Differential expression of CD126 and CD130 mediates different STAT-3 phosphorylation in CD4⁺CD25⁻ and CD25⁰high regulatory T cells

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Abstract

IL-6 is a pleiotropic cytokine involved in T-lymphocyte biology. Following IL-6 binding, the soluble IL-6R (CD126)-IL-6 complex can directly activate cells that express the signal-transducing gp130 (CD130) molecule, which mediates two distinct signals, mitogenesis by mitogen-activated protein kinase (MAPK) activation and anti-apoptosis by signal transducer and activator of transcription 3 (STAT-3) activation. This 'trans-signaling', also mediated by the soluble CD126/IL-6 fusion protein hyper-IL-6 (H-IL-6), contributes to the perpetuation of autoimmune diseases such as Morbus Crohn or rheumatoid arthritis. On the other hand, the homeostasis of cellular immune reactions and its failure leading to autoimmune diseases are critically controlled by regulatory T cells (Tregs). Here, we investigated the differential expression of CD126 and CD130 on subsets of human leukocytes in blood, tonsil and spleen. Among CD4⁺ T cells, differential expression of CD126 and CD130 was observed on the basis of CD25 expression. CD4⁺CD25⁻ T cells were strongly CD126⁺ and CD130⁺, whereas CD25⁰high Tregs expressed CD126 but little CD130. Both CD126 and CD130 were down-modulated on CD4⁺CD25⁻ T cells following ligand binding, whereas only marginal modulation was observed on Tregs. Interestingly, we observed a correlation between CD126 and CD130 expression with STAT-3 phosphorylation in CD4⁺CD25⁻ T cells compared with Tregs after stimulation with IL-6 or H-IL-6, whereas the MAPK extracellular signal-regulated kinase 1/2 were not activated by CD130 dimerization. The differential expression of CD126 and CD130 and subsequent STAT-3 phosphorylation might be relevant for the recently described role of IL-6 in the control of Treg activity.

Introduction

IL-6 is a pleiotropic pro-inflammatory cytokine produced by a variety of cells including macrophages, dendritic cells (DCs), endothelial cells, B cells and subsets of T lymphocytes. It plays an important role in the growth and differentiation of hematopoietic precursor cells, T and B lymphocytes, endothelial cells, osteoblasts and other cell types. Furthermore, IL-6 is a central factor in the initiation of the acute-phase response in the liver (1). IL-6 also has important functions in the regulation of T-cell differentiation. It promotes IL-4 gene expression and IL-4 production, and thus T₄₁₂ differentiation, through the activation of the transcription factor nuclear factor of activated T cells (2, 3). At the same time, IL-6 inhibits T₃₁₁ differentiation by up-regulating suppressor of cytokine signaling (SOCS-1, SOCS-3) (4). IL-6 also affects the regulation of survival and death of T cells. Thus, it has been reported that IL-6 prevents the death of resting T cells and the activation-induced cell death triggered by TCR stimulation (5, 6). The survival signal delivered by IL-6, however, is inhibited by T-cell activation (7).

In addition to its effects on the differentiation, survival and death of T cells, substantial evidence suggests that IL-6 also modulates the activity of regulatory T cells (Tregs). Tregs have recently attracted much interest due to their obvious role in the control of cellular immune responses and autoimmunity

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While no exclusive cell-surface markers have been described to date, CD4+ Tregs usually express high levels of the IL-2R α chain (CD25) and additional antigens such as the glucocorticoid-inducible tumor necrosis factor receptor-like molecule (GITR) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) (10, 11). Moreover, Tregs express the transcription factor FoxP3 (12) which is a transcriptional repressor of IL-2 (13). The expression of FoxP3 is functionally significant, since transfection into FoxP3-negative T cells conveys regulatory activity.

