatory effects in the joints. Unfortunately and as in previous studies [21], it was technically impossible to show local enrichment of transferred IL-17$^+$ CD4$^+$ to the joints. As KRNtg IL-17$^+$ CD4$^+$ cells prevailed in LNs draining the site of inflammation, and were only minimally increased in spleen (Fig. 2B, C), we propose that priming and Th17 differentiation may take place at local LNs draining the site where the challenge occurred. As transferred IL-17$^+$ KRNtg CD4$^+$ T cells expressed increased levels of CCR6 (Fig. 2E) we speculate this may then drive their migration to the joints, enriched in the chemokine ligand CCL20 [23]. Interestingly, transferred KRNtg CD4$^+$ T cells isolated from mesenteric LN of DSS-treated BxN.45.1 mice expressed higher CCR6 levels than KRNtg CD4$^+$ T cells isolated

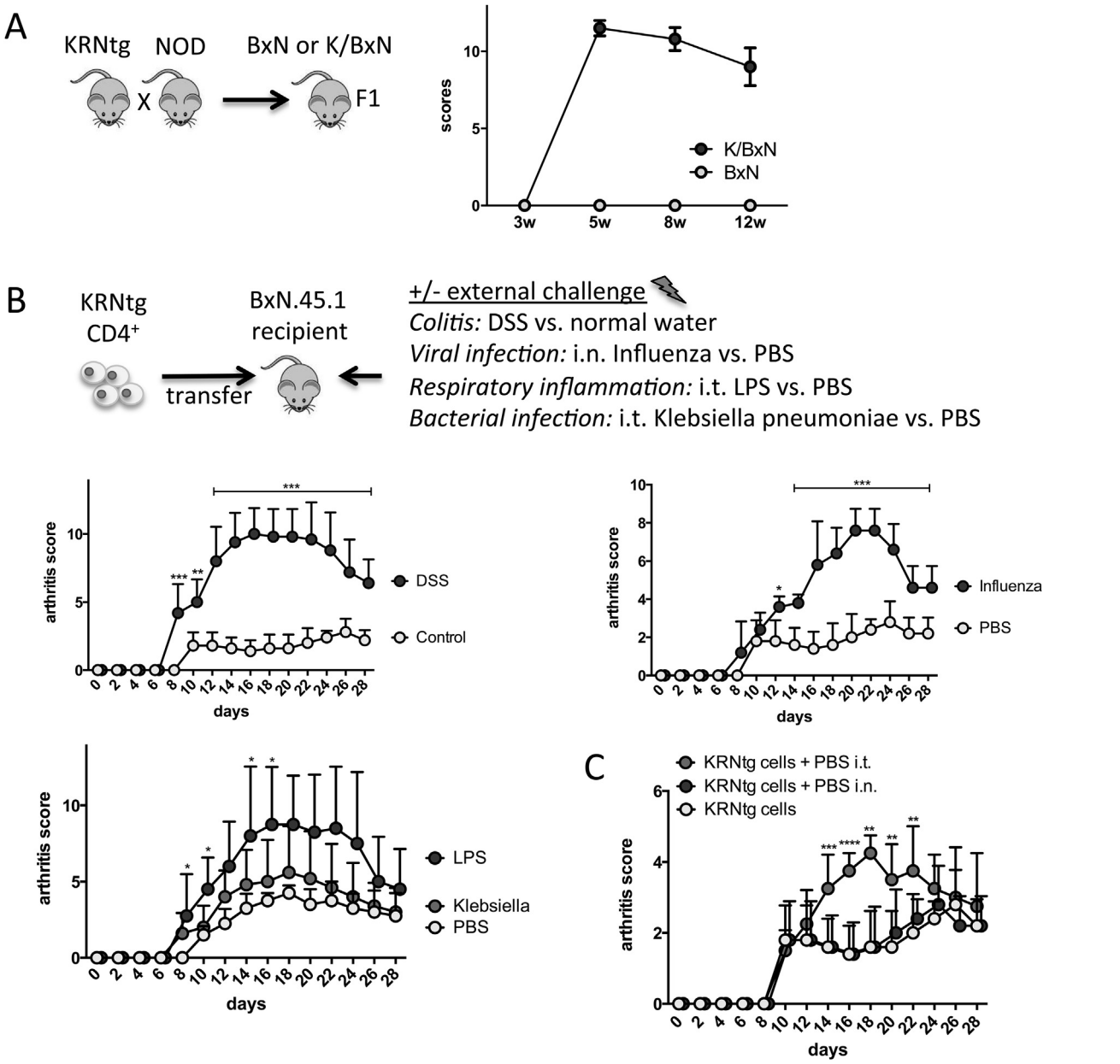

Fig. 1. Distinct challenge factors aggravate arthritic symptoms. (A) Arthritis scores of 3w, 5w, 8w and 12w old K/BxN mice and KRNtg-negative littermates (BxN), generated by crossing KRNtg and NOD mice (n > 5 mice/group). (B) Arthritis scores of BxN.45.1 recipients transferred with KRNtg cells. Where indicated mice were treated i.n. with *Influenza* versus PBS, DSS-containing versus normal drinking water, i.t. with *Klebsiella pneumoniae*, LPS or PBS (n = 4–5 mice/group; experiments were repeated independently at least twice). *P-values* were determined by *t-test*. (C) Arthritis scores of BxN.45.1 recipients transferred with KRNtg cells. Where indicated mice were treated i.t. or i.n. with PBS or they were left untreated (n = 4–5 mice/group) (n = 4–5 mice/group; experiments were repeated independently twice). *P-values* were determined by *t-test*.

