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IL-6-dependent autoimmune disease: dendritic cells as a sufficient, but transient, source[1]

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Abstract

Mice lacking IL-6 are resistant to autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), which is driven by central nervous system (CNS)-reactive CD4^{+} T cells. There are multiple cellular sources of IL-6, but the critical source in EAE has been uncertain. Using cell-specific IL-6-deficiency in models of EAE induced by active immunization, passive transfer, T cell-transfer and dendritic cell (DC)-transfer, we show that neither the pathogenic T cells, nor CNS-resident cells are required to produce IL-6. Instead, the requirement for IL-6 was restricted to the early stages of T cell activation and was entirely controlled by DC-derived IL-6. This reflected the loss of IL-6 receptor expression by T cells over time. These data explain why blockade of the IL-6 receptor only achieves protection against EAE if used at the time of T cell priming. The implications for therapeutic manipulation of IL-6-signaling in human T cell-driven autoimmune conditions are considered.

Introduction

IL-6 is a potent pro-inflammatory mediator with key roles on both acute and chronic inflammation and multiple effects on many immune and non-immune cell-types. As such tocilizumab, a blocking antibody against the IL-6 receptor has been used in clinical trials, with beneficial effects in rheumatoid arthritis (1). To provide mechanistic data, the contribution of IL-6 to pathology has been examined in a variety of experimental autoimmune models, including experimental autoimmune encephalomyelitis (EAE), the prototypic CD4^{+} T cell-driven model of organ-specific autoimmune inflammation (2). IL-6–/– mice are completely resistant to EAE induction (3, 4) and IL-6 receptor blockade can prevent the development of EAE in IL-6-sufficient mice (5). However, the key cells that are required to produce IL-6 to drive EAE have not been defined. Such information is important to provide a better understanding of how IL-6-blockade can be best targeted therapeutically. Here we have addressed this question and find that, despite their ability to produce IL-6, this is not a requirement of encephalitogenic T cells. Instead the key early source of IL-6 in vivo appears to be dendritic cells (DC), because a simple transfer of autoantigen-loaded IL-6-sufficient DC renders IL-6-deficient mice fully susceptible to EAE.

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Materials and Methods

Mice and antigens

C57BL/6, IL-6$^{-/-}$, Tg4.WT (CD45.1), Tg4.IL-6$^{-/-}$ (CD45.1), and C57BL/6×B10.PL mice were bred under specific pathogen-free conditions. Experiments were approved by the University of Edinburgh ethical review committee and were conducted under UK legislation. The myelin oligodendrocyte glycoprotein 35-55 (pMOG) peptide and the myelin basic protein (MBP) Ac1-9 and Ac1-9(4Tyr) peptides were obtained from Cambridge Research Biochemicals (Cleveland, UK).

Induction and assessment of EAE

For pMOG-induced active EAE, C57BL/6 or IL-6$^{-/-}$ mice were subcutaneously immunized with 100μg pMOG emulsified in CFA as described previously (6). Passive EAE was induced using a previously described protocol (6). For MBP-induced active EAE, C57BL/6×B10.PL host mice received an i.v. injection of 1×10^6 naïve Tg4.WT or Tg4.IL-6$^{-/-}$ CD4$^+$ T cells one day before immunization with 10μg Ac1-9(4Tyr) in CFA and administration of 200 ng pertussis toxin on the same day and two days later.

BMDC generation and transfer

Bone marrow preparations (from C57BL/6, IL-6$^{-/-}$, or C57BL/6×B10.PL mice, as indicated) were seeded into 6 well plates at 2×10^5/ml in 2ml cultures, in RMPI 1640 medium supplemented with 10% FCS, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin and 50μM 2-ME (Invitrogen Life Technologies, Paisley, UK) and 20ng/ml rGM-CSF (Peprotech). Cultures received a further 2 ml of the same medium on day three and were replenished (2ml exchanged) on day six. BMDC were harvested on day 8 and cultured overnight at 2×10^6 BMDC/ml with, 5ng/ml rGM-CSF, 1μg/ml LPS (Sigma) and 10μg/ml pMOG or Ac1-9(4Tyr) as appropriate, prior to s.c. injection of 1×10^6 peptide-pulsed BMDC into host mice.

