

Local microbleeding facilitates IL-6- and IL-17-dependent arthritis in the absence of tissue antigen recognition by activated T cells

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Cognate antigen recognition by CD4⁺ T cells is thought to contribute to the tissue specificity of various autoimmune diseases, particularly those associated with class II MHC alleles. However, we show that localized class II MHC-dependent arthritis in F759 mice depends on local events that result in the accumulation of activated CD4⁺ T cells in the absence of cognate antigen recognition. In this model, transfer of in vitro polarized Th17 cells combined with the induction of experimental microbleeding resulted in CCL20 production, the accumulation of T cells in the joints, and local production of IL-6. Disease induction required IL-17A production by transferred T cells, IL-6 and CCL20 expression, and STAT3 signaling in type I collagen-expressing cells. Our data suggest a model in which the development of autoimmune disease in F759 mice depends on four events: CD4⁺ T cell activation regardless of antigen specificity, local events that induce T cell accumulation, enhanced sensitivity to T cell-derived cytokines in the tissue, and activation of IL-6 signaling in the tissue. This model provides a possible explanation for why tissue-specific antigens recognized by activated CD4⁺ T cells have not been identified in many autoimmune diseases, especially those associated with class II MHC molecules.

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Abbreviations used: EAE, experimental autoimmune encephalomyelitis; RA, rheumatoid arthritis.

Autoimmune diseases are caused by multiple genetic and/or environmental factors and are characterized by immune hyperactivity (Marrack et al., 2001; Mathis and Benoist, 2004). Certain autoimmune diseases are thought to develop in specific tissues as a result of cognate antigen recognition by CD4⁺ T cells, particularly when these diseases are associated with class II MHC alleles, as is the case with rheumatoid arthritis (RA; Steinman, 2001; Zhang et al., 2008; Imboden, 2009). Consistent with this, joint-specific antigenic peptides have been identified in humans (derivatives of aggrecan, fibrillin, collagen, etc.; Polgár et al., 2003; Chapuy-Regaud et al., 2005; Elsaid and Chichester, 2006;

Takizawa et al., 2006; Van Steendam et al., 2010). Furthermore, immunodominant MHC class II peptides in a collagen-induced arthritis model have been found to match those seen in human RA (Andersson et al., 2010), although whether these peptides are a result rather than a cause of joint damage is unclear. Therefore, the mechanisms that determine tolerance to self-antigens are central to understanding the pathogenesis of autoimmune diseases. Despite the evidence for antigen-specific T cell activation in these diseases, tissue-specific self- or nonself-antigens recognized by activated CD4⁺

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T cells in many class II MHC-associated diseases have not been well established (Mocci et al., 2000; Skapenko et al., 2005). This raises the possibility that a breakdown in CD4⁺ T cell tolerance for a tissue-specific antigen is not always crucial for the development of autoimmune diseases. Instead, CD4⁺ T cell activation may be the consequence of events initiated by inflammation triggered by certain genetic and/or environmental factors in the affected tissues (Hirano, 1998; Matsumoto et al., 1999; Marrack et al., 2001). In these cases, direct and/or indirect cytokine production rather than cognate antigen recognition by activated CD4⁺ T cells may be critical for the resulting tissue specificity. In fact, various subsets of effector CD4⁺ T cells—for example, Th1 cells, Th2 cells, and Th17 cells, which express high levels of IFN- γ , IL-4, and IL-17A, respectively (Mosmann and Coffman, 1989; Glimcher and Murphy, 2000; Cua et al., 2003; Harrington et al., 2005; Park et al., 2005; Veldhoen et al., 2006; Zhu et al., 2006; Bettelli et al., 2007)—may initiate and drive the progression of disease even though cytokine production by T cells is dependent on TCR cross-linking.

Additionally, there exists an age-dependent increase in memory/activated phenotype CD4⁺ T cells resulting from homeostatic proliferation mediated by a reduction in T cell input from the thymus (Surh and Sprent, 2000). Moreover, it has been reported that an age-dependent reduction in naive CD4⁺ T cells in the periphery increases the likelihood of (a) weak interactions between TCRs and peptides presented by self-class II MHC molecules, which include autoantigenic peptides involved in the positive selection, and (b) cytokine consumption per CD4⁺ T cell, including the T cell survival factor IL-7 (Surh and Sprent, 2000). Furthermore, we and others have shown that homeostatic proliferation of CD4⁺ T cells is involved in the development of diabetes, arthritis, and Omenn syndrome (King et al., 2004; Jang et al., 2006; Sawa et al., 2006; Khiong et al., 2007). This process is also associated with a specific cytokine profile that includes IL-17A and IFN- γ from homeostatic proliferating CD4⁺ T cells (Gudmundsdottir and Turka, 2001; Khiong et al., 2007; Nishihara et al., 2007).

Of special interest is IL-6, which several studies have suggested has an important role in autoimmune diseases (Hirano, 1998, 2010; O'Shea et al., 2002; Sakaguchi and Sakaguchi, 2005; Awasthi and Kuchroo, 2009). The F759 knockin mouse line (F759), for example, which expresses a mutant variant of the IL-6 signaling transducer gp130 where the 759th tyrosine residue is substituted with phenylalanine (F), shows enhanced IL-6-mediated STAT3 activation as the result of a lack of SOCS3-mediated suppression (Ohtani et al., 2000; Sawa et al., 2006). As these mice age, they spontaneously develop an RA-like MHC II-associated tissue-specific disease, indicating that constitutive activation of IL-6 signaling is involved in the development of some autoimmune diseases (Atsumi et al., 2002; Sawa et al., 2006). Moreover, anti-IL-6 receptor therapy is effective for some RA patients (Nishimoto and Kishimoto, 2004), supporting the use of F759 mouse as a mouse model for RA. Furthermore, we have previously shown that MHC II-restricted CD4⁺ T cells, but not CD8⁺ T cells and B cells, enhance the development of arthritis in F759 mice (Sawa et al., 2006), whereas a subset of CD8⁺ T cells expressing Foxp3 negatively regulates arthritis development in these mice (Nakagawa et al., 2010). We have further demonstrated that excess IL-6 signaling in type I collagen⁺ cells induces the T cell survival factor IL-7, which increases the memory/activated phenotype of CD4⁺ T cells via an increase in homeostatic proliferation, a process which is critical for arthritis development in F759 mice (Sawa et al., 2006). This suggests that the interaction between nonimmune tissue (type I collagen⁺ cells in F759 mice) and immune tissue (activated CD4⁺ T cells in F759 mice) plays important roles in this model of autoimmune disease (Hirano, 2010). Moreover, IL-17A-expressing CD4⁺ T cells that express a memory/activated phenotype exist in vivo, including in F759 mice (Nishihara et al., 2007; Ogura et al., 2008), and arthritis in F759 mice is dependent on IL-17A (Ogura et al., 2008). Thus, it is plausible that an age-dependent increase in homeostatic proliferation via IL-6-mediated IL-7 expression plays a role in the accumulation of antigen-experienced memory/activated

