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Cutting Edge: Soluble IL-6R Is Produced by IL-6R Ectodomain Shedding in Activated CD4 T Cells¹

Eva M. Briso, Oliver Dienz, and Mercedes Rincon²

IL-6 trans-signaling via the soluble IL-6R (sIL-6R) plays an important role in the progression of several autoimmune diseases and cancer by providing IL-6-responsiveness to cells lacking IL-6R. However, the potential sources of sIL-6R are less understood. In this study we show that sIL-6R is produced by both naive and memory CD4 T cells upon TCR activation. The production of sIL-6R by activated CD4 T cells is mediated by shedding of the membrane-bound IL-6R, and this process correlates with the expression of the metalloproteinase ADAM17 in these cells. In contrast to CD4 T cells, CD8 T cells do not express ADAM17 and their production of sIL-6R is negligible. Thus, during an immune response CD4 T cells are an important source of sIL-6R. Production of sIL-6R by autoreactive CD4 T cells may contribute to their role in the development of autoimmune disease by conferring IL-6-responsiveness to cells lacking IL-6R such as synoviocytes. The Journal of Immunology, 2008, 180: 7102–7106.

Interleukin-6 is a cytokine produced by professional APCs, but also by cells of nonhematopoietic origin (1). IL-6 binds to the IL-6R present on the cell surface in association with gp130, the transmembrane signal transducer receptor that is shared by other cytokines of the same family (1). Neither IL-6R nor IL-6 binds to gp130 alone. A complex of IL-6R-IL6 is necessary for binding to gp130 and forms a hexamer consisting of two IL-6R, two IL-6, and two gp130 molecules (2). The gp130 receptor is broadly expressed in most cell types, but the expression of IL-6R is more restricted to liver cells and leukocytes (3). However, IL-6 has also been shown to have an effect on cells that normally lack IL-6R including cancer cells, neurons, and osteoclasts (1). This effect is mediated by a soluble form of IL-6R (sIL-6R)³ containing the extracellular domain sufficient for binding to IL-6 and gp130. Once sIL-6R is secreted, it can bind to IL-6 and the complex interacts with the ubiquitously expressed gp130 in a process that has been termed *trans-signaling* of IL-6 (4). This process makes IL-6R-lacking

cells responsive to IL-6 when there is a source that produces sIL-6R. sIL-6R and IL-6 *trans-signaling* have now been implicated in a number of pathological diseases, including rheumatoid arthritis and Crohn's disease (5–7), as well as colon cancer (5, 8).

Two major mechanisms for the production of sIL-6R have been proposed (9). In addition to the transmembrane form of the IL-6R, a mRNA transcript lacking the transmembrane domain can be generated by alternative splicing using splicing donor and acceptor sites that flank the region coding for the transmembrane domain. The alternative splicing also introduces a frameshift leading to the incorporation of 10 additional amino acids at the C terminus of the sIL-6R (10, 11). A second mechanism for the generation of sIL-6R is the proteolytic cleavage ("shedding") of the membrane-bound IL-6R at the cell surface interface through a putative proteolytic site immediately before the transmembrane domain (12). Initial studies using pharmacological inhibitors suggested that the cleavage of the membrane-bound IL-6R is mediated by metalloproteinases (13, 14). Recent studies of monocytes, fibroblasts, and cancer cell lines have shown that two specific members of the ADAM (a disintegrin and metalloproteinase domain) family of zinc-dependent metalloproteinases, ADAM17 (also named TACE for TNF- α -converting enzyme) and ADAM10, contribute to the IL-6R shedding (15, 16).

Less is known about the potential cellular sources that produce this receptor. Secretion of sIL-6R has been reported in neutrophils, monocytic and hepatocytic cell lines, and human T lymphotropic virus type I (HTLV1)-transformed, but not nontransformed, T cell lines (6, 11, 17, 18). In this study we show for the first time that human naive and memory CD4 T cells, but not CD8 T cells, produce sIL-6R upon TCR activation. The secretion of sIL-6R is primarily mediated by shedding of the membrane-bound receptor and correlates with the expression of ADAM17 in CD4 T cells.

