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Cutting Edge: IL-6–Driven Immune Dysregulation Is Strictly Dependent on IL-6R α-Chain Expression

Ilgiz A. Mufazalov,*† David Andruszewski,* Michaela Blanfeld,* Joumana Masri,* Yilang Tang,* Rebecca Schüler,*‡ Christina Eich,* F. Thomas Wunderlich,‖‡ Susanne H. Karbach,*‡‡ Jeffrey A. Bluestone,† Thomas Korn,‡‡∗§ and Ari Waisman,*‡

IL-6 binds to the IL-6R α-chain (IL-6Rα) and signals via the signal transducer gp130. Recently, IL-6 was found to also bind to the cell surface glycoprotein CD5, which would then engage gp130 in the absence of IL-6Rα. However, the biological relevance of this alternative pathway is under debate. In this study, we developed a mouse model, in which murine IL-6 is overexpressed in

Elevated levels of IL-6 have been observed in numerous pathological conditions, and several drugs are successfully used in a series of human diseases, including rheumatoid arthritis, Castleman disease, and giant cell arteritis to target IL-6 and its receptor, IL-6R α-chain (IL-6Rα) (1). For other diseases like multiple myeloma and neuromyelitis optica, the clinical program for the development of IL-6–neutralizing agents is very advanced, and drugs targeting IL-6 or IL-6Rα might be licensed for these diseases in the near future (2).

IL-6, together with IL-1, IL-27, CNTF, LIF, OSM, CT-1, and CLC, belongs to the group of cytokines that use gp130 for signal transduction (3). Since its discovery in 1990, it was believed that the IL-6–signaling complex consists of a unique IL-6–binding receptor IL-6Rα (also known as CD126) and the signal transducer gp130 (4). The assembly of IL-6, IL-6Rα, and gp130 leads to activation of STAT3-mediated intracellular signaling pathways, which control cell survival, activation, and proliferation (2). IL-6Rα also exists in a soluble form, which is generated via secretion or shedding of membrane-bound IL-6Rα. The complex of IL-6 and soluble IL-6Rα is able to bind to gp130 and then induce STAT3 phosphorylation in cells that do not express IL-6Rα themselves. This signaling modality of IL-6 was termed IL-6 trans-signaling (5). A third modality of IL-6 signaling, called IL-6 cluster signaling, is mediated by cell-bound presentation of the IL-6/IL-6Rα complex in trans by a donor cell to a receiving cell that expresses gp130 (2, 6). In both IL-6 trans-signaling and IL-6 cluster signaling, cells that express gp130, but lack IL-6Rα, still respond to IL-6.

In 2016, an alternative pathway of IL-6 signal transduction was proposed in which IL-6 binds to the membrane-anchored...
glycoprotein CD5 instead of IL-6Rα and, via gp130, initiates STAT3 phosphorylation in B cells (7). This study suggested the CD5-dependent pathway of IL-6 signaling was critical in the promotion of cancer progression (7). However, the mechanism for this novel IL-6 binding was not elucidated. In fact, soluble CD5, despite binding to IL-6, is unable to induce IL-6 trans-signaling (8). Massive overproduction of IL-6 is observed in sepsis (9) and has been reported during the cytokine release syndrome in response to CAR-T cell immune therapies (10). To test whether IL-6Rα can be functionally replaced in any IL-6-signaling modality, we used a set of genetically modified mice with IL-6 overexpression. High levels of IL-6 led to systemic inflammation, which subsequently resulted in a lethal outcome. In contrast, mice that overexpressed IL-6, but lacked IL-6Rα, did not show STAT3 phosphorylation and were completely protected from IL-6-mediated pathology, suggesting that IL-6Rα is indispensable for IL-6 signaling.