The expression of CD130 on all murine thymocytes is described to date, CD4+ Tregs usually express high levels of the IL-2R α chain (CD25) and additional antigens such as the glucocorticoid-inducible tumor necrosis factor receptor-like molecule (GITR) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) (10, 11). Moreover, Tregs express the transcription factor FoxP3 (12) which is a transcriptional repressor of IL-2 (13). The expression of FoxP3 is functionally significant, since transfection into FoxP3-negative T cells conveys regulatory activity.

IL-6 signals through a cell-surface receptor complex consisting of the ligand-binding IL-6R α chain (IL-6R and CD126) and the signal-transducing component gp130 (CD130) (17). CD130 is the common signal transducer for several cytokines including leukemia inhibitory factor, ciliary neurotropic factor, oncostatin M, IL-11 and cardiotropin-1 (18), and is almost ubiquitously expressed in most tissues. In contrast, the expression of the associated cytokine-specific α chains is restricted.

Binding of one of these cytokines to its receptor and CD130 activates Janus kinase-1, Janus kinase-2 and TYK-2, which phosphorylates tyrosine residues in the cytoplasmic domain of CD130. The transcription factor signal transducer and activator of transcription 3 (STAT-3) is recruited to the phosphorylated tyrosine residues in the YXXQ motif of CD130, where it is activated and dimerized, and subsequently enters the nucleus and regulates gene expression including anti-apoptotic molecules such as bcl-2 (18–21). The src homology 2 domain-bearing protein tyrosine phosphatase (SHP-2) is recruited to the phosphorylated tyrosine residue Y759 in CD130 where it becomes activated and forms a complex with the adapter proteins, Gab1 and Gab2, leading to activation of the Ras/mitogen-activated protein kinase (MAPK) pathway (22). SHP-2-mediated extracellular signal-regulated kinase (ERK) MAPK activation has been suggested to inhibit STAT-3-mediated biological responses (23, 24). Tyrosine residue Y759 also provides the binding site for the SOCS-3 protein, which negatively regulates the CD130 signals (25, 26). SOCS-3 induction in various immune cells depends on the de novo transcription of mRNA (27).

The membrane-expressed CD126 can be cleaved by metalloproteases from the cell surface thus giving rise to a soluble CD126 (sCD126). The sCD126–IL-6 complex or the fusion protein of this complex, hyper-IL-6 (H-IL-6) (28), can directly bind to CD130 and thereby initiate signal transduction in cells that express CD130 but lack CD126 (‘trans-signaling’). In view of the recent insights into the role of IL-6 in T-cell survival, apoptosis and escape from Treg control, we were interested to precisely characterize the expression of CD126 and CD130 on subsets of human T lymphocytes, with special emphasis on the population of CD4+ Tregs. Others have published that CD130 is expressed on all murine thymocytes throughout intrathymic maturation, whereas CD126 is acquired only at the stage of CD4+ and CD8+ single-positive thymocytes (31, 32). The expression of both CD126 and CD130 on peripheral CD4+ and CD8+ T cells is down-regulated in vivo and in vitro in response to IL-6 ligand binding or TCR-dependent cellular activation (7, 31, 32). Therefore, low or absent CD126/CD130 expression on mature peripheral T cells might indicate a non-naive, antigen-experienced stage of T-cell differentiation (31). In humans, previous studies have demonstrated low expression or absence of CD126 on B cells and NK cells and significant but variable expression on CD4+ and CD8+ T-cell subsets (33–35). In the present study, we report striking differences in CD130 and CD126 expression between peripheral blood CD4+CD25-negative T cells and CD4+CD25high Tregs. This different expression correlates with STAT-3 phosphorylation induced by IL-6 or H-IL-6. In contrast, MAPK activation was not induced under the same conditions. The possible implications of these findings with regard to the role of IL-6 and H-IL-6 in the interaction of CD4+CD25− T cells and CD4+CD25high Tregs will be discussed.