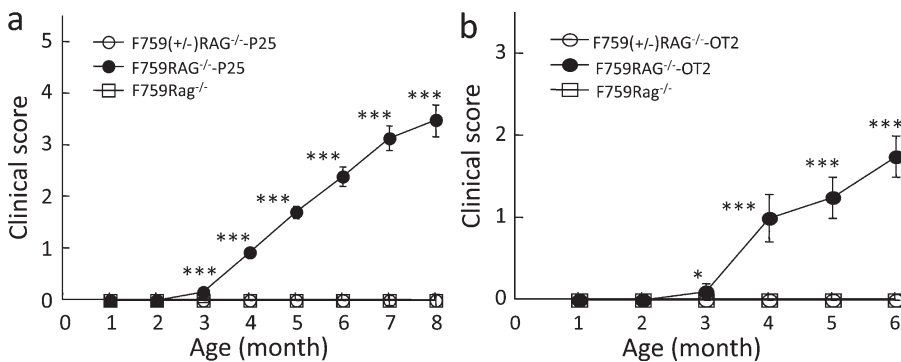


Figure 1. F759 mice expressing a single TCR variant that recognizes a nonjoint antigen develop arthritis. (a and b) Clinical arthritis scores for RAG2^{-/-} F759-P25 mice (black circles in a; $n = 76, 73, 62, 56, 46, 30, 14,$ and 7 for 1, 2, 3, 4, 5, 6, 7, and 8 mo, respectively) and RAG2^{-/-} F759-OT2 (black circles in b; $n = 5, 5, 5, 7, 8,$ and 2 for 1, 2, 3, 4, 5, and 6 mo, respectively) were determined as described in Materials and methods and compared with scores for control RAG2^{-/-} F759, RAG2^{-/-} F759^{+/+} P25, and RAG2^{-/-} F759^{+/+} OT2 mice. Control RAG2^{-/-} F759 (open squares in a and b; $n = 15$), RAG2^{-/-} F759^{+/+} P25 mice (open

circles in a; $n = 47, 47, 40, 37, 31, 20, 10,$ and 6 for 1, 2, 3, 4, 5, 6, 7, and 8 mo, respectively), and RAG2^{-/-} F759^{+/+} OT2 mice (open circles in b; $n = 36, 36, 28, 22, 17,$ and 9 for 1, 2, 3, 4, 5, and 6 mo, respectively) were also examined (RAG2^{-/-} F759^{+/+} TCR Tg mice vs. RAG2^{-/-} F759-TCR Tg mice; *, $P < 0.05$; ***, $P < 0.005$). The mean scores \pm SEM were determined as described in Materials and methods. P-values were calculated using Student's *t* tests.

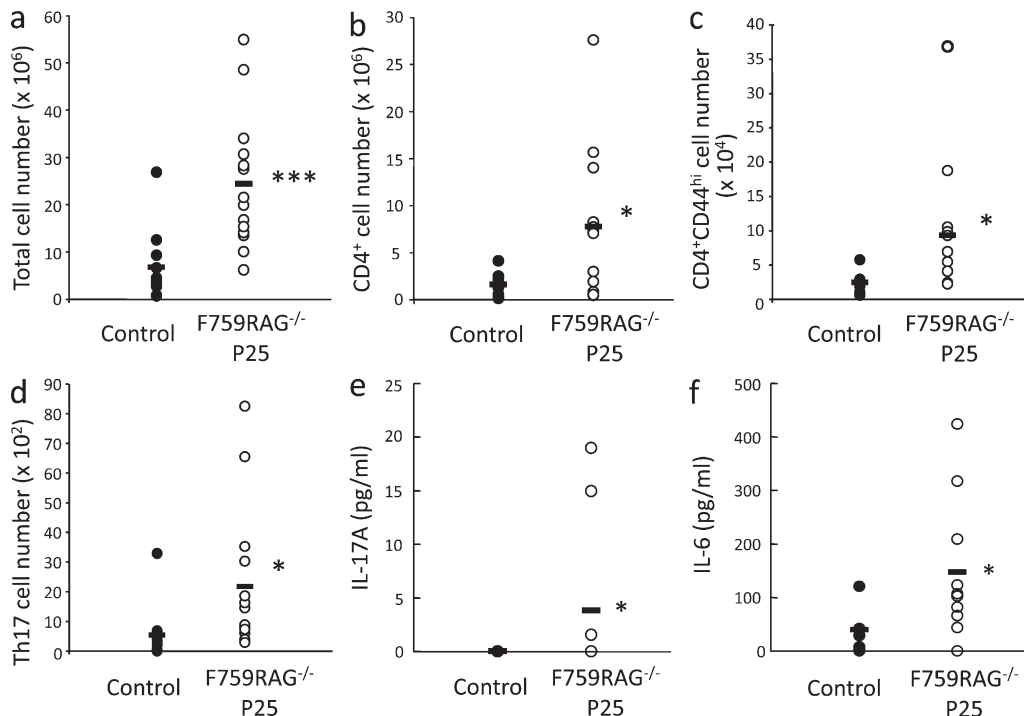


Figure 2. F759 mice expressing a single TCR variant that recognizes a nonjoint antigen show homeostatic induction of Th17 cell development.

Total cells (a), CD4⁺ T cells (b), memory/activated CD4⁺ T cells (c), and Th17 cells (d) were counted in spleen and lymph node tissues isolated from 6-mo-old RAG2^{-/-} F759-P25 mice ($n = 14$) or control mice ($n = 11$: RAGKO-P25 [$n = 3$] and F759^{+/-} RAGKO-P25 [$n = 8$]). (e and f) Serum concentrations of IL-17A and IL-6 were analyzed in 6-mo-old RAG2^{-/-} F759-P25 mice ($n = 9$ and 10, respectively) and control mice ($n = 15$ and 10, respectively). The number of total cells (***, $P < 0.005$), CD4⁺ T cells (*, $P < 0.05$), memory/activated CD4⁺ T cells (*, $P < 0.05$), and Th17 cells (*, $P < 0.05$), as well as the serum concentrations of IL-17A (*, $P < 0.05$) and IL-6 (*, $P < 0.05$), were all significantly higher in 6-mo-old RAG2^{-/-} F759-P25 mice. P-values were calculated using Student's *t* tests. Horizontal bars indicate mean.

phenotype CD4⁺ T cells expressing IL-17A. This is especially true for those F759 mice that show excess IL-6 signaling. We previously showed that an IL-17A-triggered positive-feedback loop that results in IL-6 expression, as well as chemokine expression in type I collagen⁺ cells, is enhanced in the presence of IL-6 in a manner dependent on NF- κ B and STAT3 (Ogura et al., 2008). We named this IL-17A-dependent IL-6 amplification loop “the IL-6 amplifier” (Ogura et al., 2008; Hirano, 2010). Importantly, activation of this IL-6 amplification loop is critical for the development of arthritis in F759 mice and in MOG antigen-specific T cell-mediated experimental autoimmune encephalomyelitis (EAE; Ogura et al., 2008).

In this paper, we investigate how tissue-specific disease develops in F759 mice and sought to identify the triggering factor for the IL-6 amplification loop in the joints of F759 mice. Our results demonstrate that cognate antigen recognition by effector CD4⁺ T cells is not necessary for tissue specificity in this arthritis model. Rather, we suggest that local events, including microbleeding in the affected tissue, determine the specificity via Th17 cell accumulation and activation of the IL-17-dependent IL-6 amplification loop. Based on our results, we propose that certain class II MHC-associated autoimmune diseases such as RA arise through a series of at least four steps that are independent of tissue antigen recognition by CD4⁺ T cells.

RESULTS

Cognate antigen recognition by CD4⁺ T cells is not required for the development of arthritis in F759 mice

As previously reported, F759RAG^{-/-} mice did not develop arthritis as they aged (Sawa et al., 2006; Ogura et al., 2008; Fig. 1, a and b). Arthritis did develop in F759 mice engineered to express a single TCR variant that recognizes a nonjoint antigen associated with tuberculosis (F759RAG^{-/-}-P25) or ovalbumin (F759RAG^{-/-}-OT2) but not in either F759^{+/-} RAG^{-/-}-P25 or F759^{+/-} RAG^{-/-}-OT2 mice (Fig. 1, a and b). Histology analysis confirmed these results by monitoring cell accumulation in the synovial regions (Fig. S1, a and b). Furthermore, total cell numbers from spleen and lymph node tissues, total numbers of CD4⁺ T cells, memory/activated CD4⁺ T cells, and IL-17A-expressing CD4⁺ T cells and serum concentrations of IL-17A and IL-6 were all significantly higher in F759RAG^{-/-}-P25 mice than in either control (RAG^{-/-}-P25) or F759^{+/-} RAG^{-/-}-P25 (Fig. 2, a–f). These results suggest that cognate antigen recognition by CD4⁺ T cells is not essential for the tissue specificity in F759 mouse suffering from CD4⁺ T cell-dependent arthritis.