from mediastinal LN of *Influenza*-treated BxN.45.1 mice (Fig. 2E). Whether CCR6 expression levels could have influenced the more severe disease course in DSS- compared to *Influenza*-treated animals (Fig. 1B) remains a possibility.

3.3. Inflammation/infection-induced Th17 differentiation of autoreactive KRNtg CD4$^+$ cells is triggered by IL-1β secretion

We next examined the pathways that led to IL-17 expressing autoreactive CD4$^+$ cells induced by external challenge factors. Past studies showed that IL-6 and IL-23 were not critical for disease in the murine K/BxN serum transfer model of arthritis [21]. Therefore, we hypothesized that IL-1β may play a central role, as this cytokine is required for early Th17 differentiation, by regulating expression of IRF4 and RORγt [24]. A significant increase in *IL-1β* gene transcription was observed 3 days post transfer of KRNtg cells (Fig. 3A) in lung and colon of *Influenza*- and DSS-treated animals, respectively. By means of bead-based immunoassays we were able to quantify cytokine concentrations in colon and lung tissues and also found increased IL-1β levels in DSS- and *Influenza*-treated animals (Fig. 3A). In accordance with that, IL-1β concentrations were also elevated in bronchoalveolar lavage fluid of *Influenza*-treated animals (data not shown). Detection of IL-1β in lung or colon tissues by ELISA was unsuccessful, possibly due to the low concentrations (data not shown). To further evaluate if IL-1β represents one key factor for the increased Th17 differentiation of KRNtg CD4$^+$ upon