Methods

Isolation of peripheral blood lymphocytes and CD4+CD25high (Treg) and CD4+CD25− T cells

PBMCs were isolated from buffy coats or heparinized blood obtained from healthy adult blood donors by Ficoll–Hypaque density gradient centrifugation. Informed consent was obtained from all donors. E-rosetted T cells were separated from PBMCs by rosetting with neuraminidase-treated sheep erythrocytes and subsequent density gradient centrifugation. CD4+CD25− T cells were purified from healthy buffy coats using the CD4+ T-cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and CD25 Dynabeads (Dynamag, Hamburg, Germany). Briefly, CD4+ T cells were negatively isolated by magnetic labeling of non-CD4+ T cells with a biotin–antibody cocktail and anti-biotin MicroBeads using an LD column. The effluent-pre-enriched CD4+ T cells were labeled directly with CD25 Dynabeads and separated using the magnetic particle concentrator (Dynal). The supernatant contained the CD4+CD25− T cells. The magnetic bound fraction contained the CD4+CD25high Tregs. Magnetic particles were removed from the cells by using DETACHaBEAD (Dynal).

Generation of mature dendritic cells

Mature dendritic cells (mDCs) were generated from PBMCs by positive magnetic separation of CD14+ cells using CD14 MicroBeads (Miltenyi Biotec). Isolated cells were cultured in serum-free X-VIVO-15 medium (Cambrex, Verviers, Belgium) supplemented with IL-4 (10 ng ml−1, R&D Systems, Wiesbaden, Germany) and granulocyte macrophage colony-stimulating factor (1000 U ml−1, R&D Systems) for 6 days. After washing, LPS (10 ng ml−1, Sigma–Aldrich, Taufkirchen, Germany) was added for 24 h to mature DCs.

Antibodies

For FACS analysis, we used the following FITC-conjugated mAb to detect subsets of PBMCs: CD4, CD8, CD14, CD19,
CD56, TCR-γδ (all from BD Biosciences, Heidelberg, Germany), biotinylated mAb against CD126 from BD Biosciences or from Bender Medsystems (Vienna, Austria), PE-conjugated anti-CD130 and anti-GITR/TNFRSF18 from R&D Systems, Allophycocyanin (APC)-labeled anti-CD25 mAb and PE/Cyan5.5-conjugated streptavidin were obtained from Caltag Laboratories (Hamburg, Germany), and appropriate fluorochrome-labeled control IgG from BD Biosciences. For western blot analysis, we used the monoclonal phospho-STAT-3 mAb (clone 3E2, Cell Signaling Technology, Beverly, MA, USA), the polyclonal rabbit anti-phospho-p44/42 MAPK (ERK1/2) antibody (Cell Signaling Technology) and anti-β-actin (clone AC-15, Sigma–Aldrich). To stimulate T-cell proliferation, we used the anti-CD3 mAb (clone OKT3, Orthoclone, JANSSEN-CILAG, Neuss, Germany) and the anti-CD28 mAb (clone CD82.2, BD Biosciences).

Phospho-STAT-3 and phospho-ERK1/2 western blot analysis

Freshly isolated CD4+CD25− cells and Tregs (1.5 × 10⁶) were stimulated without or with IL-6 or H-IL-6 (50 ng ml⁻¹ each; 28) or a combination of phorbol myristate acetate (PMA) (10 ng ml⁻¹, Sigma–Aldrich) and ionomycin (500 ng ml⁻¹, Calbiochem, Merck Bioscience GmbH, Germany) for 10 and 30 min. Cells were lysed in Nonidet P40 (Fluka Chemie, Buchs, Switzerland) lysis buffer [1% (v/v) of detergent in 20 mM Tris–HCl (pH 8.1), 150 mM NaCl with protease and phosphatase inhibitors]. Samples were separated on 10% SDS-PAGE, and proteins were transferred to nitrocellulose membranes (Hybond C-Extra, Amersham Biosciences, Braunschweig, Germany). Blots were blocked with 5% BSA (Sigma–Aldrich) and phosphorylated STAT-3 protein was detected with anti-phospho-STAT-3 mAb, followed by HRP-conjugated sheep anti-mouse antibody (Amersham Biosciences) and chemiluminescence detection reagent (ECL, Amersham Biosciences). After stripping, membranes were stained with polyclonal anti-phospho-ERK1/2 antibody and detected as described above. As a control of loading and transfer, membranes were stripped again and reprobed with anti-β-actin mAb.