Materials and Methods

Isolation and activation of human CD4 T cells

PBMCs were obtained from healthy volunteers according to institutional research protection guidelines. CD4 and CD8 T cells were purified by positive

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³ Abbreviations used in this paper: sIL-6R, soluble IL-6R; ADAM, a disintegrin and metalloproteinase domain; TAPI, TNF- α protease inhibitor.

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selection using MACS kits as recommended by the manufacturer (Miltenyi Biotech). Isolation of naive and memory CD4 T cells was performed by FACS sorting. Cells were activated with a plate-bound anti-CD3 mAb (OKT3; 3 $\mu\text{g}/\text{ml}$) and a soluble anti-CD28 mAb (2 $\mu\text{g}/\text{ml}$) (BD Pharmingen). Fifty μM TNF- α protease inhibitor (TAPI)-0 (Peptides International) was used.

RT-PCR

Total RNA was isolated from cells using the RNeasy kit (Qiagen). RT-PCR was performed to examine the expression of membrane-bound IL-6R and alternatively spliced sIL-6R using the IL-6R sense (5'-CATTGCCATTGTTCTGAG GTTC-3') or sIL-6R sense (5'-GCGACAAGCCTCCCAGGTTTC-3') and the shared IL-6R antisense (5'-GTGCCACCCAGCCAGCTATC-3') oligonucleotides as previously described (19). ADAM17 and ADAM10 expression was examined by RT-PCR using the previously described primers (20).

ELISA

Human soluble IL-6R production was determined by ELISA using the DuoSet kit as recommended by the manufacturer (R&D Systems). IL-2 production was determined by ELISA using the capture (2 $\mu\text{g}/\text{ml}$) and biotinylated (1 $\mu\text{g}/\text{ml}$) (BD Pharmingen) Abs.

Flow cytometry analysis

The following mAbs were used for staining: anti-CD14, -CD8, -CD4, -CD62L (Caltag), and anti-IL-6R (BD Pharmingen). Cells were analyzed using the LSR II flow cytometer (BD Bioscience). For isolation of naive and memory CD4 T cells, PBMC were stained with anti-CD4 and -CD45RO (Caltag) and anti-CD45RA (Biolegend) mAbs, and CD4 CD45RA and CD4 CD45RO populations were isolated by FACS sorting using the FACSAria instrument (BD Bioscience).

Results and Discussion

TCR signals induce the secretion of sIL-6R in CD4 T cells, but not CD8 T cells

Soluble IL-6R and IL-6 *trans*-signaling have been involved in a number of inflammatory diseases (4, 5, 9). IL-6 is known to be produced by a variety of cells. However, it remains unclear which cells produce sIL-6R. It is believed that neutrophils and macrophages are most likely the major source of sIL-6R. Because CD4 T cells play an important role in autoimmune diseases, we examined the production of sIL-6R in this population. CD4 T cells were purified from peripheral blood of healthy volunteers and activated with anti-CD3 and anti-CD28 mAbs for different periods of time. Secretion of sIL-6R into culture supernatant was determined by ELISA. Significant levels of sIL-6R were detected already 1 day after activation, and these levels increased over the activation period (Fig. 1A). No sIL-6R could be detected in CD4 T cells incubated in medium alone in the absence of TCR signals (data not shown). Unlike CD4 T cells, CD8 T cells do not seem to play a major role in autoimmune diseases. To determine whether CD8 T cells are also capable of secreting sIL-6R, we examined its production by purified CD8 T cells upon activation with anti-CD3 and anti-CD28 mAbs. In contrast to CD4 T cells, CD8 T cells produced minimal levels of sIL-6R that did not increase over the time of activation (Fig. 1B). Analysis of IL-2 production by CD8 T cells showed that these cells were activated because they produced high levels of IL-2 despite their inability to secrete sIL-6R (Fig. 1C). The purity (>95%) in both populations was comparable, and monocytes could not be detected in either purified CD4 or CD8 T cells (Fig. 1D), indicating that the differential production of sIL-6R was not due to a difference in the purity of these cells. CD4 T cells, but not CD8 T cells, are therefore a major source of sIL-6R upon TCR activation. Analysis of membrane-bound IL-6R in CD4 and CD8 T cells by flow cytometry showed increased levels of this receptor on the cell surface of CD4 T cells compared with the levels on CD8 T cells (Fig. 1E), suggesting that CD4 T cells could also help CD8 T cells by providing sIL-6R and increasing their responsiveness to IL-6.

