Although IL-6 Trans-Signaling Is Sufficient To Drive Local Immune Responses, Classical IL-6 Signaling Is Obligate for the Induction of T Cell-Mediated Autoimmunity


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Although IL-6 Trans-Signaling Is Sufficient To Drive Local Immune Responses, Classical IL-6 Signaling Is Obligate for the Induction of T Cell-Mediated Autoimmunity

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IL-6-mediated T cell-driven immune responses are associated with signaling occurring through the membrane-bound cognate receptor α-chain (mIL-6Rα). Once formed, IL-6–mIL-6Rα complexes induce the homodimerization and subsequent phosphorylation of the ubiquitously expressed signal-transducing protein, gp130. This signaling event is defined as classical IL-6 signaling. However, many inflammatory processes assigned to IL-6 may be mediated via binding a naturally occurring soluble IL-6Rα, which forms an agonistic complex (IL-6/soluble IL-6Rα) capable of evoking responses on a wide range of cell types that lack mIL-6Rα (IL-6 trans-signaling). To dissect the differential contribution of the two IL-6 signaling pathways in cell-mediated inflammatory processes, we pharmacologically targeted each using two murine models of human arthritis. Whereas intra-articular neutralization of trans-signaling attenuated local inflammatory responses, the classical pathway was found to be obligate and sufficient to induce pathogenic T cells and humoral responses, leading to systemic disease. Our data illustrate that mechanisms occurring in the secondary lymphoid organs underlying arthopathies are mediated via the classical pathway of IL-6 signaling, whereas trans-signaling contributes only at the local site, that is, in the affected tissues. The Journal of Immunology, 2010, 185: 000–000.

Cytokines are essential for orchestrating immune responses to pathogens, often dictating the potency of inflammatory outcomes. In autoimmunity, dysregulation of this cytokine network contributes to inflammation-induced tissue damage and drives progression of chronic disease (1, 2). The inflammatory cytokine IL-6 represents an integral player in these processes, as illustrated by experimental models in IL-6-deficient mice (3–5), and clinical trials in which its neutralization has pronounced therapeutic benefit for patients suffering from Castleman’s disease, rheumatoid arthritis (RA), and cancer-associated cachexia (6, 7). However, despite these numerous studies, our understanding of the mechanisms underlying IL-6–driven events remains undefined, in part, due to the complex nature of IL-6R signaling.

The IL-6R complex consists of a membrane-bound IL-6Rα (mIL-6Rα) 80-kDa cognate receptor (CD126) and a 130-kDa signal-transducing (gp130, CD130) element (8). Although CD126 expression is confined to hepatocytes and subpopulations of leukocytes, IL-6 activity is also mediated by the naturally occurring soluble IL-6Rα (sIL-6Rα) (9, 10). Together, they form a IL-6–sIL-6Rα complex that activates cells via the ubiquitously expressed gp130 (10). This process is called IL-6 trans-signaling and activates IL-6-type responses in cells lacking mIL-6Rα, including vascular endothelial cells, peritoneal mesothelial cells, and synovial fibroblasts (9, 10). This alternative activation pathway has been linked to the regulation of leukocyte recruitment, activation, and, thus, maintenance of inflammatory processes (11, 12).

The difficulty in attributing an IL-6 response to classical versus trans-signaling events is partially due to a lack of selective reagents. Whereas insightful studies using the recombinant soluble gp130-Fc fusion protein have implicated trans-signaling events in the inflammatory control of T cell recruitment (12, 13), the specificity of this construct for other gp130-binding proteins beyond IL-6 requires further investigation.

The current study extends our understanding of IL-6 involvement in T cell-mediated inflammation, as we have generated two anti–IL-6Rα mAbs (mAb 2B10 and mAb 25F10) that, through independent modes of IL-6Rα neutralization, enable the functional characteristics to be distinguished between classical versus trans-signaling. Using models of inflammation, collagen-induced arthritis (CIA) and Ag-induced arthritis (AIA), we have for the first time, to our knowledge, dissected the contribution of classical and trans-IL-6 signaling pathways on disease pathogenesis. To this end, the CIA model was used to assess the systemic role of...
IL-6 that occurs in draining lymph nodes (dLNs), and is required for priming autoaggressive T and B cell responses. The classical IL-6 pathway involving mIL-6Rα–bearing Th cells was found to be both sufficient and obligate for developing pathogenic Th17 cells. In addition, this pathway was also required for the generation of anti-Type II collagen (CII) IgG responses, associated with disease manifestation. In parallel, using the AIA model, the role of IL-6 in driving local (e.g., in the joint) inflammation was investigated. In contrast to systemic responses modeled in CIA, we observed that local blockade of the trans-signaling–driven joint inflammation is sufficient to control disease. Collectively, this study maps the in vivo dichotomy between IL-6–mediated pathways in inflammatory disease models and, thus, evokes the use of more effective therapeutic strategies targeting both classical and trans-signaling–driven events in human inflammatory diseases.

Materials and Methods

Mice

Experiments were undertaken in adult 8-wk-old male DBA/1J (Janvier Laboratories, Le Genest-St-Isle, France) and C57BL/6J male mice (Charles River Laboratories, L’Arbresle, France). All animal experimentation was conducted according to licenses from the Swiss veterinary office for animal experimentation or according to the United Kingdom Home Office–approved project license PPL-30/1820.