Materials and Methods

Mice

Mice with conditional Il6ra allele (Il6ra flox) (11) and CD11c-Cre mice (12) have been previously described. IL-6Rα full-knockout (Il6ra −/−) mice were selected from the breeding between Il6ra flox mice and CD11c-Cre mice, which displayed occasional spontaneous germine Cre activity. Principles used to generate mice with the knock-in of a transgene into Rosa26 locus have been previously described (13). Details of the generation of mice carrying murine cDNA in the CAG–(loxP)STOP(loxP)–IL-6–IRES–eGFP transgene inserted previously described (13). Details of the generation of mice carrying murine cDNA in the CAG–(loxP)STOP(loxP)–IL-6–IRES–eGFP transgene inserted in the Rosa26 locus will be reported elsewhere. For breeding strategies involving the Il6ra locus have been selected from the breeding between Il6ra flox mice and CD11c-Cre mice, +/+ and Il-6 DC-OE trans-Il6ra +/− mice were FACS sorted for CD19+CD5 +/+ cells (CD45.1) in U-button, and IL-6 DC-OE trans-Il6ra −/− mice were used in accordance with the guidelines of the central animal facility institution (Translational Animal Research Center, University of Mainz).

Mouse sample collection

Single-cell suspensions from spleen and lymph nodes were prepared by mechanical dissociation in PBS supplemented with 2% FCS. Peritoneal lavage was performed on lethally anesthetized mice with 5 ml of PBS supplemented with 3% FCS. Peripheral blood was collected from the tail vein. RBCs were removed using ACK lysis buffer.

IL-6 ELISA

A murine IL-6 ELISA Kit (BD Biosciences) was used to analyze IL-6 levels in the blood serum. Plates were measured with the Infinite M200 PRO NanoQuant reader (Tecan).

Flow cytometry

Single-cell suspensions were stained with Abs together with viability dyes (Supplemental Table I). Stained cells were acquired on FACSConto II (BD Biosciences), and data were analyzed with FlowJo software. Gating strategy (Supplemental Table I). Stained cells were acquired on FACSCanto II (BD Biosciences), and data were analyzed with FlowJo software.

Immunoblotting

To test whether IL-6Rα can be functionally replaced in any IL-6-signaling modality, we used a set of genetically modified mice with IL-6 overexpression. High levels of IL-6 led to systemic inflammation, which subsequently resulted in a lethal outcome. In contrast, mice that overexpressed IL-6, but lacked IL-6Rα, did not show STAT3 phosphorylation and were completely protected from IL-6-mediated pathology, suggesting that IL-6Rα is indispensable for IL-6 signaling.

Results and Discussion

The cytokine IL-6 uses various signaling modalities (i.e., classic signaling, trans-signaling, and cluster signaling [also called trans-presentation]) and might even signal through an alternative receptor complex composed of CD5 and gp130 (7). To assess the IL-6R signaling modality of IL-6 under conditions of exaggerated IL-6 production, we created a murine system of sterile IL-6 overexpression in vivo. To this end, we generated mice carrying a CAG–(loxP)STOP(loxP)–IL-6–IRES–eGFP construct in the Rosa26 locus. Next, we used dendritic cell (DC)–directed CD11c-Cre transgenic mice (12) to activate IL-6 overexpression (OE) from a single copy of our Il6 transgene in mice on an IL-6Rα–sufficient background (IL-6 DC-OE Il6ra +/− and IL-6 DC-OE Il6ra −/− [i.e., IL-6 DC-OE]) and on an IL-6Rα–deficient background (IL-6 DC-OE Il6ra −/−). As further controls, we used IL-6Rα–sufficient (control) and IL-6Rα–deficient (Il6ra −/−) mice without IL-6 overexpression (Supplemental Fig. 1A).

To directly assess IL-6 overexpression, we measured IL-6 levels in the serum of 5–6-wk-old mutant mice. As expected, both groups of mice with IL-6 overexpression showed elevated levels of IL-6 in comparison with wild-type controls and Il6ra −/− mice (Fig. 1A). IL-6 levels were significantly higher in IL-6 DC-OE Il6ra +/− mice than in IL-6 DC-OE Il6ra −/− mice, suggesting a positive feed-forward loop for IL-6 expression in mice sufficient for IL-6Rα. Mechanistically, transgenic IL-6 can promote IL-6 expression in IL-6 DC-OE mice, from the endogenous Il6 locus, which remained responsive to its natural regulation. In addition, IL-6 forms a complex with the soluble IL-6Rα and gp130 in the blood, which can prolong its half-life only in IL-6Rα–sufficient, but not in IL-6Rα–deficient, mice (14, 15).