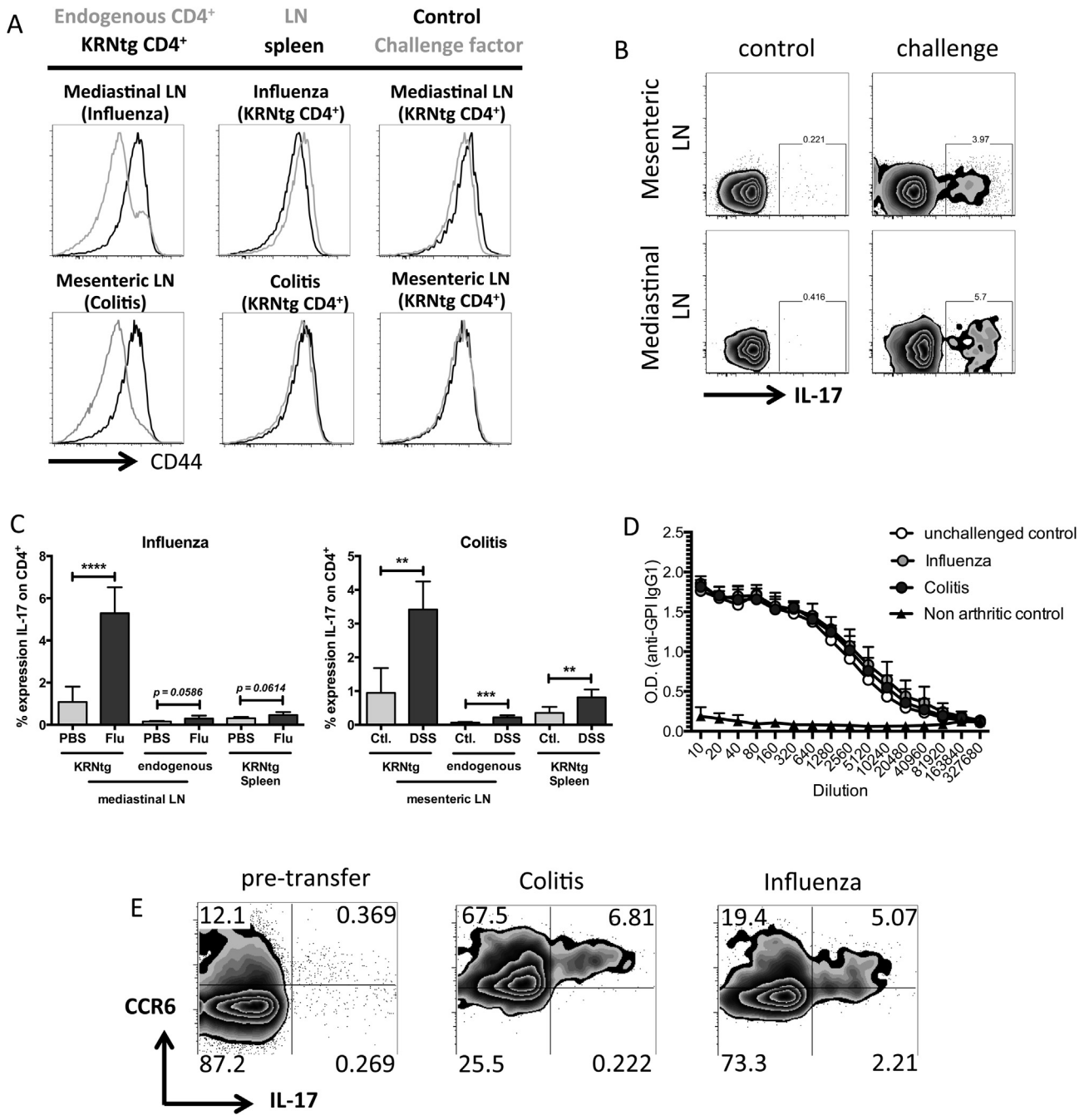

Fig. 2. CD4$^+$ activation and expression of inflammatory IL-17 in the presence of external challenge factors. (A–C) KRNtg cells were transferred into BxN.45.1 recipient animals treated i.n. with *Influenza* versus PBS (control) or with DSS-containing versus normal drinking water (control/Ctl.). 3.5 days after transfer, draining LNs (mediastinal and mesenteric LNs, respectively) and spleen were harvested and expression of CD44 (A) and IL-17 (B, C) determined for adoptively transferred CD45.2$^+$ KRNtg CD4$^+$ and endogenous CD45.2$^-$ CD4$^+$ cells (n = 4–5 mice/group, experiments were repeated independently at least twice). *P-values* were determined by *t-test*. (D) Anti-GPI IgG1 levels as determined in BxN.45.1 recipients, 28 days after transfer with KRNtg cells. Mice were treated with DSS or *Influenza* or left unchallenged. Depicted are also serum levels of non-arthritic BxN.45.1 mice that did not receive KRNtg cells (n > 5 mice/group, experiments were repeated independently at least twice). *P-values* were determined by *t-test*. (E) KRNtg cells were transferred into BxN.45.1 recipient animals treated i.n. with *Influenza* infection or with DSS-containing drinking water to induce colitis. Expression of CCR6 and IL-17 was determined on transferred KRNtg CD4$^+$ cells before and 3.5 days after transfer and isolation from draining LNs (mediastinal and mesenteric LNs, respectively) by FACS (n = 4–5 mice/group, experiments were repeated independently twice). *P-values* were determined by *t-test*.

challenge, KRNtg mice were crossed to IL-1r$^{-/-}$ mice. When CD45.2$^+$ IL-1r$^{-/-}$.KRNtg and CD45.1.2$^+$ KRNtg cells were co-transferred into *Influenza*- or DSS-treated CD45.1$^+$ BxN.45.1 recipients, IL-17 expression declined significantly in transferred IL-1r$^{-/-}$.KRNtg compared to KRNtg CD4$^+$ cells (Fig. 3B). Consistent with this, when animals were treated with IL-1β cytokine, IL-17 was significantly up-regulated in KRNtg, but not IL-1r$^{-/-}$.KRNtg CD4$^+$ cells in draining LNs (Fig. 3B). We transferred IL-1r$^{-/-}$.KRNtg cells into animals treated with DSS, *Influenza* or recombinant IL-1β, and found that arthritis severity decreased considerably, compared to transfer of KRNtg CD4$^+$ cells (Fig. 3C). We therefore conclude that distinct challenge factors triggered arthritic symptoms by