Generation of recombinant proteins and transfected cell lines

To generate the IL-6/soluble IL-6Ra fusion protein (IL-6/sIL-6Ra FP), cDNA encoding the extracellular region of the mouse mIL-6Ra (NM_010559, aa 1–364) was amplified by PCR and a synthetic DNA linker encoding the repeated motif S-(G1)-S-(G2)-S introduced at the C terminus. The resulting fragment was ligated to cDNA encoding mouse IL-6 (NM_031168, aa 25–211) and a C-terminal Avi-His-Tag motif. Recombinant proteins, sIL-6Ra and IL-6, were purchased from R&D Systems (Minneapolis, MN). Biotinylation of proteins was performed using the biotin protein labeling kit from Roche Applied Science (Rotkreuz, Switzerland). The cDNA encoding mouse IL-6Ra (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov] reference sequence: NM_010559.2) was recloned into the pCDNA3.1 mammalian expression vector (Invitrogen, Basel, Switzerland), whereas cDNA encoding mouse IL-6 (National Center for Biotechnology Information reference sequence: NM_031168.1) and IL-6mIL-6Ra FP were reclined into the pDISPLAY vector (Invitrogen). To generate stable transfectants, expression vectors described above were linearized and transfected into Chinese hamster ovary (CHO) cells by using the TransIT-LT1 transfection reagent (Mirus, Madison, WI).

Generation of mAbs to mouse IL-6Ra

Male Wistar rats (Charles River Laboratories) were immunized three times i.p. at 3-wk intervals with 10^6 CHO cells expressing high levels of mouse mIL-6Ra (for the generation of 2B10) or IL-6/mIL-6Ra FP (for 2F5), in monophosphoryl lipid A plus synthetic trehalose dycorynomycolate adjuvant (Sigma-Aldrich, St. Louis, MO), followed by a s.c. hyperboost with either 10 μg sIL-6Ra (for 2B10) or the IL-6/sIL-6Ra FP (2F5). Three days later, a fusion was performed between splenocytes and the Sp2/0 myeloma fusion partner, as previously described (14). Subsequent screening of hybridomas was performed on mock-transfected CHO cells, or CHO cells expressing mL-6Ra or IL-6/sIL-6Ra FP, using the 8200 cellular detection system (Applied Biosystems, Zug, Switzerland).

IL-6 functional assays

To evaluate classical IL-6 signaling, the murine plasmacytoma T165 cell line was used. This cell line was maintained in RPMI 1640 medium containing 10% FCS, 2 mM glutamine, 50 μM 2-ME, 50 μg/ml gentamicin, and 2 mg/ml mouse rIL-6. For the assay, cells were washed and incubated at a concentration of 10^5 cells/well in a 96-well tissue culture plate for 48 h, with 1 ng/ml mouse IL-6, and in the presence of 2B10, 2F50, or a rat IgG1 isotype control mAb (clone mAb 35; American Type Culture Collection, Molsheim, France). The colorimetric WST-1 Roche Diagnostics reagent (Mannheim, Germany) was added to each well before incubation at 37°C for 4 h, and cell proliferation was measured with an ELISA plate reader, according to the manufacturer’s instructions. For the IL-6 trans-signaling assay, the transformed human embryo kidney mono-layer epithelial (PEAK) cells (Edge Biosystems, Gaithersburg, MD), negative for mIL-6Ra expression, were used. These cells were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, and 50 μg/ml gentamicin. Cells were transfected with 0.2 μg/well STAT3-dependent and constitutive reporter plasmids using the TransIT-LT1 transfection reagent. Transfected cells were activated with 500 ng/ml IL-6/sIL-6Ra FP. Medium was recovered 18 h later, and luciferase assays were performed using the Dual-Glo System (Promega, Madison, WI) in a chemoluminescence analyzer, according to the manufacturer’s instructions.

ELISA

Immunoplates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight with 1 μg/ml 2B10, 25F10, or rat IgG1 mAbs. Washing and blocking steps were performed with PBS, containing 0.05% Tween 20. Various concentrations of biotinylated sIL-6Ra or IL-6/sIL-6Ra FP, diluted in blocking buffer, were added, and plates were incubated for 1 h at 37°C. Then plates were washed five times, and the streptavidin-HRP conjugate (Sigma-Aldrich) diluted to 1/4000 was added. After 30 min of incubation, plates were washed seven times, and 3,3',5,5'-tetramethylbenzidine substrate (T5569; Sigma-Aldrich) was added to each well. Absorption was measured at 450 nm using a microplate reader after adding H2SO4. Matched Ab pairs for quantification of IL-6 and sIL-6Ra were obtained from R&D Systems. Serum amyloid A (SAA) levels in mice sera were measured using the Invitrogen mouse SAA kit.

