Complement drives Th17 cell differentiation and triggers autoimmune arthritis

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Activation of serum complement triggers Th17 cell–dependent spontaneous autoimmune disease in an animal model. In genetically autoimmune–prone SKG mice, administration of mannan or β-glucan, both of which activate serum complement, evoked Th17 cell–mediated chronic autoimmune arthritis. C5a, a chief component of complement activation produced via all three complement pathways (i.e., lectin, classical, and alternative), stimulated tissue–resident macrophages, but not dendritic cells, to produce inflammatory cytokines including IL-6, in synergy with Toll–like receptor signaling or, notably, granulocyte/macrophage colony-stimulating factor (GM-CSF). GM-CSF secreted by activated T cells indeed enhanced in vitro IL-6 production by C5a-stimulated macrophages. In vivo, C5a receptor (C5aR) deficiency in SKG mice inhibited the differentiation/expansion of Th17 cells after mannan or β-glucan treatment, and consequently suppressed the development of arthritis. Transfer of SKG T cells induced Th17 cell differentiation/expansion and produced arthritis in C5aR-sufficient recombination activating gene (RAG)−/− mice but not in C5aR-deficient RAG−/− recipients. In vivo macrophage depletion also inhibited disease development in SKG mice. Collectively, the data suggest that complement activation by exogenous or endogenous stimulation can initiate Th17 cell differentiation and expansion in certain autoimmune diseases and presumably in microbial infections. Blockade of C5aR may thus be beneficial for controlling Th17–mediated inflammation and autoimmune disease.

There is recent evidence that IL-17–secreting CD4+ T cells (Th17 cells) play a key role in autoimmune diseases, such as rheumatoid arthritis (RA) and multiple sclerosis (Harrington et al., 2005; Veldhoen et al., 2006; Korn et al., 2009). It remains unclear, however, how pathogenic self-reactive Th17 cells are generated from naive T cells, and are activated by external or internal stimuli in autoimmune disease.

SKG mice, a mutant of the gene encoding clodronate liposome; IC, immune complex; MASP, mannose-binding lectin-associated serine protease; RA, rheumatoid arthritis; SPF, specific pathogen–free; TLR, Toll-like receptor; TNP, trinitrophenyl.

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expand or trigger the differentiation of arthritogenic Th17 cells (Yoshitomi et al., 2005). Indeed, injection of zymosan, a crude extract of yeast cell wall containing β-glucans or purified β-glucans, such as laminarin, activates innate immunity via Toll-like receptor (TLR) and Dectin-1, and drives preferential differentiation and expansion of Th17 cells, thereby triggering arthritis in SKG mice under a SPF condition (Yoshitomi et al., 2005; LeibundGut-Landmann et al., 2007). Because zymosan is also an activator of the alternative pathway of complement (Mullaly and Kubes, 2007) and β-glucan structure can be recognized by ficolin-L, an initiator of the lectin pathway (Garlatti et al., 2007), it is also likely that complement activation may contribute to triggering Th17-mediated autoimmune disease.

In this report, we show that complement activation via all three pathways (i.e., the lectin, classical, and alternative pathways) and the resulting generation of the common product C5a potently promote the differentiation/expansion of self-reactive T cells to Th17 cells that mediate autoimmune arthritis in SKG mice. The results indicate that exogenous or endogenous stimuli that activate complement can be a triggering cause of Th17-mediated autoimmune disease and that C5a is a key molecular target in controlling Th17-mediated autoimmunity as well as microbial immunity.