Flow cytometry

The expression of CD126, CD130 and GITR on subsets of PBMCs and on CD25high versus CD25-negative CD4+ T cells was determined by four-color flow cytometry using a FACSCalibur (BD Biosciences) equipped with the two lasers and the above listed combinations of conjugated mAb. A gate was set on the CD25high and CD25-negative CD4+ T cells to obtain comparable relative cell numbers. For the analysis of the modulation of CD126 and CD130, E-rosetted T cells, 0.25 × 10⁶ isolated CD4+CD25− T cells or Tregs were cultured for 6 h in the absence or presence of IL-2 (50 U ml⁻¹), IL-4 (10 ng ml⁻¹), IL-6 or H-IL-6 (50 ng ml⁻¹ each), immobilized anti-CD3 mAb in combination plus soluble anti-CD28 mAb or mDC in serum-free X-VIVO-15.

Reverse Transcription–PCR of human SOCS-3

Human SOCS-3 mRNA was analyzed using a PCR approach based on total RNA. A total of 7 × 10⁵ cells were incubated in the absence or presence of agents for the time periods detailed in Results. Total RNA was isolated using the Nucleospin RNA II Kit (Macherey und Nagel, Düren, Germany). cDNA was synthesized from 150 ng RNA using random hexamer as primer (Promega, Mannheim, Germany). PCR was performed with HotStart Taq polymerase (Qiagen, Hilden, Germany) with the following conditions: 15 min, 96°C, 30 cycles (30 s, 95°C; 30 s, 55°C; and 1 min, 72°C), 10 min, 72°C. The following oligonucleotide primers were used for human SOCS-3: sense 5’-TCA-AGA-CCT-TCA-GCT-CCA-AG-3’ and anti-sense 5’-GAG-ATG-CTG-AAG-AGT-GGC-C-3’ to yield the complete 512-bp sequence and for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense 5’-GCC-CCC-TTC-ATT-GAC-CTC-3’ and anti-sense 5’-CCA-AAG-TTG-TCA-TGG-ATG-3’ (all primer from TIB Molbiol, Berlin, Germany) to yield the complete 399-bp sequence. GAPDH PCR was performed with 58°C annealing temperature and 35 cycles. PCR products were analyzed following separation by electrophoresis in 1.5% agarose gel.

[^3]H][thymidine incorporation (suppression assay)

Highly purified, negatively selected CD4+CD25− (10⁶ per well) T cells and highly purified CD4+CD25high Tregs (2.5 × 10⁵ per well) were incubated in triplicates alone or in combination with 2 × 10⁶ MACSiBeads (Milenyi Biotec) conjugated with anti-CD3 and anti-CD28 mAbs (5 μg ml⁻¹ MACSiBeads each). Proliferation was measured by uptake of[^3]H][thymidine (Amersham Bioscience) during the last 16 h of a 5- to 6-day culture using a Wallac 1450 Microbeta β-counter (Perkin Elmer).