Flow cytometry

For cell surface or intracellular cytokine labeling, the following anti-mouse mAbs were used (from BD Biosciences, unless stated): FITC-conjugated anti-CD4 (clone IM7), anti-CD90/Pan-NK (DX5), and T and B cell activation and costimulatory agent CAM (G17); PE-conjugated anti-CCR7, anti-ICOS, and anti-CXCR3 (clone HEL3); anti-CD95 (1D3), anti-CD80 (53-67), anti-IgD (11-26c2a), and anti-B220 (RA3-6B2); PerCP-conjugated anti-CD4 (RM4.5), anti-CD19 (1D3), and anti-CD3 (145-2C11); PerCP-Cy5.5–conjugated anti-CD11b (M1/70); and allophycocyanin-conjugated anti-CD138 (281-2) and -CD4 (RM4-5). Anti-mouse neutrophil FITC (MCA717FA) and anti–IFN-γ FITC (clone 1A7) were purchased from BD Serotec (Oxford, U.K.). For intracellular staining, cells were treated with 50 ng/ml PMA, 500 ng/ml ionomycin, and 3 μM monensin (Sigma-Aldrich) for 4 h at 37°C before analysis. Cells were incubated with Fe block (BD Biosciences) to reduce nonspecific binding. Cells were incubated with anti-CD4 PerCP (RM4.5; BD Biosciences) before fixation and permeabilization in BD Cytofix/Cytoperm (BD Pharmingen). For intracellular staining, cells were purchased from ABD Serotec (Oxford, U.K.). For intracellular staining, cells were treated with 50 ng/ml PMA, 500 ng/ml ionomycin, and 3 μM monensin (Sigma-Aldrich) for 4 h at 37°C before analysis. Cells were incubated with Fe block (BD Biosciences) to reduce nonspecific binding. Cells were incubated with anti-CD4 PerCP (RM4.5; BD Biosciences) before fixation and permeabilization in BD Cytofix/Cytoperm (BD Biosciences). The intracellular localization of the cytokines IL-17A and IFN-γ was detected using an anti-IL-17A PE (clone TC11-18H10; BD Biosciences) and an anti–IFN-γ allophycocyanin (clone XMGI2.1; BD Biosciences), respectively. Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences) and analyzed by the CellQuestPro software (BD Biosciences).

Induction of CIA

Lyophilized bovine CII (MB Biosciences, Zurich, Switzerland) was dissolved overnight at 4°C in 0.1 N acetic acid at 2 mg/ml and emulsified in an equal volume of CFA (2 mg/ml Mycobacterium tuberculosis). B10 (H-2d, H-2k; Charles River, W. Ff.); B10.D2 (H-2d, H-2k; Charles River, W. Ff.; and C3H/HeJ (H-2d, H-2k; Jackson, CA). Male DBA/1J mice were immunized intradermally at day 0 with 100 μl emulsion at the base of the tail (100 μg bovine CII). After 21 d, mice received a secondary intradermal immunization with 100 μg bovine CII emulsified in IFA. The 2B10, 25F10, or control IgG1 mAbs were given i.p. on days 0, 2, and 5 following the first bovine CII immunization. Clinical scores were assessed immediately after the secondary immunization, as severity was measured as the overall assessment of inflammation on individual paws, applying a scale ranging from 0 to 4. Each paw was graded according to the following system: 0, no inflammation; 1, swelling of at least one digit; 2, swelling of all the digits
and inflammation of the paw; 3, severe inflammation of whole paw and digits or ankylosis; 4, necrosis. The sum of these four individual scores is represented as the total clinical score (i.e., with the maximal clinical score of 16 for an individual mouse).

**Induction of murine AIA**

AIA was initiated and assessed, as previously described (15). Briefly, adult C57BL/6J male mice were immunized s.c. with 100 μl 1 mg/ml methylated BSA (mBSA) emulsified in an equal volume of CFA (Sigma-Aldrich), and injected with 100 μl i.p. heat-inactivated *Bordetella pertussis* toxin (Sigma-Aldrich). These mice were boosted 1 wk later with mBSA/CFA. Three weeks later, mice received an intra-articular (i.a.) injection in the hind right limb of 100 μg mBSA with or without 20 μg 2B10, 25F10, or control rlgG1 mAbs. In all cases, a total volume of 10 μl was administered i.a.

**Histological assessment of arthritis**

In the CIA model, at least four animals per group were sacrificed at experimental end point (day 39), and the joints were collected and fixed in 10% neutral buffered formalin (Sigma-Aldrich) at room temperature for 10 d and rinsed in PBS, and fixed tissues were decalcified in an EDTA-based neutral buffered formalin (Sigma-Aldrich) at room temperature for 10 d and fixed tissues were decalcified in an EDTA-based decalcifying solution (Osteosoft, Merck, Darmstadt, Germany). Three weeks later, samples were rinsed in PBS, and fixed tissues were decalcified in an EDTA-based decalcifying solution (Osteosoft, Merck, Darmstadt, Germany). Three weeks later, samples were rinsed in PBS, dehydrated, and embedded in paraffin blocks using an automated tissue processor (Microm, STP 120). Serial mid sagittal sections (8 μm thickness) of the whole knee joint were stained with either H&E or safranin O/fast green counterstaining. Histological sections were then graded independently by two independent observers blinded to the experimental groups. A histological score was assessed using an established scoring system for synovial inflammation (0 = synovium with no hyperplasia to 3 = severe synovial hyperplasia) and for cellular infiltration in the synovium (0 = no cell infiltration to 3 = severe infiltration). To evaluate proteoglycan staining, the surface area of the whole cartilage and the surface area of the safranin O/fast green staining were calculated from serial sections of knees using Image J software. Cartilage degradation was evaluated as a percentage of loss of safranin O staining as compared with sham mice.

In the AIA model, animals were sacrificed 3 d after the Ab treatment and procured as described for the CIA model. The histological score was assessed using an established scoring system for synovial inflammation (0 = normal to 5 = extensive subsynovial leukocyte infiltration), joint exudate (0 = normal to 3 = substantial number of inflammatory cells and fibrin deposits visualized in the joint space), synovial hyperplasia (0 = normal to 3 = majority of synovial lining affected with pannus formation encroaching on to articular surface), and bone erosions (0 = normal bone architecture to 3 = severe).