Assessment of lymphoid recall responses

Cell suspensions from individual pMOG-immunized mice were cultured in 96-well flat-bottomed plates (BD, Oxford, UK) at 6×10^5 lymph node cells/well, or 8×10^5 splenocytes/well, using X-Vivo 15™ serum-free medium (BioWhittaker, Maidenhead, UK) supplemented with 2mM L-glutamine and 50μM 2-ME (Invitrogen Life Technologies, Paisley, UK). Cells were stimulated with a dose range of pMOG for 72h. IFN-γ, IL-17 and GM-CSF were quantified by ELISA.

Flow cytometric analyses

Mononuclear cells from CNS and peripheral lymphoid organs were stained with the indicated antibodies (all from e-bioscience). For pSTAT analysis ex-vivo, single cell suspensions were immediately fixed in 2% PFA for 20 mins at 37 °C prior to surface staining. Cells were then resuspended in ice-cold 90% methanol and stored overnight at −20 °C. Cells were then washed extensively and incubated with Fc-block before intracellular staining for pSTAT3 and pSTAT5 (both BD Bioscience).

Intracellular cytokine expression was determined following overnight incubation with 20μg/ml pMOG or 10μg/ml Ac1-9 as described previously (6). Data were acquired on BD LSR II and LSR Fortessa cytometers (BD biosciences, USA) and analysed with Flowjo analysis software (Treestar, USA).

Results and Discussion

Neither T effector cells nor CNS resident cells are required sources of IL-6 in EAE

Consistent with previous reports (3, 4), H-2^b mice lacking IL-6 were totally resistant to active EAE induced with pMOG (Fig. 1A). This correlated with an inability to mount a pro-inflammatory effector response, including GM-CSF production, following immunization (Fig. 1B-D). In vitro-activated effector T cells from pMOG-primed wild type (WT) mice were able to induce EAE when transferred into IL-6$^{-/-}$ hosts (Fig. 1E), unlike a previous report that suggested CNS-derived IL-6 was required for T cell infiltration in passive EAE (3). In contrast, effectors from IL-6$^{-/-}$ mice could not induce disease in WT recipients (Fig. 1F). However, this did not necessarily mean that T cells *per se* were the crucial source of IL-6. An alternative explanation would be that WT effectors had been exposed to IL-6 from a non-T cell source and that this triggered their pathogenic function, without a need for the T effectors themselves to produce IL-6. The Tg4 mouse is transgenic for a TCR recognizing the Ac1-9 peptide of MBP (7). We generated Tg4.IL-6$^{-/-}$ mice and found these to be resistant to EAE induction by immunization with the MBP peptide (data not shown). C57BL/6×B10.PL mice do not develop EAE after immunization with the MBP Ac1-9, unless they are first seeded with Tg4 T cells (EAE is driven solely by the activity of the transferred Tg4 T cell cohort) (8). Transfer of either Tg4.WT or Tg4.IL-6$^{-/-}$ T cells rendered host C57BL/6×B10.PL mice fully susceptible to disease (Fig. 2B). Tg4.IL-6$^{-/-}$ T cells showed no defects in their capacity to produce pro-inflammatory cytokines in the CNS (Fig. 2C). We can therefore conclude that the required in vivo source of IL-6 in EAE is neither the pathogenic T cells themselves, nor the innate immune cells that they draw into, and activate in, the CNS (WT T effector cells transfer EAE into IL-6$^{-/-}$ mice, Fig. 1E).

Dendritic cell expression of IL-6 determines EAE susceptibility

The requirement for IL-6 appeared to be early, in the lymph node. The obvious candidate was therefore the T cell-priming DC. The induction of EAE directly with autoantigen-loaded DC alone has been attempted in several systems, but the results have been unsatisfactory. We therefore made use of a system that we reported previously (9), in which C57BL/6 mice receive LPS-activated BMDC, that have been loaded with pMOG, seven days prior to the normal protocol for EAE induction using pMOG in CFA. This places the initial activation of pMOG-responsive T cells under the control the administered DC and allows the effects of DC gene expression to be analyzed downstream, in terms of EAE development. IL-6 was produced by CD11c^{hi} cells in WT (but not IL-6$^{-/-}$) BMDC upon LPS activation (Fig. 3).