increasing IL-1β-mediated Th17 differentiation of self-reactive KRNtg CD4$^+$ cells, and this enhanced their potential to induce joint inflammation. The probable and particular role of IL-17 produced locally by joint infiltrating self-reactive CD4$^+$ T cells is further supported by the fact that systemic injection of rIL-17 only slightly increased disease severity (Fig. 3D). However, disease severity was still enhanced in DSS-, *Influenza*- or IL-1β-treated animals transferred with IL-1r$^{-/-}$.KRNtg cells compared to untreated animals transferred with KRNtg or IL-1r$^{-/-}$.KRNtg cells (Fig. 3C). This suggests that in addition to inducing a Th17 phenotype, IL-1β may exert additional inflammatory effects, which is unsurprising as IL-1β is a cytokine with wide-ranging pro-inflammatory effects. However this could also indicate that other factors besides IL-1β could play a role, e.g. other inflammatory cytokines such as TNF and IFNγ, not addressed in this work [3,9].

3.4. Inflammation/infection-induced Th17 differentiation of autoreactive KRNtg CD4$^+$ cells and boost of arthritis are independent on Caspase-1/NLRP3 signaling

A common pathway for the production of active IL-1β is through NLRP3 inflammasome activation, which involves Caspase-1-mediated cleavage of pro-IL-1β [25]. The rationale behind this was that NLRP3 is activated by an exceptionally wide range of pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs). Also, NLRP3 mRNA levels were increased in RA synovia [26] and a NLRP3 variant allele has been associated with a lower treatment response, particularly in the RA risk group of smokers [27]. Moreover, NLRP3 may play a key role in host defense against *Influenza* infections through viral RNA sensing [28], as well as initiation of DSS-induced colitis [29]. Accordingly, we found increased NLRP3 levels in colon and lung of DSS- and *Influenza*-treated animals (Fig. 4A). However, transfer of KRNtg cells into Caspase-1$^{-/-}$.A$^{g7+/-}$ or NLRP3$^{-/-}$.A$^{g7+/-}$ mice resulted in no improvement of arthritis (Fig. 4B) nor reduced IL-17 expression by transferred self-reactive cells (Fig. 4C). Hence, our data support previous studies showing no influence of Caspase-1/NLRP3 signaling on robustness of murine arthritis [30] even though NLRP3 expression was induced.

4. Discussion

Our study identifies the IL-1β-Th17 axis as an important convergence pathway whereby diverse environmental triggers induce autoimmune inflammatory arthritis. We show that both intestinal and bronchial inflammation induced IL-1β-mediated IL-17 expression in self-reactive CD4$^+$. We suggest that IL-17 expression enhances the autoaggressive potential of self-reactive CD4$^+$ to induce inflammation in the joints, where they may migrate to after being primed in lymphoid organs draining inflammatory sites provoked by environmental challenges. This demonstrates that protective inflammatory reactions can also enhance the potential of autoreactive cells to boost joint inflammation.

In search of common downstream mechanisms for IL-1β production relevant to arthritis induction, we examined Caspase-1/NLRP3 activation. Interestingly, we could demonstrate that this pathway did not impact on inflammation-induced Th17 differentiation of autoreactive KRNtg CD4$^+$ cells, nor influence arthritis in our models. Our data support previous studies showing no influence of Caspase-1 or NLRP3 signaling on IL-1β production and arthritis severity in the K/BxN serum transfer model [30], the Ag-induced arthritis (AIA) [31] as well as the CIA model [32]. We therefore speculate that other enzymes are likely to be responsible for the cleavage and release of IL-1β in its active form. These may include neutrophil or mast cell-derived serine proteases (elastase, chymase), which appear to be particularly relevant for disease in the K/BxN model [30,33–36]. Both colitis and *Influenza* infection can lead to excessive neutrophil recruitment [37]. Hence, IL-1β cleavage may occur through neutrophil-associated proteases like PR3 and not Caspase-1. It is also possible that other Caspases are required, i.e. Caspase-8, that may induce non-canonical IL-1β maturation [38]. Caspase-8 can be activated by increased Fas signaling when macrophages are exposed to TLR-ligands, e.g. during inflammatory processes [38]. In support of this, only combined Caspase-1 and Caspase-8 deletion could protect against IL-1β-dependent osteomyelitis [39]. In view of the various contributing cell types and redundant and complex mechanisms, it is understandable that to date targeting of downstream mechanisms has not been as successful as IL-1R antagonism for the treatment of arthritis [30,37].

IL-1β has been identified as a central cytokine to human and murine arthritis pathogenesis [40,41]. Moreover, variation in IL-1β gene expression is a major determinant of genetic susceptibility in K/BxN serum-mediated arthritis in mice [42]. By means of a transfer strategy using IL-1R-deficient cells, we identify IL-1β as key factor for the induction of a Th17 phenotype in self-reactive CD4$^+$ T cells that significantly increases their autoaggressive potential. In addition, our results suggest that IL-1β exerts further direct arthritogenic effects, which accords with the complete abrogation of arthritic symptoms in IL-1r$^{-/-}$ mice upon K/BxN serum transfer [43] and the ability of IL-1β to exert direct inflammatory effects on the joints [44].