**Measurement of anti-CII Ab**

Anti-bovine CII Ab levels in sera were assessed by ELISA. Briefly, serially diluted serum samples were added to each well of bovine CII (2 μg)- precoated immunoplates (Maxisorp, Nunc, Roskilde, Denmark). After incubation for 2 h at 37°C, plates were washed with PBS/0.05% Tween 20 and bound IgG was measured using peroxidase-conjugated Ab to mouse IgG (Jackson ImmunoResearch Europe, Suffolk, U.K.). Sera from non-immunized syngenic mice were used as a negative control. Serum IgG were purified from sera of CII-immunized mice using protein G. Quantification of CII-specific IgG from 2B10-, 25F10-, and rlgG1-treated mice was performed using the mouse IgG anti-bovine CII Ab ELISA kit from MD Biosciences.

**Surface plasmon resonance analysis**

The affinity and binding kinetics of 2B10 and 25F10 mAbs were performed using a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden). mAbs were captured by using 1500 response units of a goat anti-rat IgG Fc (Jackson ImmunoResearch Europe) immobilized to 1 mg/ml methylaminopropyl) carbodiimide hydrochloride/N-Hydroxysuccinimide chem-

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**FIGURE 1.** Characterization of anti-mouse IL-6Rα mAbs. A and B, Binding of anti–IL-6Rα mAbs (2B10 and 25F10) and isotype control (rlgG1) on biotinylated sIL-6Rα (A) or IL-6/sIL-6Rα FP (B), as measured by ELISA. C, Inhibition of IL–6–dependent proliferation of T1165 cells. Cells were stimulated with 1 ng/ml IL-6 for 48 h at 37°C, and the proliferation in the presence or absence of 2B10 or 25F10 was assessed using the WST-1 reagent. Data represent percentage of inhibition of cell proliferation compared with the rlgG1 isotype control mAbs (mean ± SEM; n = 3). D, Inhibition of IL-6/sIL-6Rα–mediated STAT3 activation in PEAK cells. Cells were transfected with a vector expressing a luciferase reporter gene containing a STAT3 consensus binding site linked to a minimal thymidine kinase promoter. Cells were stimulated with an IL-6/sIL-6Rα FP (500 ng/ml) in the presence or absence of 2B10 or 25F10 for 48 h at 37°C, and STAT3 activity was monitored using a luciferase assay. Data represent the percentage of inhibition of luciferase activity as being a direct measure of STAT3 induction. In this assay, no inhibition equaled 45,000 U of fluorescence intensity, whereas 100% of IL-6 signaling blockade resulted in 8,000 U (mean ± SEM; n = 3 experiments). E, Splenocytes from naive wild-type mice were cultured with anti-CD3/anti-CD28 Abs and the indicated cytokines, IL–6 and TGF–β (upper panel) or IL-6/IL-6Rα FP and TGF–β (lower panel), for 4 d. The 2B10, 25F10, or isotype control rlgG1 (10 μg/ml) were added, as indicated. After 4 d, intracellular staining for IL-17 and IFN-γ was performed and analyzed by flow cytometry. Flow cytometry plots are representative of three independent experiments.
on a CM5 chip. The purified IgG samples were injected at 10 μg/ml for 30 min, and cells were cultured at a density of 5 × 10^6 cells/ml in RPMI 1640 with 2 mM L-glutamine plus 10% FCS, 50 μg/ml gentamicin, 1 mM sodium pyruvate, and 50 μg/ml gentamicin. Supernatants were collected after 72 h.

Cytokine measurements

The cytokines IL-6, IL-17A, and IFN-γ in cell culture supernatants were measured using the Milliplex mouse cytokine 3plex (Millipore, Billerica, MA).

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance was assessed using the one-tailed nonparametric Mann-Whitney U test (GraphPad Prism Software, San Diego, CA) or the two-way ANOVA 1. Value of p < 0.05 was considered as statistically different.

Gene expression

Total RNA from mouse dLNs was extracted with the RNeasy mini kit (Qiagen, Valencia, CA), following the manufacturer’s instructions. Reverse transcription was performed with 1 μg total RNA. Quantitative PCR was conducted in duplicates (95˚C, 10 min, then 40 cycles of 95˚C, 15 s and 60˚C, 60 s). The primer sequences were available upon request. The relative expression levels were calculated as 2^(-ΔΔCt GAPDH - Ct gene) with GAPDH RNA as the endogenous control. Five mice were analyzed per experimental group, and the data in the figures represent the mean relative expression from each group.