Results

Differential expression of CD126 and CD130 on human monocytes, B cells, NK cells and T-cell subsets

The expression of CD126 and CD130 was investigated by three-color FACS analysis on freshly isolated PBMCs from healthy adults. As shown for a representative donor in Fig. 1, we observed striking differences in CD126 and CD130 expression among subsets of PBMCs. Strong expression of both receptors was thus detected on almost all CD14+ monocytes. In contrast, CD8low cells were CD126/CD130 negative, well expressed on CD4+ and CD8+ T cells essentially lacked both CD126 and CD130 expression. CD19+ B cells, on the other hand, were mostly CD130 negative yet expressed CD126 to a significant extent, even though at much lower density than CD14+ monocytes. CD4+ and CD8+ T cells displayed variable expression of CD126 and CD130. The majority of CD4+ T cells was strongly CD126+ and CD130+, but CD126/CD130low and CD126/CD130-negative cells were clearly discernible. Among the CD8+ T-cell subset, CD126/CD130-positive and -negative cells were equally distributed. Interestingly, CD8low cells were CD126/CD130 negative, well in line with the lack of CD126 and CD130 expression on CD69+ cells, as most CD8low cells are in fact NK cells (36). Similar patterns of CD126 and CD130 expression were observed in PBMCs of 19 additional healthy adults and cells isolated from 12 tonsils as shown in Table 1. Furthermore, the expression pattern of CD126 and CD130 on cells isolated from...
15 spleens differed from that of PBMCs and tonsil cells. Fewer CD4+ and CD8+ cells expressed both receptors, and B cells expressed less CD126 when compared with PBMCs and tonsil cells. The results are summarized in Table 1.

**Table 1. CD126 and CD130 surface expression on human lymphocyte subsets**

<table>
<thead>
<tr>
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<th>Peripheral blood (n = 19)</th>
<th>Tonsil (n = 12)</th>
<th>Spleen (n = 15)</th>
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<tbody>
<tr>
<td></td>
<td>CD130</td>
<td>CD126</td>
<td>CD130</td>
</tr>
<tr>
<td>CD4+</td>
<td>79.8 ± 10.5</td>
<td>88.7 ± 14</td>
<td>86.0 ± 7.4</td>
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<tr>
<td>CD8+</td>
<td>47.8 ± 16.4</td>
<td>48.4 ± 20.2</td>
<td>42.0 ± 17</td>
</tr>
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<td>CD14+</td>
<td>97.0 ± 3.8</td>
<td>97.0 ± 3.3</td>
<td>94.9 ± 7.4</td>
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<tr>
<td>CD19+</td>
<td>11.3 ± 6.6</td>
<td>51.1 ± 20.5</td>
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</tr>
<tr>
<td>CD56+</td>
<td>3.4 ± 2</td>
<td>5.3 ± 3.3</td>
<td>11.4 ± 7.4</td>
</tr>
<tr>
<td>γδ+</td>
<td>13.9 ± 10.7</td>
<td>17.5 ± 14.9</td>
<td>15.4 ± 10</td>
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</table>

![Fig. 1. Expression of CD130 and CD126 on peripheral blood leukocyte subsets. PBMCs from a representative donor were stained with PE-labeled anti-CD130 mAb (A) or anti-CD126 mAb (B) plus FITC-conjugated mAb against leukocyte subsets as indicated. The figures indicate the percentage of positive and negative cells expressing CD130 (A) or CD126 (B) among the analyzed leucocyte subsets.](https://academic.oup.com/intimm/article-abstract/18/4/555/660548)
CD130 on CD4+CD25− T cells and CD4+CD25high Tregs by four-color FACS analysis. To this end, a gate was set on CD4+CD25− T cells (R1) and on CD4+CD25high Tregs (R2) as shown in Fig. 2 (top). As can be seen, the CD4+CD25− T cells as analyzed in the R1 gate (left) were strongly CD130+ and CD126+. The CD126 was also strongly expressed on the small subset of CD4+CD25high Tregs (R2 gate, right). In striking contrast, Tregs expressed only low levels of CD130. To obtain additional evidence that the CD4+CD25high T cells with low CD130 expression represented Tregs, we also analyzed the expression of the Treg-associated Gitr molecule on CD4+ and CD25high CD4+ T cells. As shown in Fig. 2 (bottom), Gitr was present on CD4+CD25high T cells with low CD130 expression (R2 gate), while it was essentially absent on the CD4+CD25− T cells (R1 gate). Together, these results indicate that CD25high Tregs are characterized by high CD126 and lower CD130 expression.