Like IL-1β, the cytokine IL-17 has marked pro-inflammatory properties [45]. Although IL-17 was not generally mandatory for arthritis development [46,47], it was shown to drive joint inflammation, and in many studies, IL-17 correlated with disease severity [48,49]. Failure to boost arthritis severity through transfer of IL-17^{low} IL-1r$^{-/-}$.KRNtg CD4$^+$ cells or through systemic IL-17 application, shows that the IL-17-mediated boost of arthritis was a particular feature of self-reactive CD4$^+$ T cells. This is in accordance with previous studies [21] showing that enhanced arthritic symptoms in the K/BxN serum transfer model are specifically mediated by the effects of self-reactive IL-17$^+$ KRNtg CD4$^+$ T cells, and this could be reversed by anti-IL-17 treatment.

The role of Th17 in inflammatory arthritis is multifaceted. The effects of IL-17 on germinal centre formation and autoantibody production [22,46,50] could not be confirmed by our study. We rather suggest that arthritis aggravation by self-reactive IL-17$^+$ KRNtg CD4$^+$ cells is mediated through direct inflammatory effects in the joints. Unfortunately we were unable to directly prove this hypothesis due to technical limitations to recover a sufficient number of transferred cells. Such a concept is however supported by our own results using a transfer strategy of IL-1r$^{-/-}$.KRNtg cells and studies showing high levels of IL-17 producing CD4$^+$ cells in RA synovial membranes [51,52] as well as the paws of arthritic K/BxN mice [21]. Also, IL-17 blockade had no effect on K/BxN serum transfer arthritis in the absence of transferred KRNtg CD4$^+$ T cells [21]. That IL-17 must be present locally to promote inflammatory responses is further supported by studies showing that intra-articular IL-17 application augments disease [53] while systemic IL-17 administration in our studies had hardly any effect.

Still unresolved is the question – at which site(s) does T cell priming against self-antigen occur? Given the observed preferential redistribution of IL-17$^+$ self-reactive KRNtg CD4$^+$ cells to lymphoid organs draining the site where the inflammatory challenge occurred, these most likely represent the site of priming and differentiation, from where IL-17$^+$ KRNtg CD4$^+$ cells may then migrate to the joints. This concept is supported by studies in individuals with psoriatic and rheumatoid arthritis, where compared to osteoarthritis, increased frequencies of IL-17$^+$ CD4$^+$ cells in the