Results

Generation and characterization of anti-mouse IL-6Rα mAbs

To address the contribution of IL-6–mediated pathways in vivo, neutralizing mAbs were generated. Male Wistar rats were immunized with various immunogens, including CHO-transfected cells expressing mIL-6Rα or the IL-6/sIL-6Rα FP, rIL-6Rα, and IL-6/sIL-6Rα FP. The supernatants produced from hybridomas, generated from the fusion of splenocytes and Sp2/0 myeloma cells, were screened using flow cytometry for binding to IL-6, mIL-6Rα, and IL-6/mIL-6Rα CHO transfectants. Positive clones were then expanded and tested for their capacity to neutralize IL-6 activity in vitro. Two clones, 2B10 and 25F10, were chosen for further characterization. In an ELISA format, immobilized mAbs were able to capture biotinylated sIL-6Rα with equivalent potencies (Fig. 1A), as demonstrated by the relative EC_{50} values for 2B10 (17 ng/ml) and 25F10 (33 ng/ml). These ELISA data were subsequently confirmed by Biacore analysis, which showed that the Abs had equivalent affinities for sIL-6Rα (Table I). However, only 25F10 demonstrated a binding activity for the IL-6/sIL-6Rα FP, with an EC_{50} of 158 ng/ml (Fig. 1B) and an affinity of 2.98 × 10^-9 M (Table I). To further characterize the mAbs, functional assays to distinguish signaling via the mIL-6Rα and IL-6/sIL-6Rα FP were established using cell lines that were either responsive to classical mIL-6Rα signaling (mouse T1165 placmytoma cells) or IL-6 trans-signaling (HEK 293-derived PEAK cells). Only 2B10 neutralized the IL-6–dependent proliferation of the mIL-6Rα–bearing T1165 mouse placmytoma cell line (Fig. 1C). Conversely, only 25F10 was able to neutralize gp130-mediated STAT3 activity in HEK 293-derived PEAK cells following IL-6/ sIL-6Rα FP stimulation (Fig. 1D). As IL-6/TGF-β–mediated expansion of Th17 cells is considered a STAT3-driven process (16, 17), naive CD4+ T cells were cultured in conditions to induce Th17 commitment. The 2B10 was able to suppress IL-6–dependent Th17 cell generation (0.07%) as compared with rIgG1 (4%) or 25F10 (2.5%)–cultured cells (Fig. 1E, upper panels). In contrast, 25F10 blocked Th17 generation mediated by IL-6/sIL-6Rα FP (0.6%) as compared with 2B10 (2.5%) or rIgG1 (4%) (Fig. 1E, lower panels). Collectively, these data illustrate the generation of mAbs selectively targeting either mIL-6Rα (2B10)

![Classical signaling](#) (IL-6/sIL-6Rα) and trans-signaling (IL-6/sIL-6Rα FP)

**Figure 2.** Schematic representation depicting mAb 2B10 and 25F10 mode of action. The 2B10 binds to both membrane and soluble forms of IL-6Rα, thus inhibiting IL-6–mediated activation. Therefore, mAb 2B10 blocks the classical IL-6 signaling via mIL-6Rα (left panel) and inhibits the formation of the IL-6–sIL-6Rα complex, thus neutralizing IL-6 trans-signaling (middle panel). However, 2B10 is unable to bind and, hence, neutralize trans-signaling mediated by the IL-6/sIL-6Rα FP (right panel). Conversely, whereas mAb 25F10 binds to both forms of IL-6Rα, only trans-signaling mediated by IL-6/sIL-6Rα FP is neutralized (middle and right panels). PM, plasma membrane.
or the IL-6/sIL-6R FP (25F10). Taken together, the data suggest distinct modes of action for the two mAbs targeting IL-6Rα, and a schematic representation is shown to illustrate the dichotomy (Fig. 2).

**IL-6 trans-signaling is sufficient to drive local immune responses in AIA**

Prior experiments using AIA have shown that IL-6 influences both the induction and effector phase of inflammatory arthritis (12, 18–20). These investigations have pointed toward a role for IL-6 trans-signaling in the local regulation of IL-6 activity within the inflamed synovium (12, 21, 22). Based on these observations, initial in vivo studies aimed at testing the potential of 2B10 and 25F10 to block the induction of inflammatory arthritis, the AIA model was used first. The i.a. administration of either 2B10 or 25F10 to mBSA-challenged C57BL/6 mice resulted in a significant decrease in joint swelling (Fig. 3A). Histological evaluation of joint sections showed that parameters of disease activity were significantly less severe in mice receiving 2B10 and, to a lesser extent, 25F10 as compared with the C57BL/6 mice treated with the rlgG1 isotype control mAb (Fig. 3B). Indeed, administration of 2B10 or 25F10 reduced synovial hyperplasia, synovial infiltrate, cellular exudate, and joint damage (Fig. 3C). Thus, blocking IL-6 trans-signaling locally was sufficient to avoid excessive inflammation of the synovium in AIA.

**Classical IL-6 signaling is obligate and sufficient for disease induction in CIA**

To differentiate IL-6-driven mechanisms of inflammation in a T cell-mediated disease model, 2B10 and 25F10 were tested for their capacity to protect mice from CIA. Male DBA/1J mice were immunized with bovine CII in CFA (day 0) and treated with 1 mg mAb (i.p.) on days 0, 2, and 5. Following a boost of CII in IFA at day 21, mice developed clinical signs of disease that presented inflammation of individual paws. Administration of 2B10 protected mice by significantly delaying the onset of disease and decreasing incidence and disease severity (Fig. 4A, 4B) as compared with rlgG1 and 25F10. To establish the severity of local inflammation and cartilage destruction, histological sections were prepared from knee joints obtained at day 39. To assess cellular infiltration and joint destruction, serial sections were stained with H&E or safranin O/fast green, respectively. Joints from mice treated with rlgG1 and 25F10 showed significant destruction of cartilage characterized by a loss of safranin O staining (Fig. 4C, upper panels), associated hyperplasia of the synovial tissue, and erosions of the synovium in AIA.