A local event that induces Th17 cell accumulation plays a role in the development of arthritis via the activation of IL-6 signaling

We hypothesized that local events in the joint may contribute to the determination of autoimmune disease specificity by

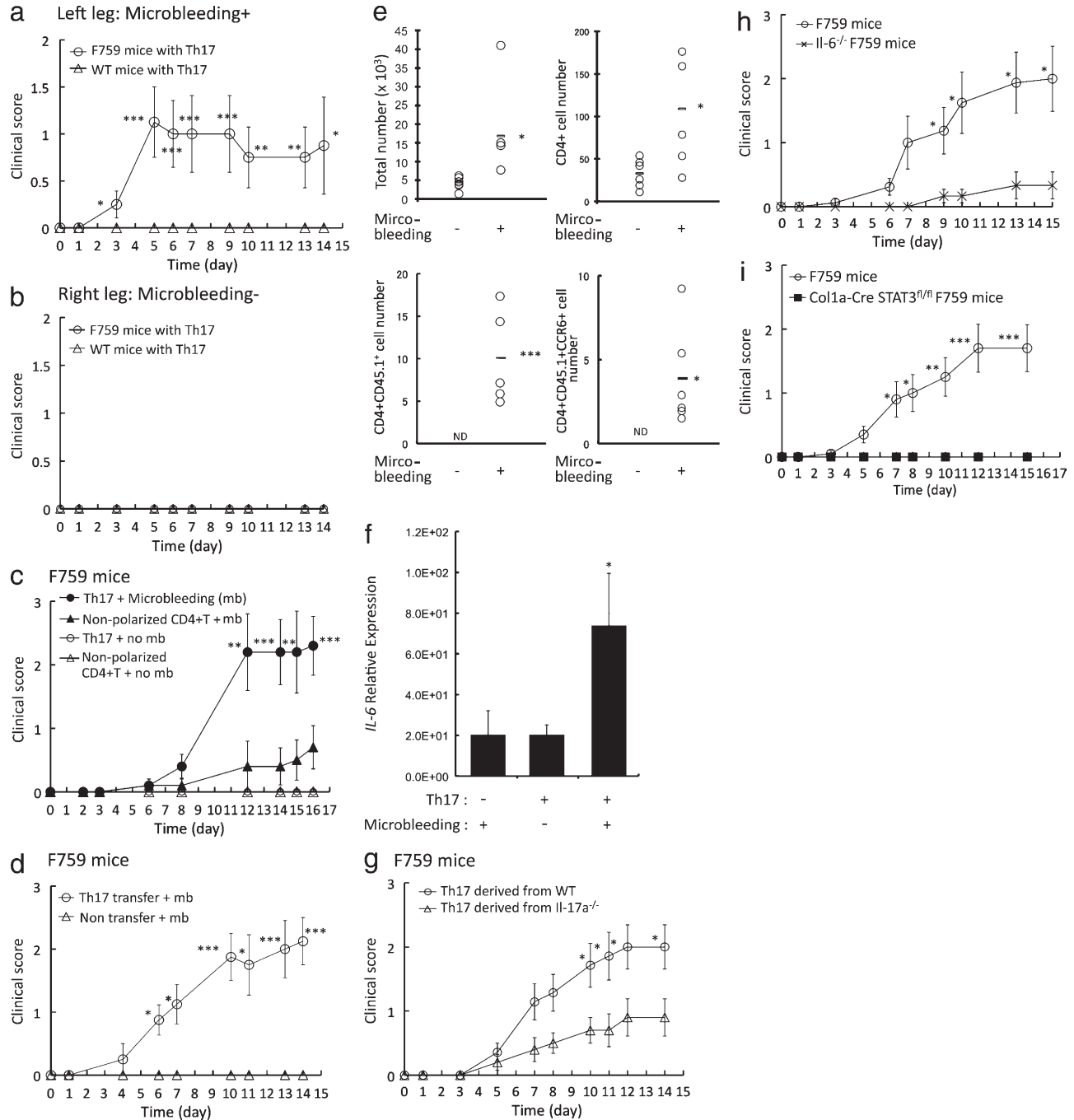


Figure 3. Microbleeding contributes to the development of arthritis in F759 mice. (a) Clinical arthritis scores from the left legs of control C57BL/6 mice (open triangles; $n = 4$) and F759 mice (open circles; $n = 4$) subjected to microbleeding and intravenously injected with 5×10^6 Th17 cells (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ for 3, 5, 6, 7, 9, 10, 13, and 14 d, respectively). (b) Clinical arthritis scores from the right legs of F759 mice (open circles; $n = 4$) or C57BL/6 mice (open triangles; $n = 4$) after injections of 5×10^6 Th17 cells without microbleeding induction. (c) Clinical arthritis scores from the legs of F759 mice injected with 5×10^6 Th17 cells and subjected to microbleeding (closed circles; $n = 5$) or F759 mice injected with 5×10^6 activated nonpolarized CD4⁺ T cells and subjected to microbleeding (closed triangles; $n = 5$). Results were compared with clinical arthritis scores from the legs of F759 mice not subjected to microbleeding (open circles: mice receiving Th17 cells [$n = 5$]; **, $P < 0.01$; ***, $P < 0.005$ for 12, 14, 15, and 16 d; open triangles: mice receiving activated, nonpolarized CD4⁺ T cells [$n = 5$]). (d) Clinical arthritis scores from the legs of F759 mice subjected to microbleeding without transfers of effector T cells are also shown (open triangles; $n = 4$) and compared with scores from the legs of F759 mice injected with 5×10^6 Th17 cells and subjected to microbleeding (open circles; $n = 4$; *, $P < 0.05$; ***, $P < 0.005$ for 6, 7, 10, 11, 13, and 14 d, respectively). (e) Number of total cells, CD4⁺ T cells, CD4⁺CD45.1⁺ T cells, and CD4⁺CD45.1⁺CCR6⁺ T cells from joint tissues of F759 mice that received transfers of 2×10^7 Th17 cells in the presence or absence of microbleeding induction (24 h after transfer) were analyzed (untreated vs. microbleeding: *, $P < 0.05$; ***, $P < 0.005$). Horizontal bars indicate mean. (f) Joint tissues of F759 mice that did or did not receive transfers of 5×10^6 Th17 cells in the presence or absence of microbleeding

triggering Th17 cell accumulation, which in turn enhances IL-6 signaling via the IL-17-triggered IL-6 amplification loop. It is known that microbleeding, which occurs even in the joints of healthy individuals, can trigger hemarthrosis in hemophilia patients because of defects in blood coagulation (Aledort et al., 1994; Manco-Johnson et al., 2007; Hakobyan et al., 2008). Interestingly, like arthritis in F759 mice, hemarthrosis most often affects the ankles (Manco-Johnson et al., 2007). Therefore, we hypothesized that experimental microbleeding in F759 mice might trigger arthritis in the presence of Th17 cells.