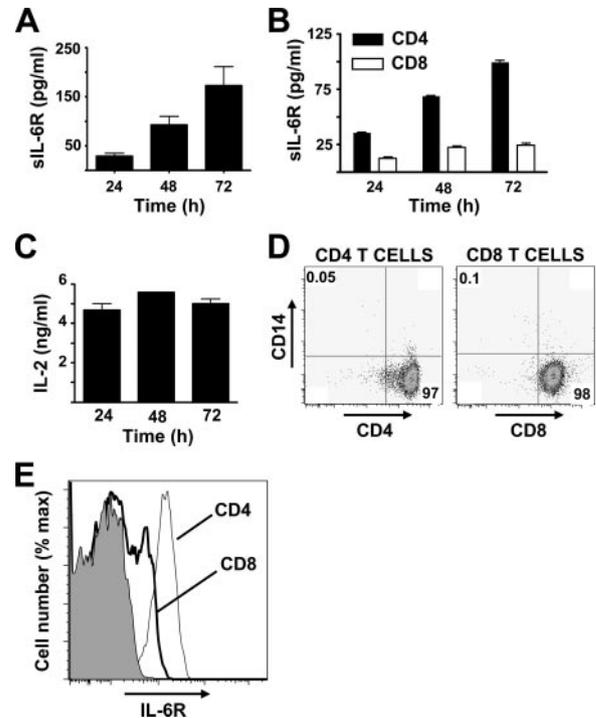


FIGURE 1. Production of sIL-6R by human CD4 T cells upon TCR stimulation. *A*, Human CD4 T cells were activated with anti-CD3 and anti-CD28 mAbs for the indicated periods of time and sIL-6R production was examined by ELISA. The mean \pm SD of three independent experiments with cells from different donors is shown. *B*, Purified CD4 and CD8 T cells were activated with anti-CD3 and anti-CD28 mAbs and sIL-6R production was examined by ELISA (data represent the mean \pm SD of triplicate determinations). A representative experiment of two performed is shown. *C*, CD8 T cells were activated as in *B* and IL-2 production was examined. *D*, CD4 and CD8 T cells were stained for CD4 or CD8 and CD14 and analyzed by flow cytometry. Numbers indicate the percentage of the corresponding population. *E*, CD4 (thin line) and CD8 (thick line) T cells were stained for CD4 or CD8 and IL-6R and analyzed by flow cytometry analysis. Histograms show the fluorescence intensity for IL-6R in each population. Filled histogram represents unstained cells.

Secretion of sIL-6R by CD4 T cells is mediated by the shedding of membrane-bound IL-6R

sIL-6R can be produced by alternative splicing of the mRNA that normally encodes for the membrane-bound form of IL-6R or by proteolytic cleavage of the membrane-bound form. To determine whether the sIL-6R secreted by CD4 T cells was produced by alternative splicing of the IL-6R mRNA induced upon activation, we examined the expression of the two isoforms (i.e., membrane-bound and secreted) by RT-PCR using RNA from CD4 T cells before and after activation. Both the membrane-bound and the soluble isoforms of IL-6R were already present in CD4 T cells before stimulation (Fig. 2A) and their expression was slightly decreased with activation (Fig. 2A), which was further demonstrated by semi-quantitative analysis (data not shown). Thus, the production of sIL-6R by activated CD4 T cells does not seem to be caused by switching from the membrane-bound to the soluble alternatively spliced isoform. In addition, because no sIL-6R was detected if CD4 T cells were incubated in medium alone in the absence of anti-CD3 and anti-CD28 mAbs (data not shown), these results also show that the presence of the alternatively spliced IL-6R mRNA is not indicative of protein expression and secretion of the sIL-6R.