**FIGURE 3.** IL-6 signaling regulates disease severity in AIA. AIA was established in C57BL/6 mice. To block IL-6 signaling locally, mBSA was administered with 100 μg of either 25F10, 2B10, or the control rlgG1. A. Joint inflammation was assessed by measurement of knee joint swelling. Results presented are mean ± SEM (*p < 0.05 for control versus 2B10 and rlgG1 versus 25F10). B. Differences in arthritis indices were determined by histological assessment of synovial infiltrate, exudate, hyperplasia, and erosion from tissue taken 3 d post-Ab treatment. C. Representative H&E-stained mid sagittal joint sections taken on day 3 after Ab treatment are shown for each group as well as sham joint receiving no mBSA, included as control of healthy tissue. The white asterisk indicates a region with substantial pannus formation and associated femoral bone erosion. Arrows indicate synovial areas with infiltrates (yellow) and exudate (blue) (original magnification ×40). AI, arthritis index.

**FIGURE 4.** Neutralization of IL-6 classical versus trans-signaling in CIA. Mice (n = 10 per group) were treated with 1 mg/mouse 2B10, 25F10, or rlgG1. Each group received three injections administered on day 0, 2, and 5 postprimary immunization with CII in CFA. A, Incidence. Results of three independent experiments. B, Clinical score. Results are the mean ± SEM of three independent experiments. C, Representative safranin O/fast green-stained (upper panels) and H&E-stained joint sections (lower panels) from experimental mice sacrificed on day 39 (experimental end point) or from nonimmunized mice (sham). Three mice were analyzed per group for each experiment. Results are representative of two independent experiments (original magnification ×5). D, Cartilage damage was evaluated as the percentage of loss of safranin O/fast green staining of the cartilage characterized by a loss of safranin O staining (Fig. 4C, upper panels), associated hyperplasia of the synovial tissue, and erosions of the synovium in AIA. E, Histological score was assessed, as described in Materials and Methods, for the joints in the 2B10- or 25F10-treated CIA mice. D and E, Results are the mean ± SEM of two independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.
an increase in leukocyte infiltration (Fig. 4C, lower panels) as compared with the sham control. The control rlgG1- and 25F10-treated mice demonstrated a 60% (±11) and 80% (±10) cartilage destruction, respectively (Fig. 4D). In agreement with joint damage, histological analysis demonstrated increased cellular infiltrates and inflammation (Fig. 4E) in rlgG1- and 25F10-treated groups. In contrast, a significant protection from both cartilage damage and inflammation was observed in the 2B10-treated mice (Fig. 4C–E). Overall, these experiments demonstrate the obligate role for the classical IL-6 signaling pathway in the induction phase of CIA leading to synovial infiltration and joint destruction.

Collagen-specific Th17 cell generation in dLN is driven by classical IL-6 signaling

Inflammation in CIA is driven by CII-specific Th1- and Th17-mediated T cell responses (23–26). We tested, therefore, whether autoreactive T cell activation in the dLNs was dependent on IL-6 signaling directly on naive CD4+ mIL-6Rα–expressing T cells or via IL-6–sIL-6Rα complex activation of CD4+ mIL-6Rα2 T cells. To address this question, single-cell suspensions from the dLNs of CII-immunized mice were prepared and cultured ex vivo with CII for 3 d. Supernatants were harvested, and inflammatory cytokines were measured. The dLN cells from the 2B10-treated mice demonstrated a significantly reduced capacity for IL-6 production at days 7 (300 pg/ml) and 14 (144 pg/ml) postimmunization as compared with the rlgG1 control (758 pg/ml at day 7 and 701 pg/ml at day 14) and 25F10-treated mice (524 pg/ml at day 7 and 440 pg/ml at day 14) (Fig. 5A). The IL-17A levels from CII-stimulated dLN cells, obtained at day 7, were markedly decreased for both 2B10 (91 pg/ml)- and 25F10 (259 pg/ml)-treated mice as compared with rlgG1 controls (692 pg/ml). However, whereas both neutralizing strategies maintained and sustained the decreased level in IL-17A production from the dLN cells isolated at day 14 postimmunization (i.e., 36 pg/ml for 2B10 and 188 pg/ml for 25F10), 25F10 administration failed to show significance as compared with rlgG1 control group. No significant differences were observed between groups concerning IFN-γ production (Fig. 5A). As these data represent the cytokine profile generated from whole lymph node cultures challenged with CII protein, it was important to ensure that all treatment groups contained equivalent numbers of distinct cell populations. Flow cytometric analysis of the dLN cell populations demonstrated that mAb treatment did not modulate the numbers of different leukocytes within the organ, including CD4+ T cells (Fig. 5B). To understand further the in vivo consequences of these blocking strategies, serum IL-6 and sIL-6Rα levels were determined. The 2B10 administration resulted in increased levels of IL-6 as compared with both 25F10 and rlgG1 mAbs (Fig. 5C). Both 2B10 and 25F10 increased the levels of circulating sIL-6Rα as compared with rlgG1 (Fig. 5C). Finally, we also investigated the role for the IL-6 pathways in liver-induced SAA, an acute-phase protein indicative of IL-6–mediated inflammation of IL-6Rα–bearing cells.
hepatocytes. Only 2B10 treatment was able to impair the induction of SAA in mice following CII immunization (Fig. 5C). These data demonstrate that blocking the IL-6 trans-signaling pathway was not sufficient to sustainably dampen the IL-17A production from DLNs nor protect from hepatocyte activation and subsequent SAA production in CIA.