We first induced the development of effector CD4⁺ T cells including Th17 cells and activated nonpolarized CD4⁺ T cells in vitro (Fig. S2). It is of note that sorting the CD4⁺ T cell population revealed that the overall TCR-V β profile was not perturbed (unpublished data). We transferred the sorted cells into young F759 mice without arthritis symptoms and experimentally induced microbleeding in one leg. This resulted in arthritis in the leg in which microbleeding was induced but not in the other legs (Fig. 3, a and b). Th17 cell transfer alone did not induce arthritis (Fig. 3 c) but resulted in disease if preceded by the induction of microbleeding (Fig. 3 c). This was confirmed by histology (Fig. S1, d and e). Transfer of nonpolarized effector CD4⁺ T cells or Th1 cells also triggered a mild form of arthritis if preceded by microbleeding (Fig. 3 c and Fig. S3), whereas microbleeding alone did not cause disease in F759 hosts (Fig. 3 d). Control C57BL/6 (WT) hosts did not develop arthritis under any condition (Fig. 3, a and b). We also showed that transferred Th17 cells maintain their phenotype by monitoring IL-17A-GFP expression in the joints after microbleeding induction (Fig. S4). Thus, we conclude that arthritis in F759 mice is triggered by local microbleeding in a manner dependent on the presence of Th17 cells in the blood and lymphoid organs. Moreover, because arthritis developed in F759 mice but not in control C57BL/6 hosts after the same treatment, enhanced sensitivity to cytokines in the given tissue is required for disease development.

After the induction of microbleeding, the number of effector CD4⁺ T cells, including CCR6⁺ T cells, increased in the joints of F759 mice that received congenic Th17 cells (Fig. 3 e). Additionally, the expression level of IL-6 increased in the joint tissues when microbleeding was accompanied by a transfer of Th17 cells (Fig. 3 f). Furthermore, F759 mice that received Th17 cells derived from IL-17A-deficient mice

showed a mild form of arthritis after microbleeding was induced (Fig. 3 g). IL-6-deficient F759 mice and F759 mice in which STAT3 was deleted in type I collagen⁺ cells did not develop arthritis even after Th17 cell transfer and joint microbleeding induction (Fig. 3, h and i). These results suggest that a local event like microbleeding induces Th17 cell accumulation and IL-6 signaling in the joints, most likely via activation of the IL-17A-mediated IL-6 amplification loop.

CCL20, a target gene of the IL-17-dependent IL-6 amplification loop in type 1 collagen⁺ cells, is required for Th17 accumulation and arthritis development

We next attempted to identify the critical target gene of the IL-6 amplification loop that induces local accumulation of effector CD4⁺ T cells, including Th17 cells, hypothesizing that chemokines like CCL20, a chemotactic factor for Th17 cells, might be involved (Williams, 2006; Hirota et al., 2007). We speculated that microbleeding in the joints initially induces a limited accumulation of Th17, which, in turn, may induce a stable level of chemokine expression. Chemokine expression may then magnify Th17 cell accumulation and enhance IL-6 signaling at the relevant site. Indeed, we found that CCL20 expression synergistically increased when type 1 collagen⁺ fibroblasts were stimulated with IL-6 and IL-17A (Fig. 4 a). Moreover, CCL20 expression levels and CD4⁺CCR6⁺ T cell numbers were higher among synovial tissues isolated from older F759 mice with arthritis compared with those from control mice (Fig. 4, b and c). Also, F759 mice that were transferred with Th17 cells developed arthritis upon injection of CCL20 into the joint (Fig. 4 d and Fig. S1 c). Arthritis did not develop in the joint of the leg subjected to vehicle injection (Fig. S5). No arthritis was observed in control C57BL/6 animals, which were similarly treated (Fig. S5 and Fig. 4 d). We also observed that microbleeding induces CCL20 expression in the joints of F759 mice that received Th17 cell transfer (Fig. 4 e). These results support the idea that local microbleeding induces CCL20 expression in the joints, which causes tissue-specific but antigen-nonspecific accumulation of CCR6⁺ Th17 cells. Furthermore, F759 mice that received IL-17A-deficient Th17 cells or Th1 cells showed lower clinical arthritis scores even after joint injections of CCL20 (Fig. 4 f and Fig. S3). Neither IL-6-deficient F759 mice nor F759 mice in which STAT3 was deleted in type I collagen⁺ cells developed arthritis, even after Th17 cell transfer followed by joint injections of CCL20 (Fig. 4, g and h). In addition,

were prepared. IL-6 mRNA expression levels were analyzed (no microbleeding vs. microbleeding in the presence of intravenous injection of Th17 cells: *, $P < 0.05$). (g) Clinical arthritis scores from the legs of F759 mice after intravenous transfer of Th17 cells derived from C57BL/6 mice (open circles; $n = 7$) or IL-17A^{-/-} mice (open triangles; $n = 5$). All mice were subjected to microbleeding (WT vs. IL-17A^{-/-} Th17 cells: *, $P < 0.05$ for 10, 11, 12, and 14 d). (h and i) Clinical arthritis scores from the legs of positive control F759 mice (open circles; $n = 8$ for IL-6^{-/-} F759 mouse experiment; $n = 10$ for type I collagen-Cre STAT3^{fllox/fllox} F759 mouse experiment) compared with those from the legs of IL-6-deficient F759 mice (crosses; $n = 6$ in h) or type I collagen-Cre STAT3^{fllox/fllox} F759 mice (closed squares; $n = 6$ in i) after intravenous transfer of 5×10^6 WT Th17 cells and microbleeding induction (IL-6-deficient F759: *, $P < 0.05$ for 9, 10, 13, and 15 d; type I collagen-Cre STAT3^{fllox/fllox} F759 mice: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ for 7, 8, 10, 12, and 15 d). The mean scores \pm SEM were determined as described in Materials and methods. P -values were calculated using Student's t tests. These experiments were performed at least twice independently. Representative data are shown.