RESULTS AND DISCUSSION
Mannan triggers autoimmune arthritis by expanding Th17 cells

We first tested whether mannan, a prototypic activator of the lectin pathway of complement activation, was able to trigger arthritis in SKG mice (Fig. 1, A–E; Fujita, 2002). A single i.p. injection of 20 mg mannan triggered self-sustained chronic arthritis within 2 wk in all of the treated SKG mice but not in BALB/c mice. A small amount (200 µg) also elicited joint swelling, but only in a few small joints and in 50% of SKG mice. IL-17−/− SKG mice were completely resistant to arthritis induction by mannan (Fig. 1 F). The ratio of IL-17+ cells among CD4+ T cells was increased significantly (approximately fourfold) in regional (e.g., popliteal) lymph nodes of mannan-treated SKG mice with arthritis (e.g., in ankles) compared with control PBS-treated SKG mice without arthritis; the ratio also increased significantly, although to a much lesser degree, in mannan-treated BALB/c mice (Fig. 1, G and H). Thus, mannan can enhance the development of arthritogenic Th17 cells and evoke arthritis in SKG mice.

C5aR is essential for driving Th17 cell differentiation and triggering arthritis

C5a, a key common product of all three complement activation pathways, is the most potent complement-derived mediator of inflammation; increases the production of IL-6, TNF, and IL-1 from TLR-stimulated macrophages; and suppresses IL-12 production (Guo and Ward, 2005; Hawlisch et al., 2005; Zhang et al., 2007). To examine possible effects of mannan treatment on C5a production via the lectin pathway, and consequently Th17 cell differentiation and expansion in SKG mice, we prepared SKG mice deficient in C5aR (CD88; Hawlisch et al., 2005). The incidence and severity of arthritis was significantly suppressed in mannan-elicited C5aR−/− SKG mice (Fig. 2 A). The measurement of serum C3a and C5a revealed that the treatment strongly triggered complement activation for the first 3 d, with persisting low level activation over 28 d in mannan-elicited arthritic SKG mice (Fig. S1). Mannan-treated C5aR+/+ SKG mice, when examined 8 wk (Fig. 2, B–E) or 2 wk (Fig. S1) after treatment, showed a marked hypertrophy of the regional lymph nodes (Fig. 2 B), which contained a much higher ratio and absolute number of IL-17+ CD4+ T cells compared with similarly
in particular can commonly activate complement to produce C5a, which critically contributes to evoking autoimmune arthritis in SKG mice. Although the lectin and alternative pathways are stimulated by microbial products such as mannan or zymosan, the classical pathway can be activated by anti-gen–antibody immune complexes (ICs; Guo and Ward, 2005). ICs not only activate complement but also deliver signal through FcγR on APCs (Sylvestre et al., 1996). As such, we asked whether an IgG3-IC, which is known to activate the classical and alternative complement pathways without the involvement of FcγR, would expand Th17 cells in a C5a/C5aR-dependent manner (Fig. 2 I; Díaz de Ståhl et al., 2003). C5aR+/+ and C5aR−/− SKG mice were i.p. injected with trinitrophenyl (TNP) hapten–conjugated BSA alone or IgG3 anti-TNP/TNP-BSA IC. The percentage of IL-17+ cells in the peritoneal CD4+ T cells was assessed on day 7 (n = 4). Horizontal bars are the means.

Figure 2. C5a drives Th17 cell differentiation and triggers arthritis. (A) Joint score of C5aR+/+ or C5aR−/− SKG mice treated by 20 mg mannan. A total of two independent experiments are shown. Error bars are means ± SD of scores. * P < 0.05. (B) Spleen and popliteal lymph nodes of C5aR+/+ or C5aR−/− SKG mice 8 wk after mannan treatment. (C) Intracellular staining of IL-17 and IFN-γ in CD4+ T cells in the popliteal lymph nodes of C5aR+/+ or C5aR−/− SKG mice, as shown in B (numbers indicate percentages). One representative staining out of nine independent experiments is shown. (D and E) Percentages (D) and absolute numbers (E) of IL-17+ cells in CD4+ T cells in each group (n = 9), as shown in C. (F) Joint scores of C5aR+/+ or C5aR−/− SKG mice 8 wk after treatment by laminarin (n = 7) or zymosan (n = 6). (G) C3 deposition assay with laminarin-, zymosan-, or mannan-coated wells incubated with 2% sera from MASP-intact or -null C57BL/6 sera. (H) C5a ELISA for the supernatant of the C3 deposition assay with BALB/c sera, as shown in G. Error bars are means ± SD. (I) C5aR+/+ and C5aR−/− SKG mice were i.p. injected with TNP-BSA alone or IgG3 anti-TNP/TNP-BSA IC. The percentage of IL-17+ cells in the peritoneal CD4+ T cells was assessed on day 7 (n = 4). Horizontal bars are the means.