To analyze cellular sources of transgenic IL-6, we assessed eGFP reporter expression driven by the CAG promoter after CD11c-Cre mediated excision of the loxP flanked STOP cassette. As expected, we found eGFP expression in DCs in IL-6 DC-OE Il6ra +/− and IL-6 DC-OE Il6ra −/− mice (Fig. 1B, Supplemental
FIGURE 1. IL-6Rα−deficient mortality of mice with IL-6 overexpression. (A) IL-6 levels in the blood serum of mice measured by ELISA. Dot plot graph shows values for individual mice and mean per group. (B) CD11c-Cre−mediated IL-6 overexpression reported by eGFP coexpression in DCs in the spleen. Data depict representative FACS plots with average frequencies per group. Three to four mice per group were used. For full gating strategy, refer to Supplemental Fig. 1B. (C) Kaplan–Meier curves depict survival of the indicated mouse strains. (D) Median fluorescence intensity (MFI) of the IL-6Rα expression on splenocytes. Bar diagram shows mean per group ± SD. Isotype control was stained on corresponding cell populations isolated from Il6ra−/− mice. Four mice per group were used. For representative IL-6Rα FACS staining, refer to Supplemental Fig. 2A. (E) Western blot analysis of STAT3 phosphorylation in response to IL-6 stimulation in vitro (10 ng/ml) of total splenocytes. Two-tailed unpaired t test was used for (A) and (D); Gehan–Breslow–Wilcoxon test was used for (C). All experiments were performed two to three times with similar results. *p < 0.05, **p < 0.01, ***p < 0.001.

Because of leakiness of the CD11c-Cre recombinase expression (16), we also noted limited reporter expression in other myeloid cells and in T and B cells of these mice (Supplemental Fig. 1C).

High systemic levels of IL-6 ultimately led to the death of IL-6DC-OE mice in an IL-6Rα−dependent manner (Fig. 1C). IL-6−overexpressing mice carrying both copies of Il6ra died within 6 wk of age, whereas deletion of one Il6ra allele (with one Il6ra allele intact in heterozygous Il6ra+− mice) extended the survival of IL-6DC-OE mice until 11 wk of age. Strikingly, null deficiency in Il6ra restored the survival of IL-6DC-OE Il6ra−/− mice with IL-6 overexpression and, thus, fully rescued the phenotype of IL-6DC-OE mice. Of note, 3 out of 41 Il6ra−/− mice died during the observation period presumably because of their immunodeficiency (17), irrespective of IL-6 overexpression.

To study the impact of IL-6Rα expression on IL-6 signaling, we isolated splenocytes from wild-type (Il6ra+×), Il6ra+−, and Il6ra−/− mice. We observed a robust IL-6Rα expression on CD4+ T cells and, to a lesser extent, on CD11b+ myeloid cells, but very limited expression on CD19+ B cells in wild-type mice (Fig. 1D). Deletion of a single Il6ra allele led to reduction in IL-6Rα expression, suggesting a haploinsufficient gene dose effect (Fig. 1D), which, together with reduced frequencies of IL-6Rα−positive cells in Il6ra−/− mice (Supplemental Fig. 2A), may contribute to the reduced mortality of IL-6DC-OE Il6ra−/− mice in comparison with IL-6DC-OE Il6ra+× mice (Fig. 1C). To determine the consequence of the lack of IL-6Rα in downstream signaling events, we stimulated total splenocytes isolated from Il6ra+×, Il6ra+−, and Il6ra−/− mice with IL-6 and assessed their STAT3 phosphorylation. We observed STAT3 phosphorylation only in wild-type and Il6ra heterozygous cells, whereas Il6ra−/− splenocytes were irresponsive to stimulation with soluble IL-6 (Fig. 1E, Supplementary Fig. 2B). FACS-sorted CD5− and CD5+ B cells, as well as CD5+ T cells, isolated from Il6ra−/− mice did not respond to soluble IL-6 either, whereas their wild-type counterparts showed a clear STAT3 activation in response to soluble IL-6 (Supplemental Fig. 2C). In contrast, IL-6Rα−deficient B cells (regardless of CD5 expression), and IL-6Rα−deficient T cells responded to IL-6 in the presence of exogenous IL-6Rα in the form of hyper–IL-6, which represents IL-6/IL-6Rα fusion protein (Supplemental Fig. 2C). These data indicate that Il6ra−/− cells retain the ability to respond to IL-6 trans-signaling, whereas IL-6Rα expression on the cell surface is nonredundant for classic IL-6 signaling. Together, this refutes the concept that alternative receptor molecules can compensate for the lack of IL-6Rα expression.