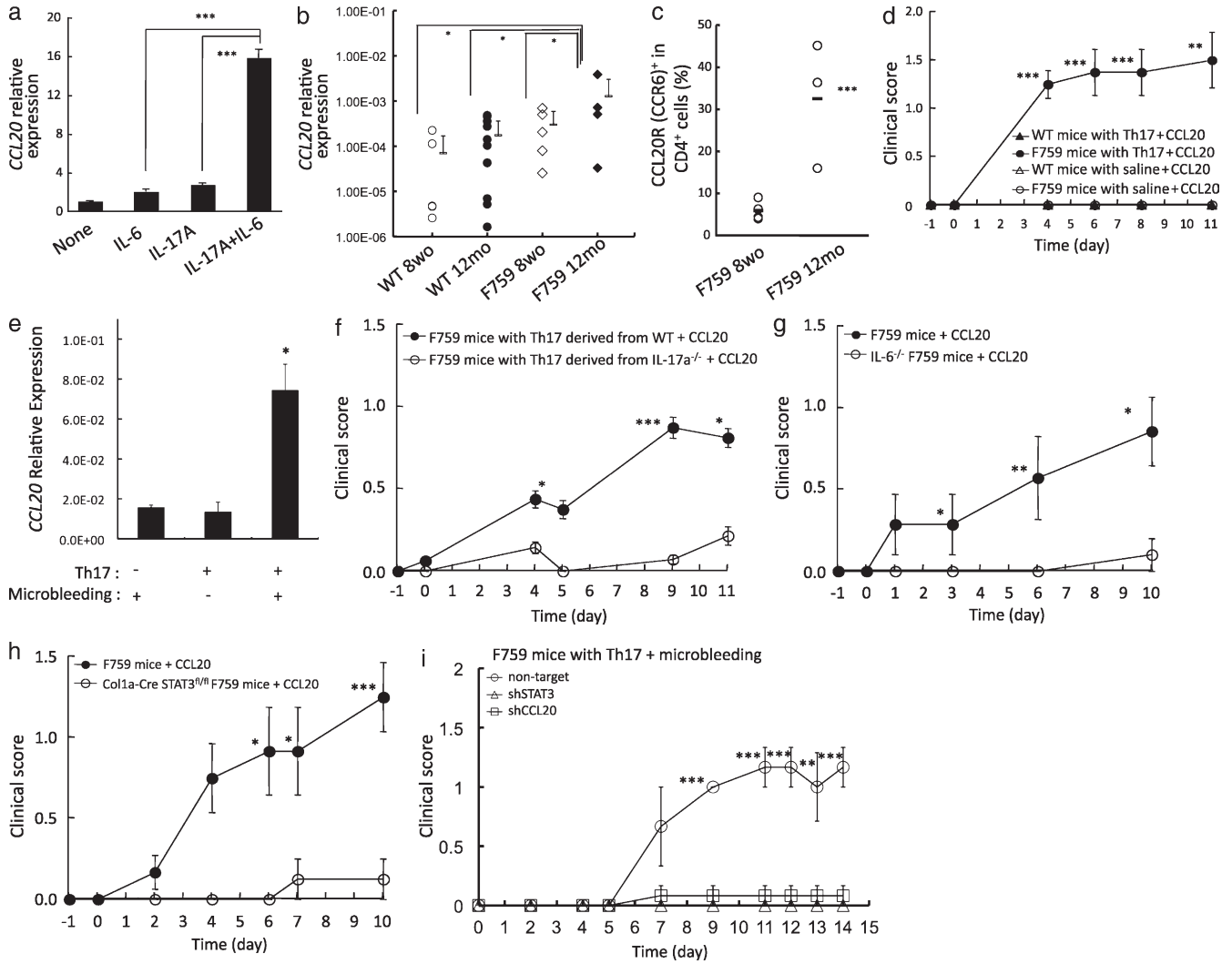


Figure 4. CCL20 contributes to the development of arthritis in F759 mice. (a) Mouse embryonic fibroblast (MEF) cells and synovial fibroblasts were prepared and stimulated with human IL-6 + soluble IL-6R and/or IL-17A for 3 h. Cells were harvested and total RNA were collected and assessed with real-time PCR for CCL20. (***) $P < 0.005$ for IL-6 and IL-17A). (b and c) Cells from F759 and C57BL/6 joints were prepared and CCL20 mRNA expression levels and CD4⁺CCR6⁺ T cell percentages were analyzed. (*, $P < 0.05$; ***, $P < 0.005$ for 8-wk-old [8wo] C57BL/6, 12-mo-old [12mo] C57BL/6, 8-wk-old F759, and 12-mo-old spontaneous arthritic F759). Horizontal bars indicate mean. (d) Clinical arthritis scores from the left legs of F759 (closed circles; $n = 8$) or C57BL/6 (closed triangles; $n = 8$) mice that received transfers of 5×10^6 Th17 cells as well as CCL20 injections. Clinical arthritis scores from the left legs of F759 (open circles; $n = 8$) or C57BL/6 mice (open triangles; $n = 8$) after CCL20 injections without Th17 cell transfers are also shown (Th17 vs. saline: **, $P < 0.01$; ***, $P < 0.005$ for 4, 6, 8, and 11 d). (e) Cells from the joint tissues of F759 mice that received transfers of 5×10^6 Th17 cells in the presence or absence of microbleeding were prepared and CCL20 mRNA expression levels were analyzed (untreated vs. microbleeding: *, $P < 0.05$). (f) Clinical arthritis scores from the left legs of F759 mice after CCL20 injections and intravenous transfer of Th17 cells derived from C57BL/6 mice (closed circles; $n = 8$) or IL-17A^{-/-} mice (open circles; $n = 7$; WT vs. IL-17A^{-/-} Th17 cells; *, $P < 0.05$; ***, $P < 0.005$ for 4, 9, and 11 d). (g) Clinical arthritis scores from the left legs of F759 mice (closed circles; $n = 7$) or IL-6^{-/-} F759 mice (open circles; $n = 5$) after intravenous transfer of 5×10^6 WT Th17 cells plus CCL20 injection (*, $P < 0.05$; **, $P < 0.01$ for 3, 6, and 10 d). (h) Clinical arthritis scores from the left legs of F759 mice (closed circles; $n = 6$) or type I collagen-Cre STAT3^{fllox/fllox} F759 mice (open circles; $n = 4$) after intravenous transfer of 5×10^6 WT Th17 cells and CCL20 injections (*, $P < 0.05$; ***, $P < 0.005$ for 6, 7, and 10 d). (i) Clinical arthritis scores from F759 mice that received transfers of 5×10^6 Th17 cells in the presence of microbleeding and joint injections of lentivirus carrying shRNA specific for CCL20 (open squares; $n = 6$) or STAT3 (open triangles; $n = 5$), or a nontarget sequence (open circles; $n = 4$; nontarget sequence vs. CCL20 specific: ***, $P < 0.005$ for 11, 12, 13, and 14 d; nontarget sequence vs. STAT3 specific: **, $P < 0.01$; ***, $P < 0.005$ for 9, 11, 12, 13, and 14 d). Mean scores \pm SEM were determined as described in Materials and Methods. P-values were calculated using Student's *t* tests. These experiments were performed at least twice independently. Representative data are shown.

local microbleeding failed to induce arthritis in F759 mice with Th17 cell transfer if either CCL20 or STAT3 expression was knocked down in the joints (Fig. 4 i). Thus, our results

indicate that microbleeding, which induces Th17 cell accumulation, enhances IL-6 signaling in type I collagen⁺ cells in joints. This then gives rise to the development of CD4⁺