Modulation of CD130 and CD126 expression

In view of the different patterns of CD126 and CD130 expression on CD4+CD25− T cells and CD25high Tregs, we investigated the modulation of the CD126/CD130 expression in response to IL-6, H-IL-6 and activated mDCs which can produce IL-6. E-rosetted T cells were incubated in the absence or presence of IL-6, H-IL-6, IL-2 or IL-4. After 6 h, the expression of CD126 and CD130 was analyzed on gated populations of CD4+CD25− and CD4+CD25high T cells. As shown in Fig. 3, CD126 and CD130 were down-regulated on CD4+CD25− T cells and CD25high Tregs by IL-6 stimulation. Comparable results were obtained when cells were co-cultured with mDCs. We analyzed the supernatant of mDCs and found a high concentration of IL-6 (>1000 ng ml−1, data not shown). As expected, H-IL-6 modulated CD130 but not CD126 expression on both cell populations. In contrast to IL-6, IL-2 and IL-4 did not modulate the expression of CD126 and CD130 on CD4+CD25− T cells or Tregs within this short-term culture period. Comparable results were obtained with magnetically purified CD4+CD25− T cells and CD25high Tregs by IL-6 stimulation. Comparable results were obtained with magnetically purified CD4+CD25− T cells and CD25high Tregs by IL-6 stimulation. The potential up-regulation of CD25 after TCR stimulation and thus the difficulty to discriminate between activated CD25+ T cells and CD25high Tregs was negligible due to the usage of isolated cells and the short 6 h stimulation period.

STAT-3 and ERK1/2 phosphorylation and SOCS-3 mRNA expression in CD4+CD25− cells and CD4+CD25high Tregs

As shown above, CD4+CD25high Tregs and CD4+CD25− cells differ in their expression of CD130 signal transduction unit of the IL-6R. Therefore, we asked whether the reduced CD130 expression on Tregs resulted in a reduced responsiveness to IL-6/H-IL-6. As shown in Fig. 4(A), incubation with IL-6 or H-IL-6 for 10 or 30 min induced a strong STAT-3 phosphorylation in CD4+CD25− T cells but only weak STAT-3 activation in Tregs. In line with the different CD130 expression, PMA plus ionomycin did not induce STAT-3 phosphorylation but resulted in a rapid and strong ERK1/2 phosphorylation, whereas IL-6 and H-IL-6 had no effect on ERK1/2 activation. To examine a possible correlation between CD130 expression, STAT-3 phosphorylation and SOCS-3 induction, we investigated SOCS-3 mRNA expression in response to IL-6 and H-IL-6 by reverse transcription (RT)–PCR. As shown in Fig. 4(B), SOCS-3 mRNA was up-regulated after stimulation with IL-6 and H-IL-6 both in CD4+CD25− T cells as well as in CD25high Tregs.
Effect of IL-6 and H-IL-6 on Tregs induced T-cell suppression

We tested the role of IL-6 and H-IL-6 in our feeder-free Treg suppression assay based on anti-CD3 and anti-CD28 mAb-coated beads. This assay avoids interference due to cytokine production by contaminating accessory cells. Isolated CD4+CD25− T cells were cultured with or without CD25high Tregs with anti-CD3/anti-CD28 mAb-coated Microbeads in the presence or absence of IL-6 or H-IL-6. The proliferation of CD4+CD25− T cells was significantly suppressed by Tregs (Fig. 5). Moreover, IL-6 partially restored the proliferation in three of five experiments. More striking, H-IL-6 completely abolished suppression in two of four tested donors (Fig. 5).