The disease phenotype proved to be totally dependent on the genotype of the DC used for the initial transfer (Fig. 4A,B). Thus, administering pMOG-loaded WT DC was sufficient to render IL-6$^{-/-}$ mice fully susceptible to EAE upon subsequent immunization. Conversely, administering pMOG-loaded IL-6$^{-/-}$ DC to WT mice rendered them resistant to EAE (Fig. 4B). Perhaps predictably for mice that developed no EAE, IL-6$^{-/-}$ mice that had first received IL-6$^{-/-}$ DC had no discernable inflammatory infiltrate in their CNS (data not shown). CNS CD4$^+$ T cells from WT DC→WT mice produced IFN-γ and GM-CSF, but low numbers of T cells stained for IL-17 after culture with pMOG (Fig. 4C). The CNS of WT mice that had been "protected" from EAE by the initial transfer of IL-6$^{-/-}$ DC did contain inflammatory infiltrates, but only low numbers of T cells producing inflammatory cytokines (Fig. 4C) In contrast, IL-6$^{-/-}$ mice in which EAE susceptibility had been "restored" by the initial administration of pMOG-loaded WT DC had high numbers of CD4$^+$ cells producing IFN-γ and GM-CSF (Fig. 4C). Although no IL-17 production was evident in CNS samples from these mice, their splenocytes did produce IL-17 on challenge with pMOG (Fig. 4D).

These data indicate that the only point at which IL-6 determines the ultimate pathological outcome in EAE is the initial exposure of T cells to their autoantigen in the lymph node. This can be controlled experimentally by a single administration of DC. The persistence of DC after transfer is difficult to follow accurately, but is believed to be in the order of 48-72h. This would predict that T cells must receive all the IL-6-signaling required to imprint pathogenic activity within this period.

Loss of T cell CD126 expression after in vivo antigen stimulation

In vitro studies have reported that T cells lose expression of their IL-6 receptors within 24h of TCR stimulation (10), an observation that we have also made. However, we had no data on whether the kinetics of IL-6 receptor loss were similar in vivo. We seeded C57BL/6×B10.PL mice with naïve Tg4.WT T cells, prior to s.c. administration of MBP peptide-loaded WT DC (Fig. 5A). At three days, donor T cells showed ex vivo evidence of STAT5 phosporylation, indicative of IL-2 signaling and, importantly, STAT3 phosphorylation indicative of recent exposure to IL-6 (Fig. 5B). At day 6, frequencies of Tg4 T cells expressing T-bet, and producing IL-17, IFN-γ and GM-CSF were elevated compared to host T cells (Fig. 5C). Focusing on IL-6 receptor expression, there was no clear evidence for down-regulation of CD126 by Tg4 cells at day three, but this did become evident at day six (Fig. 5D). We can therefore conclude that downregulation of T cell expression of IL-6 receptors is a consequence of their in vivo activation but, at least for the model system used here, this takes significantly longer than has been predicted by previous in vitro experiments.

Others have reported that H-2^b mice lacking T cell expression of gp130 (the signal-transducing chain of the IL-6 receptor) generate pMOG-responsive adaptive Treg in response to immunization (11). This would make some sense given the paradigm in which exposure to IL-6 is the key checkpoint determining Treg versus Th17 differentiation (12). However, in our hands, pMOG-responsive naïve TCR transgenic T cells did not show a conversion to Foxp3-expression when placed in IL-6$^{-/-}$ hosts. There was also no dominance of Foxp3$^+$ cells in the CNS CD4$^+$ infiltrate of WT mice that were protected from EAE by the initial transfer of IL-6$^{-/-}$ DC and we found no evidence for an expansion in the frequency of CD4$^+$Foxp3$^+$ cells in Tg4.IL-6$^{-/-}$ mice that had been immunized with the MBP peptide in CFA. It is of course worth noting that gp130 is also the signaling chain for a number of other cytokines and so it is possible that the observations of that previous report (11) reflected a cumulative deficiency in cytokine signaling that we did not mimic by using mice or cells that lacked only IL-6. We saw little IFN-γ (consistent with previous reports) (4, 5) or GM-CSF production by IL-6−/− mice, indicative of a more general inhibition of T cell priming, rather than Th17 in particular.