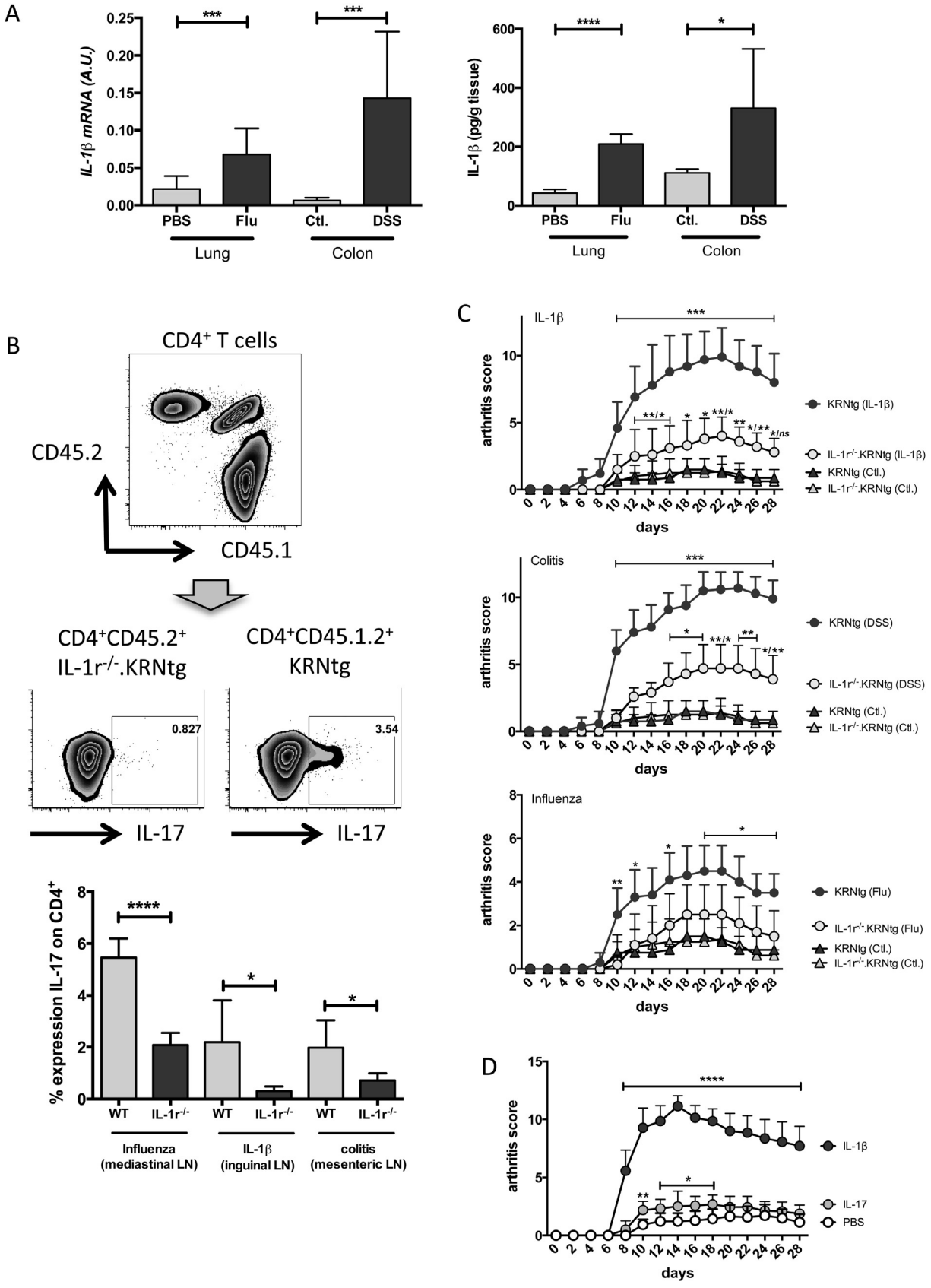

Fig. 3. Environmental challenge factors trigger arthritis through IL-1β-mediated Th17 differentiation of autoreactive KRNtg CD4$^+$ cells. (A) KRNtg cells were transferred into BxN.45.1 recipient animals treated i.n. with *Influenza* versus PBS or with DSS-containing versus normal drinking water (Ctl.). 3.5 days after transfer, colon and lung were examined for *IL-1β* mRNA expression by RT-PCR and IL-1β protein levels by means of bead-based immunoassays. (B) CD45.1.2$^+$ KRNtg and CD45.2$^+$ IL-1r$^{-/-}$.KRNtg cells were co-transferred into CD45.1$^+$ BxN.45.1 recipients treated with DSS, *Influenza* or IL-1β (100 ng). IL-17 expression was determined 3.5 days later on transferred CD4$^+$ cells in draining LNs (mesenteric,