C5a acts on tissue-resident macrophages to drive Th17 cell differentiation

C5aR was highly expressed on neutrophils (as CD11bhighGr-1low/F4/80− cells) and monocytes/macrophages (as CD11bhigh/F4/80+Gr-1low− cells) in the peritoneal cavity or the spleen of nontreated SKG mice (Fig. 3 A), and in arthritic joints of mannan-treated SKG mice (Fig. 3 B). To determine the cell types that received C5aR signaling and drove Th17 cell differentiation, we cultured naive BALB/c CD4+ T cells with various types of APCs and stimulated them with anti-CD3 in the presence or absence of C5a and/or TGF-β. Notably, treated C5aR−/− SKG mice (Fig. 2, C–E) or BALB/c mice (not depicted).

Similar to mannan treatment, arthritis elicitation by laminarin or zymosan was significantly suppressed in C5aR−/− SKG mice (Fig. 2 F). The C3 deposition assay, in which the amount of activated C3c was quantified by ELISA, showed that laminarin and zymosan activated complement (Fig. 2 G). The C3c deposition was reduced by the use of mannose-binding lectin-associated serine protease (MASP)–null serum, indicating the involvement of the lectin pathway (Fujita, 2002; Iwaki et al., 2006; Takahashi et al., 2008). C5a was indeed detected in the supernatant sera of the C3 deposition assay after complement activation (Fig. 2 H). These results collectively indicate that laminarin, zymosan, and mannan
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(Fig. S2), even in the presence of TGF-β. Without TGF-β, CD4+ T cells cultured with DCs differentiated primarily into IFN-γ+ cells. Addition of C5a slightly decreased the percentage of IFN-γ+ cells but did not evoke Th17 cell differentiation (Fig. 3 D).

To determine whether the induction of Th17 cells by C5a in CD4+ T cell/macrophage co-culture depended on a direct effect of C5a on CD4+ T cells or macrophages, we performed criss-cross co-cultures with C5aR+/+ or C5aR−/− CD4+ T cells and C5aR+/+ or C5aR−/− macrophages in the presence of TGF-β (Fig. 3 E). Intracellular cytokine staining of CD4+ T cells cultured with C5aR+/+ or C5aR−/− macrophages suggested that cytokines produced by C5a-stimulated macrophages were responsible for inducing Th17 cell differentiation. Indeed, C5a elicted a dose-dependent production of large amounts of IL-6 and, to a lesser degree, TNF and IL-1β from resident peritoneal macrophages (Fig. 3 F). Production of IL-23, which is essential for the survival and expansion of Th17 cells, was up-regulated at the mRNA level in C5a-treated macrophages, although the cytokine was below the detection limit (30 pg/ml) of ELISA (Fig. 3 F). C5a also up-regulated co-stimulatory molecules such as CD86 on C5aR+/+ macrophages but not on C5aR−/− macrophages (Fig. 3 G; Strainic et al., 2008).

C5a drives Th17 cell differentiation in synergy with GM-CSF or TLR signaling

Next we asked whether the in vitro robust expansion of Th17 cells was mediated by C5a alone or by a synergy of C5a and LPS, because the recombinant C5a we used contained a trace amount of contaminated LPS, which could synergistically...
act to drive Th17 cell differentiation (see Materials and methods; Fang et al., 2009). To dissect TLR-dependent and -independent effects, we used macrophages from TLR4−/− or MyD88−/− mice. Although a deficiency of TLR4−/− or MyD88−/− in macrophages substantially reduced the C5a-mediated expansion of Th17 cells, a significant proportion of Th17 cells still developed (Fig. 4 A). Further, C5a derived from LPS-free human plasma expanded Th17 cells (unpublished data; Köhl, 1997). Notably, when TLR4−/− or MyD88−/− macrophages alone were stimulated by C5a, IL-6 production was nearly abolished (Fig. 4 B). However, in co-culture with anti-CD3-stimulated IL-6−/− CD4+ T cells, C5a significantly enhanced IL-6 production by TLR4−/− or MyD88−/− macrophages, although much less potently compared with wild types (Fig. 4 B).