As a consequence of IL-6 overexpression, CD11b+ myeloid cells massively infiltrated secondary lymphoid organs, resulting in splenomegaly of IL-6Rα−sufficient IL-6DC-OE mice (Supplemental Fig. 3). The most dramatic increase in response to transgenic IL-6 was observed in Ly-6G+ neutrophils and Ly-6Ch+ monocytes/macrophages (Fig. 2A). Notably, the systemic increase in these myeloid cell subsets was completely abrogated in IL-6DC-OE Il6ra−/− mice lacking IL-6Rα. It is likely that the systemic inflammatory response in IL-6DC-OE mice is dependent on the massive expansion of myeloid cells.

Initially, IL-6 was named “B cell hybridoma growth factor,” to acknowledge its stimulatory function on B cells (18). Although we did not detect robust expression of IL-6Rα on the surface of B cells (Fig. 1D), IL-6 can pair with soluble IL-6Rα and act on B cells expressing gp130. Importantly, a recent study suggested that IL-6 can bind to CD5 on B cells and activate STAT3 independently of IL-6Rα (7). Among B cells, CD5 expression is rather restricted to B-1a cells, identifying this subset as a primary target for the IL-6–CD5-signaling module (19). Of note, mice with IL-6 overexpression on an IL-6Rα−sufficient background exhibited a dramatic reduction in B-1a cells in the spleen (Fig. 2B), whereas IL-6Rα deficiency completely prevented the IL-6–triggered loss of B-1a cells in IL-6DC-OE Il6ra−/− mice (Fig. 2B).
Because CD4⁺ T cells expressed high levels of IL-6Rα (Fig. 1D), we analyzed the T cell compartment in mice with activated IL-6 pathway. We noted a significant shift toward CD44⁺CD62L⁻ effector CD4⁺ T cells in the spleen of IL-6DC-OE mice in comparison with the other groups tested (Fig. 2C). However, the frequencies of effector CD4⁺ T cells in response to exaggerated IL-6 expression became normal again after IL-6Rα deletion. In accordance with a previous report (20), we found abundant CD5 expression on CD4⁺ T cells, however, irrespective of their IL-6Rα status (Fig. 2D).

To test STAT3 activation in specific lymphocyte subsets in mice with IL-6 overexpression, we performed immunoblotting with lysates from CD19⁺ B cells and CD4⁺ T cells. Both cell types isolated from IL-6 DC-OE mice displayed STAT3 phosphorylation already in steady state without additional in vitro IL-6 stimulation (Fig. 2E). Importantly, the steady-state STAT3 phosphorylation (and also STAT3 phosphorylation in response to exogenous IL-6 stimulation in vitro) was absolutely dependent on the presence of at least one copy of Il6ra in CD19⁺ B cells and in CD4⁺ T cells (Fig. 2E).

In the current study, we observed a lethal immunopathology in mice that overexpressed IL-6 as long as they were kept on an IL-6Rα-sufficient background. Among several genetic models with IL-6 overexpression (reviewed in Ref. 21), we activated murine IL-6 overexpression driven by CAG promoter in a CD11c-Cre–dependent manner. By genetic ablation of Il6ra, the phenotype of profound immune dysregulation in mice with transgenic IL-6 was completely rescued. This argues against the biological significance of any IL-6 signaling systems that would be independent of IL-6Rα. In our model, we cannot draw a definite conclusion as to which IL-6 signaling modality (i.e., classic IL-6 signaling, IL-6 trans-signaling, or IL-6 cluster signaling [trans-presentation]), is the most relevant IL-6–signaling modality for the fatal immune dysregulation in IL-6DC-OE mice. All three signaling modalities depend on IL-6Rα, either on the side of the receiving cell (classic IL-6 signaling) or on the side of the donating cell in a soluble manner (IL-6 trans-signaling) or in a cell-bound manner (IL-6 trans-presentation) (2, 6). In steady state, CD4⁺ T cells and CD11b⁺ myeloid cells express relatively high levels of the membrane-bound IL-6Rα. Thus, we speculate that, at least initially, IL-6 overexpression is operational in lymphocytes and myeloid cells through classic IL-6 signaling. The massive expansion of granulocytes in IL-6DC-OE mice was a robust
observation. However, it remains to be determined whether the expansion of Ly-6G+ cells in IL-6\textsuperscript{DC-OE} mice is a direct effect of IL-6. Recently, concerns have been raised as to whether circulating granulocytes can directly respond to IL-6 (22).