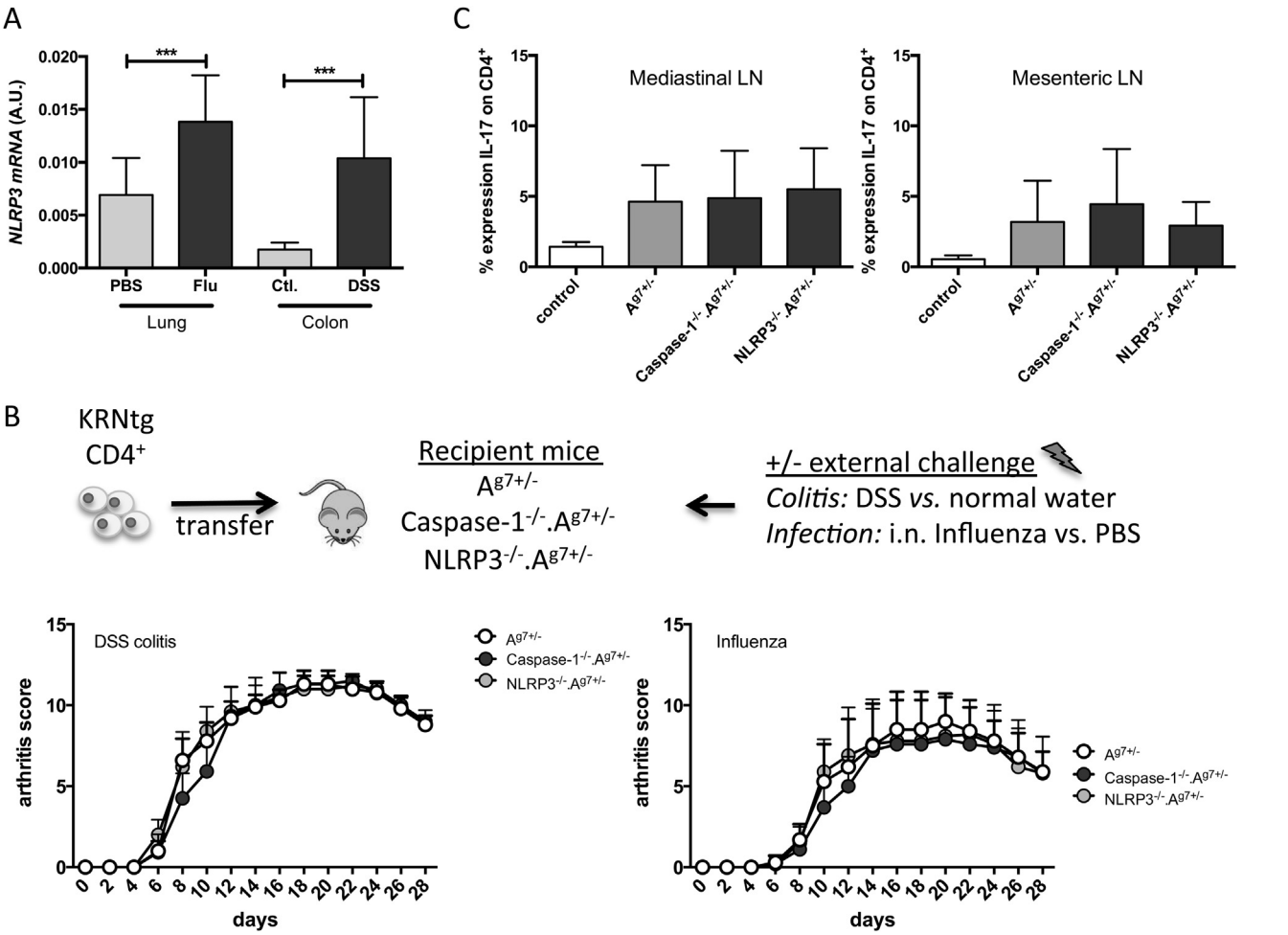

Fig. 4. Caspase-1 and NLRP3 neither affect arthritis severity nor Th17 differentiation of KRNtg CD4 T cells. (A) KRNtg cells were transferred into BxN.45.1 recipient animals that were treated i.n. with *Influenza* versus PBS or with DSS containing versus normal drinking water (Ctl.). 3.5 days after transfer, colon and lung were examined for *NLRP3* mRNA levels by quantitative RT PCR. (B + C) KRNtg cells were transferred into WT $A^{g7+/-}$, Caspase-$1^{-/-}$.$A^{g7+/-}$ and $NLRP3^{-/-}$.$A^{g7+/-}$ recipient animals that were treated with *Influenza* or DSS. (B) Arthritis scores were measured over a period of 28 days. (C) IL-17 expression was determined 3.5 days after transfer in draining LN (mesenteric LN and mediastinal LN, respectively) on adoptively transferred cells. Depicted is also the IL-17 expression on untreated controls (control). N = 4–5 mice/group, experiments were repeated independently at least twice. *P-values* were determined by *t-test*.

joints were associated with a significantly higher Th17:Th1 ratio in the synovial fluid, compared to peripheral blood [51]. What exactly drives these cells to the inflamed joints, and whether Th17 differentiation can also occur within inflamed joints, will need to be addressed in future studies. A role has been attributed to the chemokine receptor CCR6 which is highly expressed on synovial Th17 cells and found to be up-regulated in IL-17^{+} KRNtg CD4 T cells in our studies [51]. This may be indicative of selective migration of Th17 cells to the joints, which are enriched in the CCR6 chemokine ligand, CCL20 [23].

Having identified the IL-1β-Th17 axis as an important link between intestinal and bronchial inflammation and autoinflammatory arthritis, the question arises if further environmental factors might also use this convergence pathway. Moreover, there may be an important role for the composition of the gut microbiota, which influences $CD4^{+}$ T cell differentiation [54–57]. For K/BxN arthritis, the composition of gut-resident bacteria may influence arthritic symptoms through regulation of Th17 or T follicular helper cells [22,47]. In addition to arthritis, commensal microbes and Th17 cells impact on various other autoimmune disorders [48,49,58–60]. It is likely that the inflammation-induced IL-1β – Th17 axis has a pathogenic role beyond autoimmune inflammatory arthritis.