Analysis of the IL-6R expression on the cell surface of CD4 T cells during activation by flow cytometry showed that the levels of IL-6R decreased significantly upon activation (Fig. 2B). This

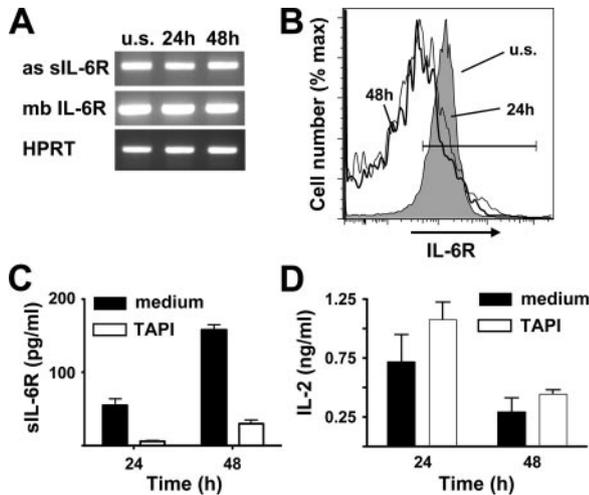


FIGURE 2. Production of sIL-6R by CD4 T cells is due to shedding of the membrane-bound IL-6R. *A*, Total RNA was isolated from unstimulated CD4 T cells (u.s.) and from cells activated with anti-CD3 and anti-CD28 mAbs. Expression of membrane-bound IL-6R (mb IL6R) and sIL-6R generated by alternative splicing (as sIL-6R) was examined by RT-PCR. Hypoxanthine phosphoribosyltransferase (HPRT) was also examined as an endogenous control. A representative experiment of four performed is shown. *B*, Cell surface expression of membrane-bound IL-6R was examined in unstimulated CD4 T cells (filled histogram) and in CD4 T cells activated with anti-CD3 and anti-CD28 mAbs for 24 h (thin line) or 48 h (thick line) by flow cytometry analysis. Horizontal line denotes the gate relative to unstained cells. *C*, CD4 T cells were activated with anti-CD3 and anti-CD28 mAbs in the absence or presence of TAPI. sIL-6R production was determined by ELISA. A representative experiment of four performed is shown. *D*, CD4 T cells were activated as described in *C*. IL-2 production was examined by ELISA. The mean \pm SD of three independent experiments is shown.

down-modulation of IL-6R on the surface during activation could be associated with the production of sIL-6R by shedding of the membrane-bound form. Cleavage of the membrane-bound sIL-6R is mediated primarily by specific members of the ADAM family of metalloproteinases. To determine whether the production of sIL-6R by CD4 T cells could be due to the shedding of membrane-bound IL-6R, we examined the effect of TAPI, an inhibitor of metalloproteinases that has been shown to prevent IL-6R shedding in other cell types (13, 14). CD4 T cells were activated in the presence or absence of TAPI and the production of sIL-6R in the supernatant was measured by ELISA. The presence of TAPI during activation practically abolished the production of sIL-6R (Fig. 2C). No effect of TAPI on survival and/or cell expansion was observed (data not shown), indicating that the effect was not due to the toxicity of TAPI in these cells. Furthermore, analysis of IL-2 production showed that the levels of IL-2 in the supernatant were not decreased by the presence of TAPI (Fig. 2D). Together, these results indicate that CD4 T cells produce high levels of sIL-6R primarily by metalloproteinase-mediated shedding of the membrane-bound IL-6R form. The progressive increase in the levels of sIL-6R during the activation is probably due to the continuous expression and shedding of membrane-bound IL-6R. Because low levels of sIL-6R could still be detected in the presence of TAPI, we cannot rule out the possibility that a minimal fraction of the secreted sIL-6R may also be derived from the expression of the alternatively spliced form.