Discussion

IL-6 is a multi-faceted cytokine with important functions in the immune system and, more generally, in cell biology. The cellular receptor for IL-6 consists of the ligand-binding α chain (CD126) and the CD130 molecule which is shared by several cytokines as the common signal-transducing receptor (17, 37). The distribution of CD126 and CD130 on leukocyte subsets has been previously investigated in humans and mice (31–35). In view of the recently discovered role of IL-6 in the regulation of Treg activity (16), we have re-evaluated the expression of CD126 and CD130 on subsets of human peripheral blood T cells. Pasare and Medzhitov identified IL-6 as one of at least two crucial factors in the supernatant of activated murine DCs that helped to overcome the suppressive activity of Tregs on the activation of CD4+CD25− T cells (16). In order to understand whether IL-6 (plus ‘factor X’; 16) acts on Tregs (to inhibit their suppressive activity) or on CD4+CD25− T cells (to render them resistant against the suppressive activity of Tregs), it is important to investigate the expression of CD126 and CD130 on the various T-cell subsets.

In line with previous investigations, we observed strong expression of CD126 and CD130 on essentially all CD14+ peripheral blood monocytes, and lack of both receptors on CD56+ NK cells (34). Differential expression of the two receptors was detected on CD19+ B cells. While CD130 was present on only a few B cells, more B cells expressed CD126, even though at low density. Together, however, this pattern is consistent with the previously described poor expression of CD126 on human B cells as revealed by IL-6 binding (34) or antibody staining (35).
With regard to subsets of human T cells, we confirm the expression of CD126 on the majority of CD4+ T cells and a sizeable yet smaller population of CD8+ T cells (33–35). Furthermore, our results reveal a close correlation between CD126 and CD130 expression on human peripheral blood T cells, which has not been investigated before. More importantly, we observed a differential expression of CD126 and CD130 on subsets of CD4+ T cells defined on the basis of their IL-2R-α (CD25) expression. Thus, CD4+CD25low T cells were strongly CD126+ and CD130+, while CD4+CD25hi T cells expressed CD126 but little CD130. High-level expression of CD25 among ex vivo isolated CD4+ T cells is a characteristic feature of Tregs (11, 38) which, in addition, express FoxP3 (12), GITR (10) and intracellular and cell-surface CTLA-4 (39). In line with their characterization as Tregs, CD4+CD25hi T cells as analyzed in our study co-expressed GITR and exerted suppressive activity on CD4+CD25low T cells when positively isolated by a two-step magnetic bead-based procedure. The differential expression of CD130 on CD4+CD25low T cells versus CD25hi Tregs might be functionally relevant. As reported by Pasare and Medzhitov, IL-6 produced by DCs in response to bacterial products, together with an unknown factor, inhibited the suppressive activity of Tregs. Indirect evidence suggested that IL-6 acted on CD4+CD25low T cells rather than on CD25hi Tregs in these experiments (16). Our results seem to support this notion as CD25low but not CD25hi CD4+ T cells were found to strongly express CD130, the signal-transducing receptor of the CD126 complex. CD130 expression on CD4+CD25low T cells and CD25hi Tregs within T cells was down-modulated to a similar extent after 6 h culture with IL-6, IL-6-producing mDCs or H-IL-6. However, CD130 modulation was more pronounced in negatively isolated CD4+CD25low T cells when compared with positively isolated CD25hi Tregs. Moreover, CD126 was strongly down-regulated on CD4+CD25low T cells when E-rosetted T cells or isolated CD4+CD25low T cells were cultured for 6 h in the presence of IL-6 or IL-6-producing mDCs or H-IL-6. CD130 expression on CD25hi Tregs was only minimally affected in the very same cultures. Doganci et al. (40) observed a direct effect of IL-6 on lung Tregs in a murine experimental asthma model, suggesting that IL-6 controls the balance between effector cells and Tregs. In contrast to our results, they found that CD4+CD25hi Tregs but not CD4+CD25low lung T cells selectively expressed CD126 mRNA. Furthermore, they showed IL-6-dependent STAT-3 phosphorylation in spleen Tregs, which was blocked by anti-CD126 antibodies. It is possible that murine and human Tregs differ with respect to CD126 expression. Our work with isolated CD25low and CD25hi CD4+ T cells, and additional resting cells or pre-activated T-cell lines (Fig. 4A and data not shown), showed a clear correlation between CD126/CD130 surface expression and STAT-3 phosphorylation. The low CD130 expression on Tregs resulted in a lower STAT-3 phosphorylation compared with CD4+CD25low T cells with a strong
CD126 and CD130 expression on human T cells