Many cells can produce IL-6. Murine B cells are a major source following TLR-triggering (13, 14) and we have recently reported that B cell-derived IL-6 can contribute to EAE severity (15). However, it is not a critical component because EAE is exacerbated, rather than ameliorated, in mice that are genetically deficient in B cells (16).

Others have reported that administration of recombinant IL-6 to IL-6$^{-/-}$ mice can allow the development of EAE following immunization with pMOG, but only if treatment is commenced at the time of immunization (17). Consistent with this, anti-IL-6R antibody could effectively prevent EAE if given at the time of immunization, but could not reverse established disease (5). Our data make sense of this, because T cells are sensitive to IL-6 at the time of their initial activation in the lymph node, but not when they are executing their pathogenic roles in the CNS. All CD4$^+$ T cells found in the inflamed CNS during EAE display profound reductions in their expression of both CD126 and gp130, rendering them unresponsive to IL-6 classical or *trans*-signaling (18). Although CNS T cells are insensitive to IL-6, they can produce IL-6 themselves (19) and the innate immune cells in the CNS do

express IL-6 receptors. However, given that IL-6R-blockade does not reverse EAE (5) and that we can confer EAE susceptibility when the only source of IL-6 is a small cohort of short-lived DC, we can be confident that IL-6 production in the CNS makes no critical contribution to pathology.

Nevertheless, IL-6-blockade can be highly effective in the clinic, particularly in rheumatoid arthritis (1). How can we square this with our data? In both mouse and man, expression of the IL-6R seems to be restricted to naïve and central memory T cells (20). Loss of IL-6R following TCR-mediated activation of effector T cells correlates with their loss of CD62L and CCR7. IL-6 might therefore influence T cell function during the generation of new autoaggressive cells from the naïve T cell pool, or during the reactivation of central memory populations that might drive exacerbation of chronic human conditions. Of course a simpler alternative is that IL-6 blockade functions by inhibiting IL-6 signaling in pathogenic non-T cell populations which are less susceptible to activation-induced loss of IL-6R (21). Further detailed exploration is warranted to test these possibilities under different immunopathological scenarios.

In conclusion, our study pin-points the key early requirement for IL-6 in the provocation of autoaggressive CD4^{+} T cells and that DC are entirely sufficient as the source of the IL-6 instructing this function.