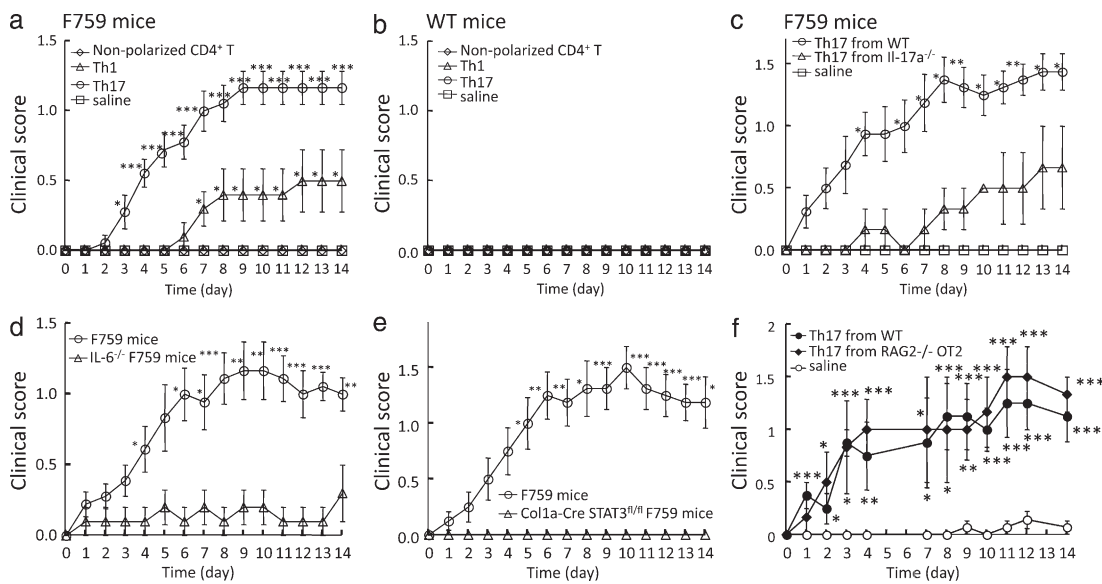


Figure 5. Th17 cell-mediated induction of the IL-6 amplification loop in joints is critical for arthritis development in F759 mice. (a) Clinical arthritis scores from the left legs of F759 mice after left leg joint injections of 5×10^6 Th17 cells (circles; $n = 9$; *, $P < 0.05$; ***, $P < 0.005$ for 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 d), 5×10^6 Th1 cells (triangles; $n = 4$; *, $P < 0.05$ for 7, 8, 9, 10, 11, 12, 13, and 14 d), and 5×10^6 activated nonpolarized CD4⁺ T cells (diamonds; $n = 6$) compared with those from the left legs of F759 mice after left leg joint injections of saline (squares; $n = 9$). (b) Clinical arthritis scores from the left legs of C57BL/6 mice after left leg joint injections of 5×10^6 Th17 cells (circles; $n = 7$), 5×10^6 Th1 cells (triangles; $n = 4$), 5×10^6 activated nonpolarized CD4⁺ T cells (diamonds; $n = 4$), or saline (squares; $n = 7$). (c) Clinical arthritis scores from the left legs of F759 mice after left leg joint injections of Th17 cells derived from C57BL/6 mice (circles; $n = 8$), IL-17A-deficient mice (triangles; $n = 3$), or saline (squares; $n = 5$; WT vs. IL-17A-deficient Th17 cells: *, $P < 0.05$; **, $P < 0.01$ for 4, 6, 7, 8, 9, 10, 11, 12, 13, and 14 d). (d) Clinical arthritis scores from the left legs of F759 mice (circles; $n = 9$) or IL-6-deficient F759 mice (triangles; $n = 5$) after left leg joint injections of 5×10^6 Th17 cells (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ for 4, 6, 7, 8, 9, 10, 11, 12, 13, and 14 d). (e) Clinical arthritis scores from the left legs of F759 mice (circles; $n = 8$) or type I collagen-Cre STAT3^{fl/fl} F759 mice (triangles; $n = 3$) after left leg joint injections of 5×10^6 Th17 cells (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ for 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 d). (f) Clinical arthritis scores from the left legs of F759 mice after left leg joint injections of Th17 cells derived from C57BL/6 mice (closed circles; $n = 4$), OT2 RAG2^{-/-} mice (diamonds; $n = 3$), or saline (open circles; $n = 7$; OT2 RAG2^{-/-}-derived Th17 cells vs. saline: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ for 2, 3, 4, 7, 8, 9, 10, 11, 12, and 14 d). Mean scores \pm SEM were determined as described in Materials and methods. P-values were calculated using Student's *t* tests. These experiments were performed at least twice independently. Representative data are shown.

T cell-dependent tissue-specific diseases in hosts having a sufficient number of effector CD4⁺ T cells and tissues with enhanced sensitivity to T cell-derived cytokines.

Local accumulation of Th17 cells contributes to the development of arthritis in F759 mice by acting as a source of IL-17A

Lastly, we examined whether direct injection of Th17 cells or IL-17A into the joint bypasses local events to directly induce arthritis in F759 mice. Indeed, injections of Th17 cells but not nonpolarized CD4⁺ T cells or Th1 cells induced arthritis (Fig. 5 a). However, none of these treatments elicited arthritis in WT mice (Fig. 5 b). Joint injections of Th17 cells derived from IL-17A-deficient mice into F759 mice resulted in lower clinical scores than injection of Th17 cells from control C57BL/6 mice (Fig. 5 c). Injections of Th17 cells from control mice into IL-6-deficient F759 (Fig. 5 d) or F759 mice in which STAT3 was deleted from type I collagen⁺ cells also resulted in lower clinical scores (Fig. 5 e). These results strongly suggest that Th17-mediated IL-6 expression in type I collagen⁺ cells plays a role in arthritis development in F759 mice. Injections of Th17 cells from OT2-RAG^{-/-} mice also

induced arthritis in F759 mice (Fig. 5 f), confirming that the accumulation of Th17 cells in the joint is sufficient for arthritis development and that antigen recognition is not required. Finally, we showed that direct injection of IL-17A into one joint induces arthritis in F759, but not control C57BL/6, mice (Fig. 6, a and b; and Fig. S1 f). Additionally, arthritis did not develop in the leg joints of F759 mice that did not receive injections of Th17 cells or IL-17A (Fig. S6, a-e). All these data are consistent with the idea that local accumulation of Th17 cells contributes to the development of arthritis in F759 mice by serving as a synovial source of cytokines (e.g., IL-17A) and by enhancing IL-6 signaling independent of antigen recognition.

DISCUSSION

It is clear that the recognition of specific antigens by autoreactive T cells contributes to the tissue specificity of several autoimmune diseases (Marrack et al., 2001; Mathis and Benoist, 2004). In many cases, however, causative tissue-specific antigens recognized by memory/activated CD4⁺ T cells have not been established, even for diseases associated with class II MHC alleles (Mocci et al., 2000; Skapenko et al., 2005).

RA and arthritis in F759 mice are such examples (Falgarone et al., 2009; this paper). Interestingly, several lines of evidence support a pathogenic role for CD4⁺ T cells in both diseases. These include associations with class II MHC and CD4 molecules, increased numbers of memory/activated CD4⁺ T cells as the disease progresses, and improved outcomes in response to suppressions and/or deficiencies in class II MHC molecules, CD4⁺ T cells, and the T cell survival cytokine IL-7. IL-7 further increases the homeostatic proliferation of CD4⁺ T cells followed by the development of arthritis in F759 mice (Sawa et al., 2006; Ogura et al., 2008). These observations led us to ask how arthritis is triggered in the joints of F759 mice, and tissue-specific CD4⁺ recognition of tissue antigen is a likely explanation. However, our results do not support this model. Rather, we found that an accumulation of activated CD4⁺ T cells in the joint is critical for arthritis development in F759 mice. In fact, local microbleeding-mediated CCL20 expression induced such an accumulation, resulting in the development of arthritis. This, then, may argue for the existence of RA subgroups, which would include RA, that are mainly dependent on either autoantibodies (e.g., anti-citrullinated antibodies), tissue-specific antigen-mediated immune responses, or inflammatory cytokines like IL-6 and TNF. Consistent with this notion, therapies against certain molecules like IL-6 receptor or TNF do not cure all cases of RA. Thus, certain class II MHC-associated tissue-specific autoimmune diseases, including some RA subtypes, may be at least partly induced by local events that cause an antigen-independent accumulation of effector CD4⁺ T cells followed by the induction of enhanced IL-6 signaling via activation of the IL-6 amplification loop in the affected tissue. This is triggered by CD4⁺ T cell-derived cytokines.

To explain the development of tissue-specific MHC class II-mediated autoimmune diseases, including RA, we propose a four-step model. This model describes the development of certain MHC II-mediated autoimmune diseases that are independent of tissue-specific antigens. The steps include: (1) T cell activation regardless of antigen specificity, (2) local events inducing tissue-specific accumulation of activated T cells, (3) enhanced sensitivity to T cell-derived cytokines in a population of cells in the affected tissue, and (4) activation of an cytokine-dependent IL-6 amplification loop, which is triggered by CD4⁺ T cell-derived cytokines such as IL-17A. It is likely that each step interacts with the others, and the

degree of contribution of each to pathogenesis varies with the disease.

The number of cytokine-secreting effector/memory CD4⁺ T cells increases with age, as the result of an accumulation of pathogen-specific memory T cells and homeostatic proliferation of CD4⁺ T cells. The resulting memory/activated CD4⁺ T cells are increasingly localized in parenchymal organs like the alimentary tract rather than lymphoid organs. Therefore, even at steady state, memory/activated CD4⁺ T cells may sometimes migrate and/or stay in tissues at risk for autoimmune diseases. Consistent with this, these diseases are more prevalent in older patients who have a large population of memory/activated CD4⁺ T cells, some of which secrete cytokines because of homeostatic proliferation and/or chronic inflammations (Hasler and Zouali, 2005; Larbi et al., 2008). Therefore, the first and second steps in the four-step model will occur to some degree even in healthy people, particularly in elderly individuals who have a larger population of memory/activated T cells. Autoimmune diseases, including RA, however, do not develop in all individuals. The relatively low rates of these disorders may reflect the fact that the factors that increase the risk of enhancement of local IL-6 signaling in affected tissues (the third step) are rare. These factors may include products made by viruses or bacteria (Münz et al., 2009). For instance, HTLV1 infection is a significant risk factor for arthritis (Uchiyama, 1997), whereas the transgenic expression of p40 Tax, a product of HTLV1 which activates NF- κ B, causes an RA-like disease in mice (Iwakura et al., 1991). Indeed, forced expression of p40 Tax in F759 mice has been seen to enhance disease development (Ishihara et al., 2004). Moreover, many viral proteins, including hepatitis C virus Core protein and EBNA2 from Epstein-Barr virus, are strong STAT3 activators (Yoshida et al., 2002; Muromoto et al., 2009). Products from pathogens are also known to stimulate Toll-like receptors, which leads to NF- κ B activation. Because viruses and bacteria also have their own preferential target cells and/or tissues, their infections could determine the tissue specificity of the disease by enhancing cytokine sensitivity in the given tissue. Consistent with this idea, autoimmune diseases are sometimes induced after infections that also increase the number of activated pathogen-specific cytokine-secreting CD4⁺ T cells (Kivity et al., 2009). Tissue sensitivity to mechanical stress induced by deterioration through age provides another potential trigger or enhancer for autoimmune diseases.