A phosphorylation signal (Fig. 4A and results of four additional experiments) and high expression of CD126 and CD130.

In contrast, SOCS-3 was readily induced at the mRNA level in both CD25+ and Tregs. Further analysis of the molecular interdependence of SOCS family members, STAT-3 phosphorylation and functional effects of IL-6 or H-IL-6 which require larger numbers of Tregs and thus in vitro expansion of Treg lines is currently underway in our laboratory.

In support of a functional significance of the observed CD126/CD130 expression, IL-6 and even more potently H-IL-6 reduced the suppression in CD4+CD25+ and Treg co-cultures. The stronger effect of IL-6/soluble IL-6R fusion protein H-IL-6 suggest that it is the soluble IL-6R (in addition to IL-6) which might have been present in the supernatants of DCs described by Pasare and Medzhitov (16). It should be mentioned, however, that only partial inhibition of Treg activity by H-IL-6 was observed when CD4+CD25+ and Tregs were co-cultured at 1:1 ratios. It is obvious that in addition to the IL-6 signaling pathway, other pathways such as Toll-like receptors are involved in the control of Treg activity (41).

In addition to the demonstration of differential CD130 expression on CD4+CD25+ T-cells and Tregs, the present results revealed remarkable differences in the CD126 and CD130 expression between αβ and γδ T cells. Only a minor fraction of freshly isolated γδ T cells expressed these receptors. This suggests that the vast majority of circulating γδ T cells is unable to directly respond to IL-6, in contrast to most αβ T cells. While the significance of this observation is presently unknown, the nearly absent expression of CD126 and CD130 on most circulating γδ T cells might reflect the non-resting activation stage of the dominant Vγ9Vδ2 γδ T-cell population, which recognize microbial phosphoantigens (42, 43). The Vγ9Vδ2 T cells in the peripheral blood of adults display features of pre-activated or memory T cells, i.e. they express CD45RO, serine esterases and certain chemokine receptors such as CCR5 (44–47). The stronger effect of IL-6/soluble IL-6R fusion protein H-IL-6 might have been present in the supernatants of DCs described by Pasare and Medzhitov (16). It should be mentioned, however, that only partial inhibition of Treg activity by H-IL-6 was observed when CD4+CD25+ and Tregs were co-cultured at 1:1 ratios. It is obvious that in addition to the IL-6 signaling pathway, other pathways such as Toll-like receptors are involved in the control of Treg activity (41).

Supplementary data are available at International Immunology Online.

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Abbreviations
APC  aliphophycocyanin
CTLA-4 cytotoxic T-lymphocyte antigen 4
DC dendritic cell
ERK extracellular signal-regulated kinase
GAPDH glyceraldehyde-3-phosphate dehydrogenase
GITR glucocorticoid-inducible tumor necrosis factor receptor-like protein
H-IL-6 hyper-IL-6
MAPK mitogen-activated protein kinase
mDC mature dendritic cell
PMA phorbol 12-myristate 13-acetate
RT reverse transcription
sCD126 soluble CD126
SHP-2 src homology 2 domain-bearing protein tyrosine phosphatase 2
SOCS suppressor of cytokine signaling
STAT-3 signal transducer and activator of transcription 3
Treg regulatory T cell

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