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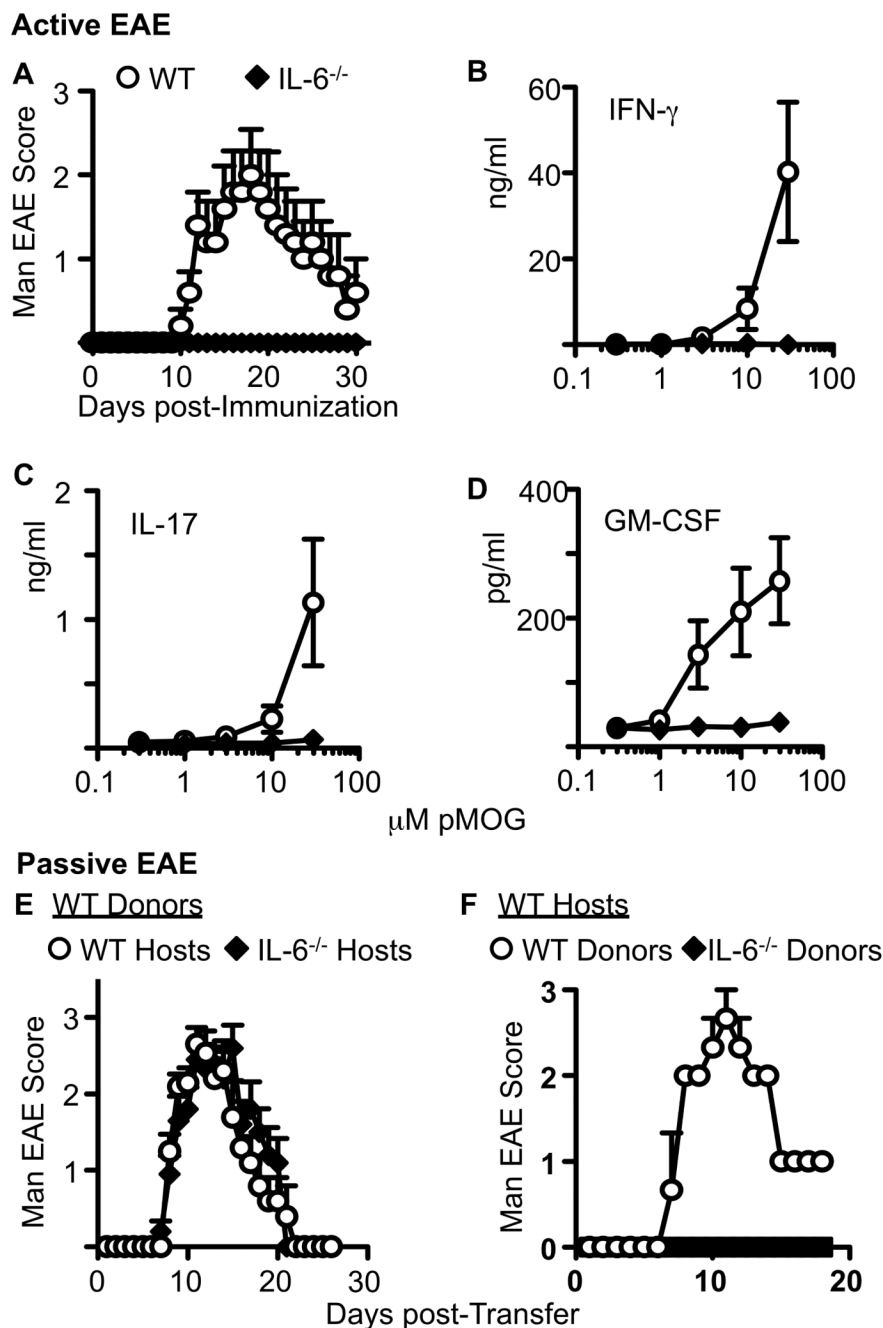
Figure 1. IL-6 is not required from CNS innate immune cells in EAE