Differential expression of ADAM17 and ADAM10 in CD4 and CD8 T cells

ADAM10 and ADAM17 have been shown to contribute to the shedding of IL-6R and the production of sIL-6R in monocytes,

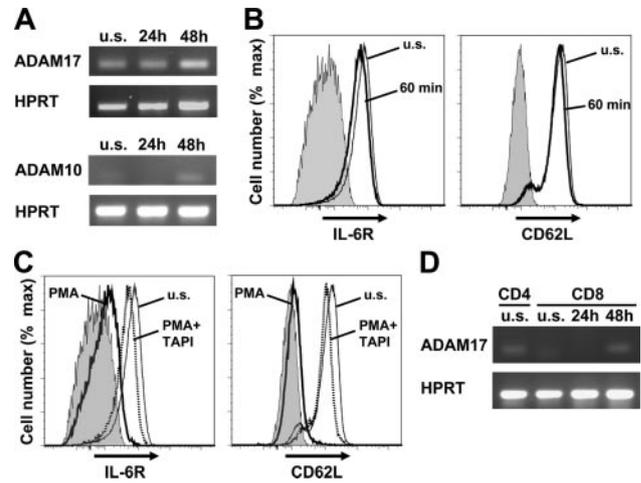


FIGURE 3. Expression of ADAM17 and ADAM10 in CD4 and CD8 T cells. *A*, ADAM17 and ADAM10 mRNA levels were examined in unstimulated CD4 T cells (u.s.) and CD4 T cells stimulated with anti-CD3 and anti-CD28 mAbs by RT-PCR. A representative experiment of three performed is shown. HPRT, Hypoxanthine phosphoribosyltransferase. *B*, CD4 T cells were treated (thick line) or nontreated (thin line) with anti-CD3 and anti-CD28 mAbs for 60 min. IL-6R and CD62L were examined by flow cytometry analysis. Unstained cells are shown by the filled histograms. *C*, CD4 T cells were treated with medium alone (thin line), PMA (thick line), or PMA and TAPI (dashed line) for 60 min. IL-6R and CD62L were examined by flow cytometry. *D*, Expression of ADAM17 in unstimulated CD4 and CD8 T cells, as well as in CD8 T cells activated as in *A*, was examined by RT-PCR.

fibroblasts, or osteogenic cell lines. The expression of ADAMs is not well characterized in CD4 T cells. Thus, to determine whether these two metalloproteinases could also be the target of TAPI in CD4 T cells, we examined their expression in these cells by RT-PCR. ADAM17 mRNA was readily detected in freshly isolated CD4 T cells and its expression was slightly up-regulated during T cell activation (Fig. 3A), which was further demonstrated by semi-quantitative analysis (data not shown). The levels of ADAM10 mRNA in CD4 T cells before stimulation were almost undetectable but appeared to increase later during activation (Fig. 3A). Thus, secretion of sIL-6R by shedding in CD4 T cells is most likely mediated by ADAM17, but ADAM10 may also contribute.