To analyze how T cells contributed to the C5a-induced IL-6 production by macrophages, we assessed the effect of co-stimulatory molecules and T cell–derived cytokines that could alter macrophage function (Grabstein et al., 1986). IL-6 production was partially inhibited by blockade of CD40L (Fig. S3; Hirota et al., 2007). When TLR4−/− or MyD88−/− macrophages alone were stimulated with C5a in the presence or absence of various cytokines (e.g., IL-17, IL-21, IFN-γ, and GM-CSF), only GM-CSF significantly enhanced IL-6 production even at a low concentration (e.g., 1 ng/ml; Fig. 4 C and Fig. S3; Sonderegger et al., 2008). Although freshly iso-

Figure 4. C5a drives Th17 cell differentiation in synergy with TLR or GM-CSF. (A) C57BL/6 CD4+ T cells were cultured with TLR4−/−, MyD88−/−, or wild-type C57BL/6 macrophages in the presence of TGF-β with or without C5a. (B) Macrophages alone from these mice or co-cultured with anti-CD3-stimulated CD4+ T cells from IL-6−/− mice were stimulated with C5a overnight. IL-6 in the supernatant was determined by ELISA. (C) MyD88−/− macrophages were stimulated by C5a overnight in the presence or absence of cytokines at the indicated doses. IL-6 in the supernatant was determined by ELISA (triplicates). Error bars are means ± SD. (D) Freshly isolated BALB/c or SKG splenic CD4+ T cells were stained for intracellular IL-17 and GM-CSF. (E) BALB/c CD4+ T cells were cultured with macrophages in the presence or absence of C5a and/or TGF-β, stimulated with anti-CD3, and stained for intracellular cytokines on day 3. Results in A–E represent three independent experiments. Numbers in A, D, and E indicate percentages.

Collectively, the results in Fig. 3 and Fig. 4 indicate that C5a acts on macrophages in the joint and other tissues to provoke their production of IL-1, IL-6, TNF, and IL-23, and together with tissue TGF-β, promotes the differentiation and expansion of self-reactive T cells into Th17 cells. C5a stimulates macrophages to produce IL-6 in at least two ways: via synergy with TLR signaling and via T cell–macrophage interaction. In the latter, GM-CSF produced by activated T cells enhances IL-6 production by C5a-stimulated macrophages, indicating a novel pathway of promoting Th17 cell differentiation and expansion.

Macrophages are required for in vivo Th17 expansion and induction of arthritis

To determine the role of macrophages in vivo, we treated SKG mice with clodronate liposome (CL), which specifically depletes monocytes and macrophages (Solomon et al., 2005). The treatment indeed efficiently depleted C5ar1+ monocytes/macrophages without affecting neutrophils or DCs in SKG mice (Fig. 5 A). CL administration before mannan injection markedly attenuated the development of arthritis (Fig. 5 B) and reduced the expansion of Th17 cells (Fig. 5 C).