5. Conclusion

Our study reveals the IL-1β-Th17 axis as a mechanism linking disparate external challenges to exacerbation of autoimmune arthritis (Fig. 5). We show that excessive triggering of innate and adaptive immunity, designed to protect the organism from

mediastinal or inguinal LNs, respectively). (C) Arthritis scores of BxN.45.1 mice transferred with KRNtg or IL-$1r^{-/-}$.KRNtg cells and treated with DSS, *Influenza* or IL-1β (100 ng). Depicted are also scores of unchallenged (Ctl.) BxN.45.1 animals transferred with KRNtg or IL-$1r^{-/-}$.KRNtg cells. Indicated are the statistical differences between challenged BxN.45.1 mice transferred with KRNtg vs. IL-$1r^{-/-}$.KRNtg cells (top) as well as between challenged BxN.45.1 mice transferred with IL-$1r^{-/-}$.KRNtg cells and unchallenged BxN.45.1 mice transferred with KRNtg/IL-$1r^{-/-}$.KRNtg cells (below). (D) Arthritis scores of BxN.45.1 mice transferred with KRNtg cells and treated with IL-1β (100 ng) or IL-17 (100 ng) versus PBS. N = 4–5 mice/group, experiments were repeated independently at least twice. *P-values* were determined by *t-test*.

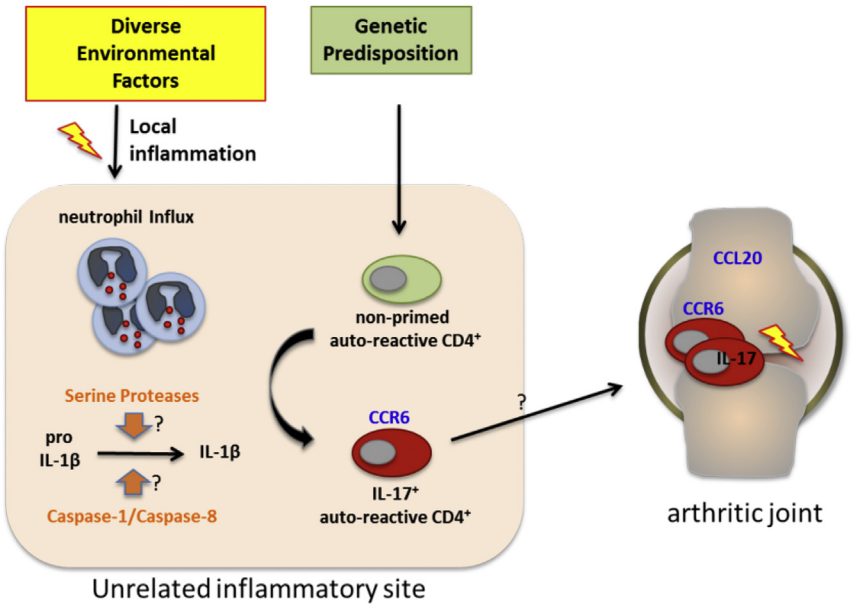

Fig. 5. Hypothetical model. Development of autoimmunity involves a complex interplay between genetics and environmental factors. Diverse environmental challenges (e.g. infections, intestinal inflammation, bronchial stress etc.) can provoke inflammatory responses distinct from the joints. This can result in locally increased IL-1β levels what induces IL-17 expression in autoreactive CD4 T cells in genetically susceptible individuals and increases their autoreactive potential. As IL-17$^+$ autoreactive CD4 T cells express high levels of CCR6 we speculate this may drive their migration to the joints, enriched in the chemokine ligand CCL20. Joint-infiltrating IL-17$^+$ autoreactive CD4 T cells may then aggravate synovial inflammation and increase arthritic symptoms. We did not observe any influence of Caspase-1/NLRP3 signaling on locally increased IL-1β production and arthritis severity. We therefore speculate that serine proteases (e.g. cathepsin G, neutrophil elastase or proteinase 3), secreted by locally infiltrating neutrophils, may be able to trigger Caspase-1-/NLRP3-independent IL-1β activation. Alternatively, Caspase-8 (possibly in concert with Caspase-1) may induce non-canonical IL-1β maturation.

pathogens, can potentiate the priming milieu allowing self-reactive cells to enhance their autoaggressive potential. Circulating autoreactive T cells appear to be a feature of normal healthy individuals [61]. Excessive IL-1β/Th17 production following infections or damage in different tissues may tip the balance in genetically susceptible individuals, over-ride regulatory control, and precipitate autoimmunity. A similar phenomenon may explain flares that are observed in arthritis patients. The cause of autoimmune arthritis in humans likely rests with multiple environmental factors, however IL-1β/Th17 represents an important convergence pathway whereby diverse environmental challenges, on top of genetics, precipitates disease.

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