It is believed that the shedding mediated by ADAM17 in T cells occurs very rapidly upon activation, because the cell surface expression of CD62L (another ADAM17 target) can be down-regulated within 30 to 60 min of activation (21–23). Analysis of IL-6R expression in CD4 T cells upon 60 min of activation with anti-CD3 and anti-CD28 mAbs showed no significant reduction in the cell surface levels (Fig. 3B). However, no reduction in CD62L cell surface levels was observed either (Fig. 3B). Similarly, minimal changes in the cell surface levels for either molecule could be found after 4 h of activation (data not shown). Because most studies showing a rapid shedding of CD62L were performed using PMA as stimulus, we also examined the expression of IL-6R and CD62L after 60 min of treatment with PMA. Interestingly, PMA almost abrogated cell surface expression of both CD62L and IL-6R, and this effect was blocked by TAPI (Fig. 3C). Thus, shedding of IL-6R in CD4 T cells is rapidly induced by PMA, but it seems to be delayed and progressive in response to TCR-mediated signals.

Because CD8 and CD4 T cells differ in their ability to produce sIL-6R, we examined the expression of ADAM10 and ADAM17 in CD8 T cells. The levels of ADAM17 in CD8 T cells before stimulation were almost undetectable compared with the levels in

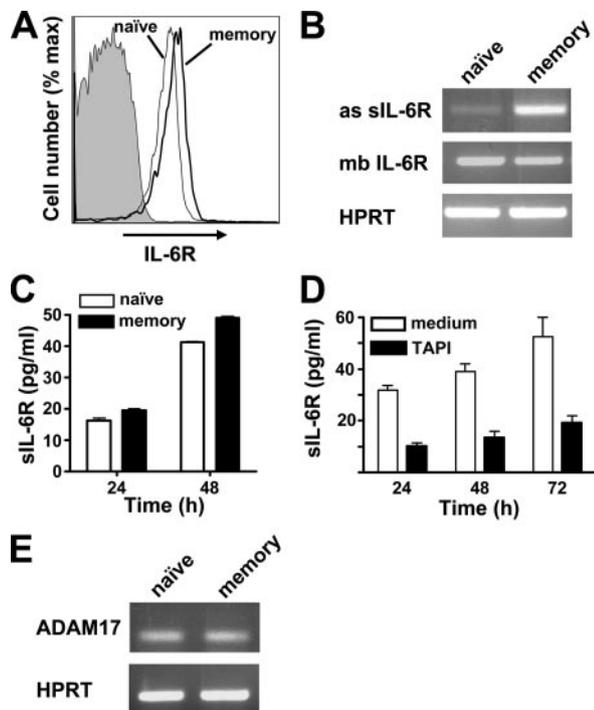


FIGURE 4. Naive and memory CD4 T cells produce sIL-6R upon activation. *A*, PBMCs were stained for CD4, CD45RO, CD45RA, and IL-6R and analyzed by flow cytometry. Profiles show cell surface expression of IL-6R in gated CD4 CD45RA (naive) (thin line) and CD4 CD45RO (memory) (thick line) cells. Filled histogram represents unstained cells. *B*, Naive and memory cells were purified by FACS sorting. The expression of sIL-6R generated by alternative splicing (as sIL-6R) and membrane-bound IL-6R (mb IL-6R) was examined by RT-PCR. A representative experiment of three performed is shown. HPRT, Hypoxanthine phosphoribosyltransferase. *C*, FACS-sorted naive and memory cells were activated with anti-CD3 and anti-CD28 mAbs, and sIL-6R production was determined by ELISA. A representative experiment of three performed is shown. *D*, FACS-sorted naive cells were activated with anti-CD3 and anti-CD28 mAbs in the presence or absence of TAPI, and sIL-6R production was determined by ELISA. *E*, Expression of ADAM17 in FACS-sorted naive and memory CD4 T cells was examined by RT-PCR.

CD4 T cells (Fig. 3*D*). ADAM17 expression however seems to be induced later during the activation of CD8 T cells (Fig. 3*D*). The expression of ADAM10 in CD8 T cells was also practically undetectable, similar to the levels in CD4 T cells (data not shown). The lack of ADAM10 and ADAM17 in CD8 T cells could explain the inability of these cells to cleave the membrane-bound IL-6R and produce sIL-6R upon activation.