**IL-6 classical signaling affects B cell responses**

Autoimmune pathogenesis in CIA mice is mediated by CII-specific T cells, which trigger germinal center (GC) responses and development of anti-CII IgG titers. Thus, serum was collected from CII-challenged mice at day 14 following the first immunization, and analyzed for the presence of CII-specific IgG from 2B10-, 25F10-, and rlgG1-treated mice. A marked reduction was observed in total anti-CII IgG titers from 2B10-treated mice as well as isotype-specific IgG2a, IgG1, and IgG2b end point titers (Fig. 6). Interestingly, the decreased production of CII-specific IgG responses following a primary CII immunization in 2B10-treated mice was not a consequence of altered T follicular helper cell (Tfh), GC B cell, and plasma cell (PC) numbers (Fig. 5B and data not shown). The failure to alter Tfh and GC B cell numbers in 2B10-administered CIA mice led us to investigate the relative affinities of the CII-specific IgG produced by days 14 and 39. Binding analysis by Biacore of sera on immobilized bovine CII demonstrated no significant difference between groups at either time point (Fig. 6F and data not shown).

**Discussion**

A large body of evidence has demonstrated a key role for IL-6 in the development of immune responses and, in particular, T cell-dependent inflammation (13, 27–29). Indeed, the biological activities assigned to IL-6 include, but are not restricted to, T cell differentiation, granulocyte/macrophage differentiation, T cell priming, and osteoclast activation (30, 31). A central role for IL-6 also has been described for the generation of the GC reaction, Ig somatic hypermutation, affinity maturation (5, 32), and plasma cell differentiation (33). In autoimmunity, inflammation driven by IL-6 is often the hallmark of the pathophysiology associated with diseases, such as RA, multiple sclerosis, and inflammatory bowel disease (7, 19, 28, 34–36). Animal models of these human conditions have demonstrated a role for IL-6 in the generation of autoimmune T cells (in particular Th17) and humoral responses leading to joint destruction in CIA (34), brain lesions in experimental autoimmune encephalitis (37, 38), and gut inflammation in intestinal bowel disease (7).

Attempts to dissect a signaling mechanism for IL-6–mediated inflammatory events have been complicated by the ability of IL-6 to orchestrate immune responses through both membrane-bound and soluble forms of IL-6R. Therefore, the studies in this work were designed to determine the relative spatial and temporal impact of IL-6 classical signaling and IL-6 trans-signaling on autoimmune responses in vivo. Using two experimental models of arthritis, we demonstrate that mIL-6R drives the systemic development of T cell-dependent processes, whereas sIL-6R governs local IL-6–signaling events within the inflamed joint.

To address these issues, mAbs were generated that disrupted the classical IL-6 pathway or inhibited IL-6 trans-signaling. Abs targeting the classical IL-6 pathway in mice have been described and used successfully in several animal models of disease (39). The key feature of the mAbs used in these studies is their capacity, when bound to mIL-6R, to inhibit IL-6 engagement to its cognate receptor. In agreement with this mode of action, the 2B10 mAb described in this work is able to potently bind mIL-6R and inhibit IL-6–induced proliferation of the mouse plasmacytoma cell line T1165. However, the ability of 2B10 to prevent ligand binding precluded the capacity for this Ab to bind to, or inhibit the activity of a preformed IL-6–IL-6R ligand–receptor complex. Considering the 7.94 nM affinity of 2B10 for sIL-6R and the affinity of IL-6 for sIL-6Rα to be ~2 nM (40), it is conceivable that 2B10 is actively competing for the ligand binding site. Thus, the data suggest that 2B10 inhibits formation of IL-6–sIL-6R complexes (Fig. 2). The predication from this hypothesis, therefore, would be that the in vivo administration of 2B10 would lead to increased levels of circulating IL-6 due to its decreased clearance via mIL-6R. Indeed, this is the case, as observed in CIA mice administered 2B10 (Fig. 5C). Consistent with these observations, in vitro differentiation of IL-17A–secreting CD4+ T cells from naive splenocytes in the presence of IL-6 and TGF-β was abrogated by 2B10, but not 25F10, suggesting that this mAb recognized a different epitope outside the cytokine binding domain (Fig. 2). Consequently, when IL-6 was replaced with a IL-6/ sIL-6Rα FP in the Th17-polarizing conditions, 25F10 was able to block IL-17 secretion. Thus, 25F10 represents what, to our knowl-
edge, is a novel reagent that specifically targets IL-6 trans-signaling and provides a valuable tool in dissecting the relevance of this pathway in homeostatic and pathophysiological settings.

Although high concentrations of IL-6 occur in the serum and synovial fluids of patients with RA, cell types resident in the joint (chondrocytes, synoviocytes, fibroblasts, and endothelial cells) lack expression of the mIL-6R (6). Thus, it was not so surprising that in vitro studies demonstrated an involvement of sIL-6R in regulating synovial fibrosis and bone degradation (41–43). The first question, therefore, was to address the contribution of IL-6 trans-signaling in local joint inflammation. For this, we employed the monoarthritis AIA model, which has been used previously to illustrate that IL-6 has an effect on both the induction and effector phase of arthritis (19). Administration (i.a.) of mBSA into primed mice leads to a thickening of the synovial lining, cellular hyperplasia, and increased leukocyte infiltration and joint destruction. The lack of mIL-6R—expressing cells in the joint infers that these are unlikely to contribute to synovial sIL-6R concentrations. As mIL-6R expression is for the most part restricted to hepatocytes and leukocytes (8, 44), increases in synovial sIL-6R levels may originate from infiltrating leukocytes. In this respect, synovial sIL-6R levels directly correlate with leukocyte numbers found within the inflamed synovium, whereas various inflammatory mediators and activation events lead to sIL-6R release from leukocytes (6, 11, 45, 46). Consequently, locally generated sIL-6R is considered a rate-limiting event affecting IL-6 control of inflammation (47).