C5ar1 signaling promotes spontaneous differentiation of CD4+ T cells to Th17 cells via homeostatic proliferation

Similar to innate immune stimulation by microbial products, aseptic stimulation of SKG self-reactive T cells (e.g., via homeostatic proliferation in a lymphopenic environment) evokes
spontaneous differentiation of Th17 cells and triggers autoimmune arthritis (Hirata et al., 2007). Because T cell–APC interaction induces local complement activation (Liu et al., 2008; Strainic et al., 2008), we asked whether such intrinsic complement activation would contribute to Th17 cell differentiation and arthritis induction. In RAG2−/− mice that developed arthritis after transfer of SKG CD4+ T cells, the joint tissue actively transcribed C3 and IL-6 mRNA, indicating complement activation and IL-6 production in the affected joint (Fig. 6 A). When CD4+ T cells from C5aR+/+ or C5aR−/− SKG mice were transferred to C5aR+/+ or C5aR−/− RAG2−/− mice, both CD4+ T cell populations induced arthritis at equivalent incidences and severities in C5aR+/+ RAG2−/− mice but were significantly lower in C5aR−/− RAG2−/− mice (Fig. 6 B). Thus, C5aR expression by the recipient cells, not donor CD4+ T cells, was required for disease induction. In accordance with the joint scores, the generation of Th17 cells from transferred CD4+ T cells was significantly suppressed in C5aR−/− recipients (Fig. 6 C and D). The generation of IFN-γ-secreting cells was not significantly affected, although there was a tendency toward a higher proportion of IFN-γ+ cells in C5aR−/− recipients. Inhibition of Th17 cell differentiation by C5aR deficiency was not restricted to SKG CD4+ T cells, as Th17 cell differentiation of BALB/c CD4+ T cells during homeostatic proliferation was also suppressed in C5aR-deficient recipients (Fig. 6 E). Collectively, these results indicate that interactions between self-reactive T cells and APCs can preferentially drive the differentiation of the former into Th17 effector cells via complement activation and the resulting C5a action on APCs.

Thus, extrinsic and intrinsic complement activation promotes Th17 cell differentiation and expansion, evoking autoimmune arthritis in SKG mice (Fig. 6 F). It was noted, however, that C5aR deficiency significantly suppressed but did not completely inhibit arthritis development triggered by laminarin, zymosan, or mannan (Fig. 2). This incomplete inhibition could be attributed to the fact that these microbial products not only activate complement but also directly stimulate macrophages and DCs via cell surface–expressed pattern recognition receptors such as TLR and C-type lectin receptors (e.g., Dectin-1, mannose receptor, and DC-SIGN; Yoshitomi et al., 2005; Robinson et al., 2006; Sheng et al., 2006). Indeed, in our coculture experiments with macrophages, zymosan, laminarin, or mannan at a high dose (1 mg/ml) expanded Th17 cells, presumably via IL-6 production in an MyD88-dependent and –independent manner (Fig. S4). In addition, we observed a synergy between the signals from C5aR and MyD88 for cytokine production by macrophages (Fig. 4 A; Fang et al., 2009). Yet, it is of note that the prototypic TLR agonist LPS or CpG alone failed to elicit arthritis in SKG mice, in contrast to successful arthritis induction by mannan, a prototypic complement activator (Yoshitomi et al., 2005). This indicates that complement activation and C5a production can be a major pathway for driving Th17-dependent autoimmune arthritis in SKG mice.

Because C5a, IL-17, and GM-CSF are commonly capable of enhancing granulopoiesis and neutrophil recruitment (Höpken et al., 1996; Korn et al., 2009), together they would cause robust neutrophil accumulation (Fig. 3 B) and form a positive feedback loop of Th17-mediated inflammation (Fig. 6 H; Sonderegger et al., 2008). In addition, the cartilage surface lacks several complement inhibitors, which might render the joint highly susceptible to complement activation (Matsumoto et al., 2002). It is thus likely that transient synovial inflammation may frequently occur when an individual is exposed to complement-activating microbial products, ICs, autoantibodies, or physical trauma. Such synovial inflammation per se may not be sufficient to trigger chronic arthritis in normal individuals. Yet, if an individual harbors potentially arthritogenic CD4+ T cells (e.g., because of genetic predisposition), such complement-induced synovial inflammation may promote the differentiation/expansion of arthritogenic Th17 cells and instigate chronic arthritis. It is worth noting in this regard that genetic susceptibility to RA is in part determined by the polymorphism of the genes encoding C5 (TRAF1-C5), PTPN22 (which affects TCR proximal signaling, as observed with the SKG ZAP-70 mutation), or STAT4, which might alter Th17 cell function (Vang et al., 2005; Plenge et al., 2007; Remmers et al., 2007). These genetic polymorphisms could promote the production of arthritogenic T cells and their Th17 cell differentiation via
complement activation. Although C5aR signaling plays a complex role depending on the cell types (e.g., macrophages or DCs) and additional receptors (e.g., TLRs; Guo and Ward, 2005; Weaver et al., 2010), this report provides evidence that complement activation and C5a production is critically involved in the initiation of certain autoimmune disease, and presumably microbial immunity, by driving Th17 development.