Naive and memory CD4 T cells can produce sIL-6R upon activation

Whereas naive CD4 T cells produce very limited amounts of effector cytokines such as IL-4 or IFN- γ , memory cells have the ability to produce large amounts of a variety of cytokines very rapidly upon activation. We determined whether only memory or both naive and memory populations contribute to the production of sIL-6R observed in total CD4 T cells. We first examined the cell surface expression of membrane-bound IL-6R in naive (CD45RA⁺) and memory (CD45RO⁺) CD4 T cells by flow cytometry analysis. The levels of membrane-bound IL-6R were comparable in both populations (Fig. 4*A*). To examine the presence of the alternatively spliced sIL-6R in each population, naive and memory CD4 cells were isolated from PBMCs by FACS sorting. Similar to the expression on the surface, no difference in mRNA levels for membrane-bound IL-6R was observed between naive and memory CD4 cells (Fig. 4*B*). In contrast, increased levels of

the alternatively spliced form of sIL-6R were detected in memory relative to naive CD4 T cells (Fig. 4*B*).

We therefore examined the production of sIL-6R in both CD4 T cell populations upon activation. FACS-sorted naive and memory CD4 cells were activated and sIL-6R production was examined. The levels of sIL-6R produced by naive CD4 cells were comparable to those produced by memory CD4 T cells (Fig. 4*C*). These results indicate that both populations were capable of producing sIL-6R despite the differential expression of the alternatively spliced form and suggested that shedding of the membrane-bound IL-6R is the major mechanism that contributes to the production of sIL-6R in naive CD4 T cells. Analysis of sIL-6R production in naive cells activated in the presence of TAPI further showed that sIL-6R production in these cells is primarily due to the shedding of membrane-bound IL-6R (Fig. 4*D*). We also examined the expression of ADAM17 in naive and memory CD4 T cells by RT-PCR. ADAM17 was similarly expressed in both cell populations (Fig. 4*E*). No expression of ADAM10 was detected in either population (data not shown), further supporting the probability that the shedding of sIL-6R is primarily mediated by ADAM17.

In summary, our data show for the first time that both naive and memory human CD4 T cells secrete sIL-6R upon TCR activation. In addition, we also demonstrate that despite the constitutive expression of the alternatively spliced form of the sIL-6R in CD4 T cells, shedding of the membrane-bound IL-6R is the major mechanism that contributes to the secretion of sIL-6R by these cells. Secretion of sIL-6R by shedding of the membrane-bound IL-6R correlates with the presence of ADAM17 in CD4 T cells before stimulation.

Although macrophages and neutrophils are believed to be the major source of sIL-6R, our data indicate that CD4 T cells are also capable of producing sIL-6R upon TCR activation. While several studies have examined the effect of sIL-6R and IL-6 *trans*-signaling in CD4 T cells (24, 25), this is the first report showing that CD4 T cells indeed represent an additional and likely critical source of sIL-6R during an immune response. The ability of producing sIL-6R, however, is restricted to CD4 T cells because CD8 T cells did not secrete significant levels of sIL-6R. Although naive CD4 T cells display limited ability as effector cells in that they cannot produce significant amounts of cytokines, we show here that they can produce substantial amounts of sIL-6R. Thus, naive CD4 T cells may also act as effector cells by providing responsiveness to IL-6. Because IL-6 plays a critical role in generating IL-17-expressing Th17 cells (26), the sIL-6R produced by activated CD4 T cells may enhance IL-6 responsiveness and therefore Th17 differentiation of naive CD4 T cells.

The role of sIL-6R in arthritis has been well described (7), but it remains unclear which cells are responsible for the generation of sIL-6R. CD4 T cells are clearly involved in the development and progression of rheumatoid arthritis. It is therefore possible that autoreactive CD4 T cells present in the joints, instead of macrophages, are the main source of sIL-6R, while macrophages and/or fibroblasts could be the major source of IL-6.

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Disclosures

The authors have no financial conflict of interest.

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