(A) WT C57BL/6 and IL-6$^{-/-}$ mice were immunized with pMOG for active EAE (5 mice per group, data from one of three experiments giving consistent results), or for LN recall responses to pMOG (B-D) (5 mice per group, data from one of three experiments giving consistent results). (E,F) For passive EAE, donor mice were immunized with pMOG+CFA, draining LN were isolated and restimulated in vitro prior to transfer. (E,) WT versus IL-6$^{-/-}$ hosts received transfers from WT donors (5 mice per group, data from one of three experiments giving consistent results). (F) WT hosts received transfers from WT versus IL-6$^{-/-}$ donors (5 mice per group, data from one of two experiments giving consistent results).
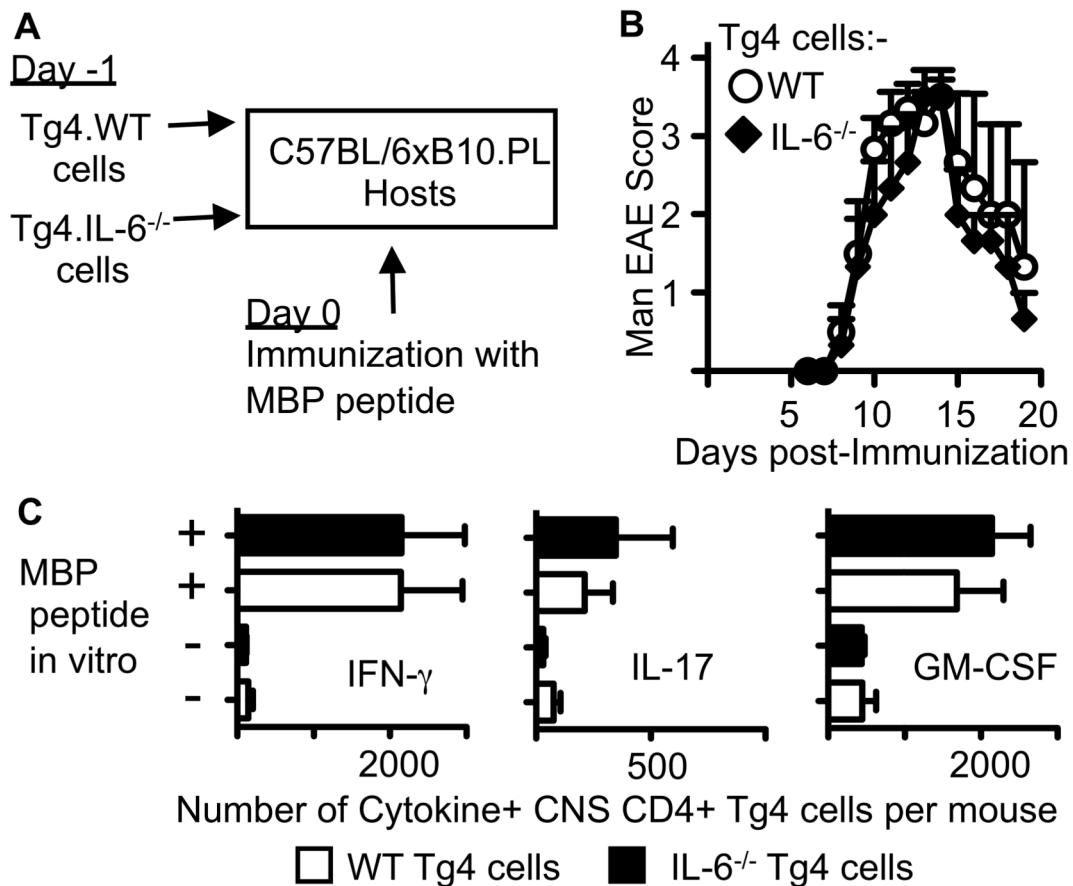
Figure 2. Encephalitogenic T cells do not need to produce IL-6
(A,B) C57BL/6×B10.PL mice were seeded with 1×10^6 naïve CD4$^+$ T cells from Tg4.WT or Tg4.IL-6$^{-/-}$ prior to induction of EAE by immunization with MBP Ac1-9(4Tyr) peptide in CFA. (C) At day 14, CNS mononuclear cells were isolated, stained ex vivo for surface markers, or cultured with or without MBP Ac1-9 peptide prior to intracellular cytokine staining. Numbers of cytokine-positive Tg4 cells per mouse were calculated from the % cytokine-positive CD45.1$^+$CD4$^+$ cells and the numbers of CD45.1$^+$CD4$^+$ cells on ex vivo analysis. Data (6 mice per group, 3 analyzed at day 14) are from one of two experiments giving consistent results.
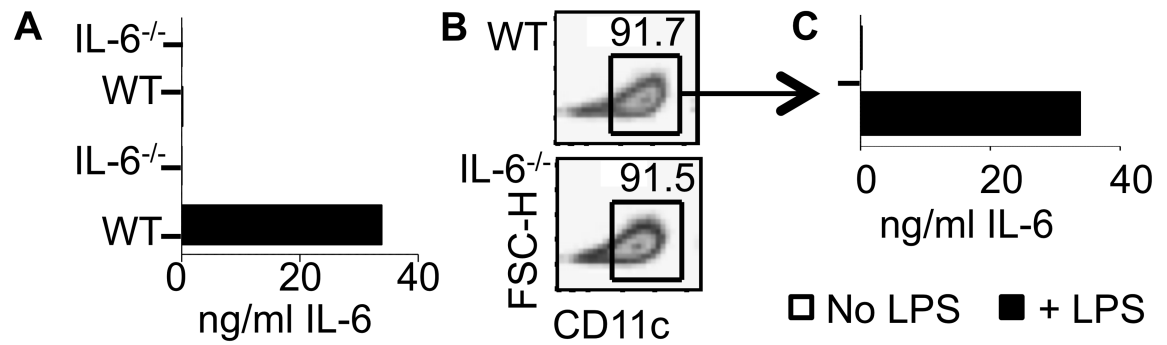
Figure 3. LPS drives IL-6 production by WT CD11c⁺ DC