MATERIALS AND METHODS

Mice. C5aR−/−, IL-17−/−, IL-6−/−, RAG2−/−, TLR4−/−, and MyD88−/− mice (Iwaki et al., 2006; Takahashi et al., 2008) were described previously (Hawlisch et al., 2005; Hirota et al., 2007). MASP-null mice were generated by crossing MASP1/3 (Yoshitomi et al., 2005; Hirota et al., 2007) and MASP2/sMAP (Zhang et al., 2007). Splenic DCs were sorted for CD11chigh cells in the Institute for Frontier Medical Sciences, Kyoto University.

Reagents. Recombinant mouse C5a was purchased from R&D Systems. Mannan from Saccharomyces cerevisiae was purchased from Sigma-Aldrich, and was dissolved in 200 µl PBS before i.p. injection. The endotoxin level in the endotoxin level in the reagents used in the current study was 0.44 EU/ml for 1 µg/ml C5a and 6.14 EU/ml for 1 mg/ml mannan, as determined by the LAL test (Wako Chemicals USA, Inc.). Plasma-derived C5a was purified from human EGTA-plasma as previously described (Köhler, 1997). CL was a gift from Roche, and the liposomal preparation was prepared as previously described (Solomon et al., 2005).

Antibodies. For flow-cytometric analysis, anti–CD4 (RM4-5), anti–IL-17 (TC11-18H10.1), anti–IFN-γ (XMG1.2), anti–GM-CSF (MIP-1-22E9) was purchased from BD; anti-C5aR (20/70) was purchased from Cedarlane; and anti-F4/80 (A3-1) was purchased from AbD Serotec.

C3 deposition assay. Maxi-Plates (Thermo Fisher Scientific) were coated with 100 µg/ml laminarin, zymosan, or mannan. After blocking, wells were incubated with 2% mouse sera diluted with TBS/Tween/Ca (0.1% BSA, 0.05% Tween 20, 5 mM CaCl2) at 37°C for 30 min. C3c deposited on the well surfaces was detected by anti–human C3c (Dako) followed by horseradish peroxidase–conjugated secondary antibody (Dako), and assessed by ELISA. Supernatants from the C3c deposition assay were assessed for the concentration of C5a by ELISA and expressed as the concentrations in the original sera.

Quantitative RT-PCR. Total RNA of peritoneal macrophages was extracted 24 h after incubation with 500 ng/ml C5a using the RNeasy column.
Total RNA of joint tissues was extracted by Isogen (Wako). Gene expression was normalized to expression of the HPRT gene.

**Clinical assessment of joint scores, intracellular cytokine staining, and preparation of synovial cells.** These were performed as described previously (Hirota et al., 2007).

**Statistical analysis.** The in vivo joint scores were analyzed by the Mann-Whitney U test. Unless otherwise mentioned, the Student’s t test was used for statistical analysis. P < 0.05 was considered significant.

**Online supplemental material.** Fig. S1 shows C3a/C5a production and early Th17 cell expansion after manifold treatment. Fig. S2 depicts the dependency of C5a/TGF-β–induced Th17 cell development on the type of APCs. Fig. S3 shows the effect of co-stimulation and T cell–derived cytokines on IL-6 production by C5a-stimulated macrophages. Fig. S4 depicts TLR-dependent IL-6 production by lammarin, zymosan, or mannan. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092301/DC1.

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