Taken together, although we did not aim to dissect direct and indirect effects of IL-6 overexpression in developing devastating inflammation, our data compellingly support the absolute requirement for IL-6Rα in mediating IL-6 effects in mice. The fact that our study did not confirm the IL-6–CD5-signaling module proposed for B cells (7) challenges the biological significance of IL-6 signals transduced by an IL-6/CD5/gp130 complex. Our conclusion is in accordance with the recent finding that soluble CD5, despite binding of IL-6, is unable to induce STAT3-mediated signal transduction in gp130-expressing cells (8).

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental materials

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Supplemental Figures 1-3

Supplemental Table I
Supplemental Figure 1. Generation and analysis of mice with IL-6 overexpression (Related to Figure 1).

A – Breeding scheme to generate different groups of mice with IL6 overexpression. Tg - transgene; + - wild type (wt) allele; - - knock out allele; DC - dendritic cells; OE - overexpression.

B – Gating strategy used for Figure 1B. After defining VD live cells autofluorescence in FITC channel was excluded by using “NOT Boolean Gating” FlowJo algorithm.

C – CD11c-Cre-mediated IL-6 overexpression followed by eGFP co-expression across different cell types in lymph nodes. Data depict representative FACS plots with average frequencies per group. Three to five mice per group were used. LN - pooled inguinal, brachial and axillary lymph nodes. All experiments were performed two to three times with similar results.
Supplemental Figure 2. Analysis of IL-6 signaling modalities (Related to Figure 1).

A – FACS analysis of IL-6Rα expression across different cell types in the spleen. Data depict representative FACS histograms indicating IL-6Rα+ cells within the respective parental population. Bar diagram shows mean per group + SD. Isotype control was stained on corresponding cell populations isolated from Il6ra+/- mice. Four mice per group were used. Total cells defined as VD− live cells; CD4+ T cells defined as VD−CD19− TCRβ+CD4+ cells; CD19+ B cells defined as VD−TCRβ−CD19+ cells, CD11b+ cells defined as VD−CD19− TCRβ−CD11b+ cells.

B – Western blot analysis of STAT3 phosphorylation in total splenocytes in response to IL-6 stimulation in vitro (300 ng/ml).

C – FACS analysis of STAT3 phosphorylation in response to in vitro stimulation with IL-6 (50 ng/ml) and hyper-IL-6 (125 ng/ml). Mean fluorescence intensity (MFI) of p-STAT3 was normalized to the condition without stimulation (time point 0) per genotype. CD5+ and CD5+ B cells were isolated from the peritoneal cavity, and CD5−CD4+ T cells were isolated from the spleen of Il6ra+/- and Il6ra-/- mice, respectively.

*p < 0.05, ***p < 0.001, N.S. – not significant; two-tailed unpaired t-test. All experiments were performed two to three times with similar results.
Supplemental Figure 3. Analysis of different cell types in mice with IL-6 overexpression (Related to Figure 2).

A – Fractions and absolute numbers of different leukocyte subsets in the spleen and lymph nodes of the indicated mouse strains.

B – Cellularity in the spleen and lymph nodes of the indicated mouse strains.

Dot plot graphs (A-B) show values for individual mice and mean per group. Three to five mice per group were used. LN - pooled inguinal, brachial and axillary lymph nodes; VD - Viability dye; cDC - conventional dendritic cells, defined as VD\(\text{CD19-TCR}\beta\text{CD11b-CD11c}\)+ cells; moDC - myeloid-derived dendritic cells, defined as VD\(\text{CD19-TCR}\beta\text{CD11b+CD11c}\)+ cells.

\(\ast p < 0.05, \ast\ast p < 0.01, \ast\ast\ast p < 0.001, \text{N.S.} – \text{not significant}; \text{two-tailed unpaired t-test. All experiments were performed two to three times with similar results.}\)
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**Supplemental Table I. FACS antibodies and viability dyes used in the study.**