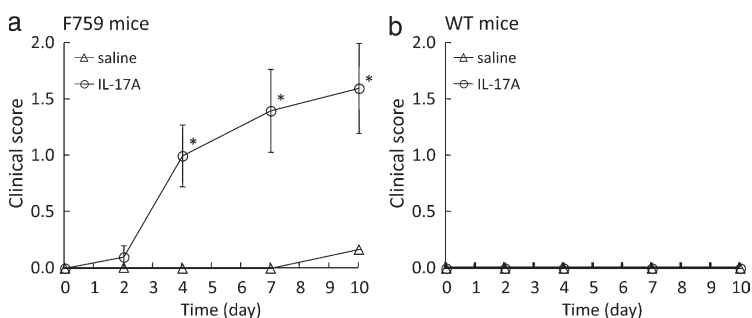


Figure 6. IL-17A in the joints induced the development of arthritis in F759 mice.

(a) Clinical arthritis scores from the left legs of F759 mice after left leg joint injections of 0.1 μ g IL-17A on days 0, 1, and 2 (circles; $n = 5$; *, $P < 0.05$ for 4, 7, and 10 d) or F759 mice after left leg joint injections of saline (triangles, $n = 3$). (b) Clinical arthritis scores from the left legs of C57BL/6 mice after left leg joint injections of 0.1 μ g IL-17A (circles; $n = 4$) or saline (triangles, $n = 4$) on days 0, 1, and 2. These experiments were performed at least three times independently. Representative data are shown.

Such stress can induce local events such as microbleeding and/or IL-6 expression via activation of NF- κ B. Moreover, these diseases might be associated with various genetic aberrations, including a somatic mutation in gp130 molecules, which induces STAT3 hyperactivation and numerous mutations in NF- κ B regulators that activate NF- κ B signaling (Lenz et al., 2008; Compagno et al., 2009; Rebouissou et al., 2009). In addition, de novo mutations in STAT3 have been identified in patients with hyperimmunoglobulin E syndrome (Minegishi et al., 2007). Thus, activation of STAT3 and/or NF- κ B regulators via local pathogen infections, accumulated pathogen products, and/or genetic mutations may play a role in enhancing the sensitivity of affected tissues to T cell-derived cytokines.

Microbleeding in the joints may result in the accumulation of many different cell types, including red blood cells, neutrophils, macrophages, and dendritic cells, as well as memory/activated CD4⁺ T cells including Th17 cells. In this paper, we focused on IL-17A expression from Th17 cells accumulating in the joints. However, it is reasonable to argue that microbleeding could accelerate the inflammatory reaction by other means. For example, the environment of the joint synovium or the presence of dead cells may lead to the release of intracellular stimulants such as heme and/or danger-associated molecular patterns (DAMPs) including mitochondria (Zhang et al., 2010). These stimulants secondarily enhance cell death by heme's toxic effect. They might also induce IL-6 and/or CCL20 expression based on the fact that DAMPs from dead cells directly and/or indirectly activate the NF- κ B pathway (Bianchi, 2007; Sims et al., 2010).

Interestingly, activation of the IL-17-triggered IL-6 amplification loop is also involved in the development of EAE (Ogura et al., 2008). Because the model for EAE is dependent on a tissue-specific MOG-derived peptide, these results suggest that antigen specificity by the effector CD4⁺ T cells and enhanced IL-6 signaling in the affected tissue via the IL-6 amplification loop do not always function independently of each other in tissue-specific autoimmune diseases. If this is the case, cognate antigen recognition by the effector CD4⁺ T cells could occur upstream of the enhanced IL-6 signaling in the affected tissue. The antigen specificity of the effector CD4⁺ T cells may function initially to target these cells around the affected tissues. In other words, the antigen specificity might bypass initial local events (like microbleeding in F759 mouse) that induce tissue-specific accumulation of activated T cells, although some local events might increase the efficacy of the tissue accumulation. The resulting pool of activated CD4⁺ T cells around the specific tissues could enhance local IL-6 signaling as a source for cytokines via the IL-6 amplification loop. Thus, regardless of the stimulus for the local accumulation of effector CD4⁺ T cells, the resulting inflammatory disease is associated with class II MHC molecules if cytokines from the activated CD4⁺ T cells are involved in the disease development. Cytokines other than IL-17A may also contribute to class II MHC-associated diseases to enhance IL-6 signaling in type I collagen⁺ cells (fourth step), as nonpolarized activated CD4⁺ T cells, Th1, or IL-17A^{-/-} Th17 cells induced a mild form of arthritis. TNF, for example,

may contribute to the localized class II MHC-associated autoimmune diseases via NF- κ B activation followed by an enhancement of IL-6 signaling in the affected tissues. In support of this idea, numerous studies have demonstrated the efficacy of targeting TNF for treating RA and other chronic autoimmune diseases, the majority of which involve class II MHC molecules (Feldmann and Maini, 2001). Furthermore, activated CD4⁺ T cells are known to express TNF (Cherwinski et al., 1987; Constant and Bottomly, 1997; Williams et al., 2008), whereas we have found that a lack of TNF attenuates arthritis in F759 mice (unpublished data). Moreover, it is interesting that LPS administration around the joints induces arthritis in mice that have an excess number of Th1 cells (Nickdel et al., 2009). This may suggest that LPS-mediated IL-6 production around the joints induces the local accumulation of Th1 cells followed by activation of the IL-6 amplification loop. Consistent with this, combined treatment with IL-6 and IFN- γ synergistically induces IL-6 production, at least in vitro (unpublished data). We also found that IL-22 enhances IL-6 expression in the presence of IL-17A in type I collagen⁺ cells (Fig. S7 a). However, joint injections of shRNA specific for the IL-22 receptor hardly suppressed arthritis development after joint injection of Th17 cells in F759 mice (Fig. S7 b). Therefore, it is likely that IL-22 from Th17 cells plays a minimal role, at least for Th17 cell-driven arthritis in F759 mice.

To summarize, in this paper we describe how MHC class II-associated tissue-specific autoimmune arthritis arises in F759 mice and we propose a four-step model to explain the process. This model provides a possible explanation for why tissue-specific antigens recognized by activated CD4⁺ T cells have not been identified in many tissue-specific autoimmune diseases associated with class II MHC molecules, including RA. We expect that our four-step model will provide new and important insight into the immunological mechanisms driving autoimmune disease development.