Several studies have highlighted a role for sIL-6R in the regulation of chemokine and adhesion molecule expression (48), whereas sIL-6R—mediated signaling appears to control the transition from the early neutrophilic stage of acute inflammation to the more sustained mononuclear cell influx (11, 44). Indeed, blockade of sIL-6R activity by its natural antagonist, soluble gp130, inhibits arthritis progression (12). In line with these studies, we demonstrate that when mBSA was administered with either 25F10 or 2B10, arthritis indices, with either mAbs, as determined by histological assessment of synovial infiltrate, exudate, hyperplasia, and erosion, were significantly limited. Similarly, both mAbs protected from substantial pannus formation and associated bone erosion.

Induction of CIA requires systemic T cell-mediated inflammatory responses that drive the development of Abs against CII (49, 50). To dissect the contribution of the IL-6–directed T cell responses in this experimental model, mice were administered 2B10 or 25F10 during the priming phase of the response. Only 2B10 afforded protection from disease by significantly decreasing the incidence and diminishing the clinical manifestations. In agreement with a necessity of classical IL-6 signaling for the development of inflammatory T cell-derived cytokines, 2B10 treatment of mice resulted in decreased IL-6 and IL-17A ex vivo responses. This observation was indeed a reflection of the diminished potential of cells to generate the cytokines and not a result of decreased T cells in the dLN of 2B10-treated mice. In addition, the blockade of the classical IL-6 signaling pathway decreased Th17 IL-17A and IL-17F mRNA gene expression from cells obtained from the dLNs of mice at day 7 (Supplemental Fig. 1). Taken together, classical IL-6 activation of membrane-positive IL-6Rα cells in the dLNs is obligate for the development of autoreactive Th17 cells.

The involvement of IL-6 in the generation of the T cell-dependent GC reaction has been well documented (4, 5, 51). Furthermore, recent work has established a role for IL-6 in the development of the T FH cells, as determined by flow cytometry, was not affected in dLNs (albeit at the time point assessed). Histological analysis confirmed the presence of GC structures in all treated groups (data not shown). Despite these results, total bovine CII-specific IgG, IgG1, IgG2a, and IgG2b end point titers significantly decreased at day 14 in 2B10-treated mice as compared with both 25F10- and rlgG1-treated animals. These data suggest that despite the absence of classical IL-6 signaling, T FH cell and GC B cell number is unaltered in dLNs. However, the potential benefit afforded by 2B10 administration on decreasing IL-21 mRNA expression (Supplemental Fig. 1) cannot be excluded. Indeed, IL-21 has been implicated in regulating GC B cell differentiation (53), and blockade of the IL-21/IL-21R axis has been shown to ameliorate disease in CIA (54). Nonetheless, the decreased serum titers may in fact be a consequence of decreased secretion in the bone marrow as IL-6 is an important PC survival factor (55, 56). Whereas not conclusively demonstrated in our study, this latter hypothesis would predict that despite the lowered detectable serum Ig titers to CII, the binding affinities of serum anti-CII IgG would be similar across the groups. Indeed, purified serum IgG from day 14-treated 2B10, 25F10, and rlgG1 mice demonstrated relatively equivalent binding kinetics, as measured by Biacore analysis. Together, our data point to the key role for the classical and not trans–IL-6 signaling in the early development of autoimmune via adaptive immune mechanisms. The blockade of IL-6 pathways in a therapeutic setting (i.e., treatment with mAbs at day 21 coinciding with the secondary CII immunization), however, failed to alter the disease pathogenesis in CIA (data not shown). This may suggest redundancy and a role for other inflammatory cytokines following the secondary boost with CII. Indeed, TNF and IL-17 have been demonstrated to be implicated in driving this later phase of disease (57). More probable, however, is that i.p. administration of mAbs precludes their access to the inflamed joints in this study. Indeed, administration of mAbs 2B10 and 25F10 (i.a.) in the AIA demonstrated protection from bone and cartilage degradation, suggesting this route of mAb treatment may afford protection in both CIA and other autoimmune models. We are currently testing the therapeutic potential of locally targeting IL-6 trans-signaling in other inflammatory disease settings. Nonetheless, how IL-6 classical and trans-signaling in human and mouse have any differential physiological function in the pathogenesis of human disease remains to be further explored.

A plethora of preclinical animal models and, more importantly, successful clinical studies have validated targeting the IL-6 pathway in inflammatory-mediated diseases (58). Our study, via the use of what we believe are new differentiating reagents, has brought what we consider to be novel insights as to how T cell-dependent responses are governed by neutralizing the different modes of IL-6 activation. Furthermore, our study has further established the control of systemic immune processes regulated by classical IL-6 signaling and highlighted that local inflammatory responses do rely on trans-signaling events.

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References
Signal transduction and inflammation.