MATERIALS AND METHODS

Mouse strains. C57BL/6 mice were purchased from Japan SLC. F759 mice were backcrossed with C57BL/6 mice for >10 generations. IL-6-deficient mice (provided by M. Kopf, Max-Planck-Institute of Immunobiology, Freiburg, Germany) were backcrossed with C57BL/6 mice for >10 generations and were then crossed with F759 mice. Type I collagen-Cre mice (provided by G. Karsenty, Baylor College of Medicine, Houston, TX) were crossed with STAT3^{flax/flax} mice (provided by S. Akira, Osaka University, Osaka, Japan; Takeda et al., 1998). IL-17A-deficient mice on a C57BL/6 background (Iwakura and Ishigame, 2006) were crossed with F759 mice. The C57BL/6/P25 TCR-Tg mouse line was established in our laboratory (Tamura et al., 2004). C57BL/6 OT2 TCR-Tg mice were a gift from W.R. Heath (University of Melbourne, Melbourne, Australia). IL-17A-GFP knockin mice were purchased from Biocytogen. C57BL/6 SJL (CD45.1) mice were purchased from The Jackson Laboratory. All mice were maintained under specific pathogen-free conditions according to the protocols of Osaka University Medical School. All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committees of the Graduate School of Frontier Biosciences and Graduate School of Medicine, Osaka University. The protocols for animal experiments used here were approved by the Institutional Animal Care and Use Committees of the Graduate School of Frontier Biosciences and Graduate School of Medicine, Osaka University.

Clinical assessment of arthritis. Mice were inspected and assessed for signs of arthritis as described previously (Atsumi et al., 2002; Ishihara et al., 2004; Sawa et al., 2006). In brief, the severity of the arthritis was based on two bilaterally assessed parameters: (1) swelling of the ankle or wrist and (2) restricted mobility of the ankle or wrist joints. The severity of each parameter was graded on a scale of 0–2: 0, no change; 1, mild change; and 2, severe change. The severity score was the sum of the scores for the two parameters, resulting in a maximum possible score of 16 for each mouse. In some experiments, means for a single point in one leg ankle joint from each mouse were used. In these cases, the severity score was the sum of the bilateral scores for swelling of the ankle and restricted mobility in the ankle joint. In general, because in the majority of experiments we treated only one leg joint from each host mouse, the maximum possible score for each leg was 4.

Histological analysis. Joints were fixed in 4% paraformaldehyde, decalcified for 12 h in Morse's solution (22.5% borynol formate and 10% sodium acid citrate solution), followed by 12 h in 4% paraformaldehyde, and embedded in paraffin. Sections were stained with hematoxylin-eosin (Sawa et al., 2006).

Intracellular cytokine staining. The number of Th17 cells *in vivo* was determined as previously described (Nishihara et al., 2007). In brief, T cells from spleen and lymph nodes were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of GolgiPlug (BD) for 6 h. Intracellular IL-17A and IFN- γ were labeled with anti-IL-17A and anti-IFN- γ antibodies after surface staining, fixation, and permeabilization.

Antibodies and reagents. The following antibodies were used for staining: APC-conjugated anti-CD8 (eBioscience), anti-TCR- β (eBioscience), anti-IFN- γ (eBioscience), and control IgG1 (eBioscience); FITC-conjugated anti-CD8 (eBioscience), anti-CD19 (eBioscience), anti-CD44 (BioLegend), anti-B220 (BD), anti-NK1.1 (eBioscience), and anti-I-A/I-E (BioLegend); PE-conjugated anti-CD25 (eBioscience), anti-IL-17A (eBioscience), control IgG2a (eBioscience), anti-I-A/I-E (BioLegend), anti-B220 (BD), anti-CD45.1 (eBioscience), and anti-CD62L (eBioscience); PE-Cy5-conjugated anti-CD4 (BioLegend); PE-Cy7-conjugated anti-CD4 (BioLegend); Pacific blue-conjugated anti-CD44 (eBioscience), and anti-CD8 (eBioscience); and Alexa Fluor 647-conjugated anti-CCR6 (BD).

ELISA. IL-6 and IL-17A levels in serum or cell culture supernatant were determined using ELISA kits (BD, eBioscience, or R&D Systems).

Flow cytometry. For cell surface labeling, 10^6 cells were incubated with fluorescence-conjugated antibodies for 30 min on ice. The cells were then analyzed with CyAn flow cytometers (Beckman Coulter). The collected data were analyzed using Summit software (Beckman Coulter) and/or FlowJo software (Tree Star, Inc.).

Induction of activated nonpolarized CD4⁺ T cells, Th1 cells, and Th17 cells. Lymph nodes and spleens from WT mice or IL-17A-deficient mice were harvested and CD25⁺CD44⁺ naive CD4⁺ T cells were sorted using a MoFlo cell sorter (Beckman Coulter). The cells were cocultured for 4 d with bone marrow-derived dendritic cells plus 1 μ g/ml of anti-CD3 antibodies for activated nonpolarized CD4⁺ T cells, in the presence of 20 ng/ml of recombinant mouse (rm) IL-12 (PeproTech) for Th1 cells and in the presence of 1 μ g/ml of recombinant human (rh) IL-6 (Toray), 5 ng/ml rhTGF- β (PeproTech), 10 ng/ml rmIL-23 (R&D Systems), and 10 μ g/ml anti-IFN- γ antibodies for Th17 cells.

Joint injections. Th1, Th17, or activated nonpolarized CD4⁺ T cells prepared *in vitro* were washed extensively with saline and then injected into the joints of mice as described previously (Maffia et al., 2004). IL-17A (R&D Systems) or saline and a lentivirus carrying shRNA specific for CCL20, STAT3, or a nontargeting sequence (Sigma-Aldrich) were injected into the joints as described previously (Maffia et al., 2004).

Microbleeding induction. Microbleeding was induced in the joints as described previously (Hakobyan et al., 2008). In brief, the knee joint capsule of each mouse was punctured with a 30-g needle below the patella to induce microbleeding.

Real-time PCRs. A GeneAmp 5700 sequence detection system (ABI) and SYBER green PCR Master Mix (Sigma-Aldrich) were used to quantify the levels of CCL20 mRNA and HPRT mRNA. Total RNA was prepared from mouse embryonic fibroblast cells and synovial tissues using a Gen-Elute Mammalian Total RNA kit (Sigma-Aldrich) and DNase I (Sigma-Aldrich). The PCR primer pairs used for real-time PCRs were as follows: mouse HPRT primers, 5'-GATTAGCGATGATGAACCAGGTT-3' and 5'-CCTCCCATCTCCTTCATGACA-3'; mouse IL-6 primers, 5'-GTGG-CAGGTAGAGCAGGAAG-3' and 5'-CCACCTGAAAGGCACTCTGT-3'; and CCL20 primers, 5'-ACAGTGTGGGAAGCAAGTCC-3' and 5'-CCGT-GAATCCTTTGACCAT-3'. The conditions for real-time PCR were 40 cycles at 94°C for 15 s followed by 40 cycles at 60°C for 60 s. The relative mRNA expression levels were normalized to the levels of HPRT mRNA.

Statistical analysis. Student's *t* tests (two-tailed) were used for statistical analysis of differences between two groups.

Online supplemental material. Fig. S1 shows histology analysis that confirmed arthritis development. Fig. S2 shows cytokine expression in activated nonpolarized CD4⁺ T cells, Th1 cells, and Th17 cells induced *in vitro*. Fig. S3 shows clinical arthritis scores from the left legs of F759 mice transferred with Th1 or with Th17 cells, followed by microbleeding induction or by joint injection of CCL20. Fig. S4 shows the number of IL-17A-expressing Th17 cells presented in the joints of F759 hosts after microbleeding induction. Fig. S5 shows clinical arthritis scores from the right legs of F759 mice or C57BL/6 mice after intravenous injections of Th17 cells followed by CCL20 in the left leg joints. Fig. S6 shows clinical arthritis scores from the right legs in various mouse strains after injections into left leg joints of activated nonpolarized CD4⁺ T cells, Th1 cells, or Th17 cells. Fig. S7 shows that IL-6 expression in type 1 collagen⁺ cells after stimulations with IL-17 and/or IL-22 and clinical arthritis scores from the left legs of F759 mice joint injected with Th17 cells in the presence of lentivirus carrying shRNA for non targeting sequences, STAT3, or IL-22 receptor α . Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20100900/DC1>.

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