A) IL-6 was measured in the supernatants of WT or IL-6–/– BMDC conditioned with or without LPS for the final 18 hours of culture. B) CD11c expression on WT and IL-6–/– BMDC. C) After 8-day culture of WT BMDC, CD11c⁺ cells were isolated by FACS sorting. IL-6 was measured in the supernatants after a further 18 hours of culture with or without LPS.
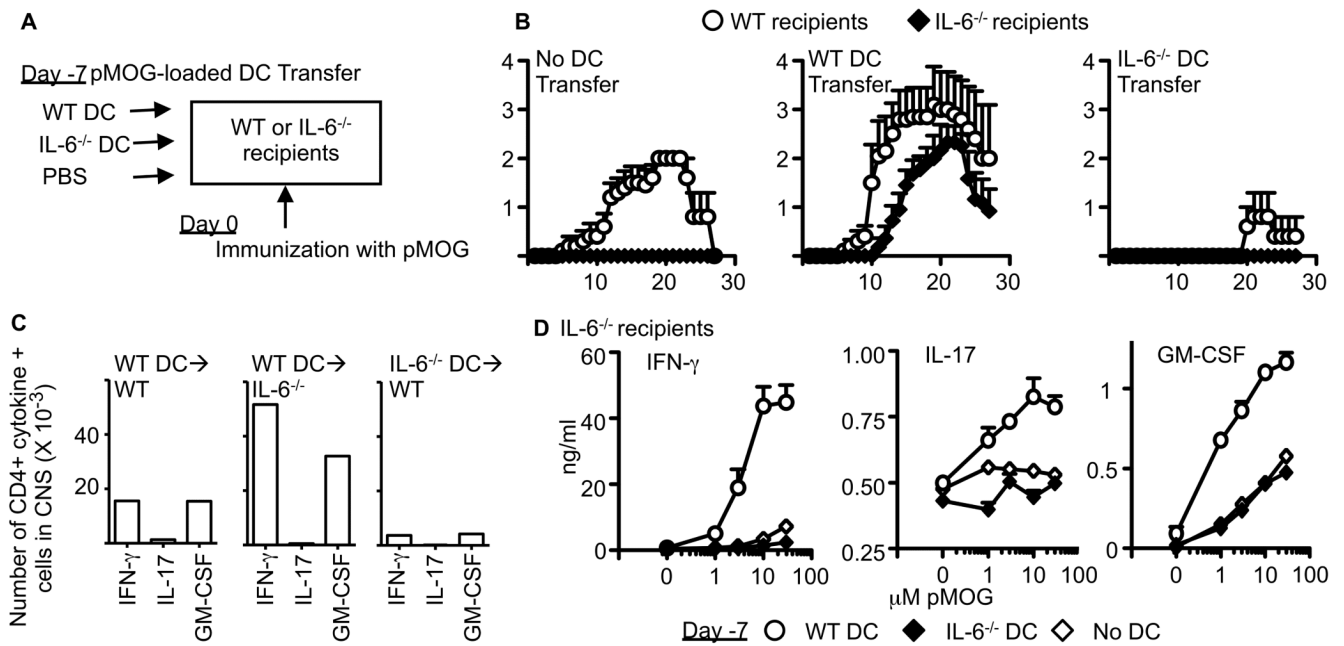
Figure 4. DC-derived IL-6 determines clinical outcome in EAE

(A,B) WT C57BL/6 or IL-6$^{-/-}$ mice (ten per group) received pMOG-loaded BMDC of the indicated genotype seven days before the induction of active EAE by immunization with pMOG+CFA. (C,D) At day 18, CNS mononuclear cells were isolated from five mice per group and stained individually for surface markers. Low numbers of cells from the IL-6$^{-/-}$ DC→WT group required samples to be pooled in advance of in vitro stimulation with pMOG and intracellular cytokine staining. Numbers shown in (D) were calculated from % cytokine-positive CD4$^+$ cells and the mean ex vivo number of CD4$^+$ cells from the CNS. (E) Splenocytes isolated from IL-6$^{-/-}$ hosts on day 18 were cultured with pMOG prior to assessment of IFN-γ, IL-17 and GM-CSF by ELISA. Data are from one of three experiments giving consistent results.
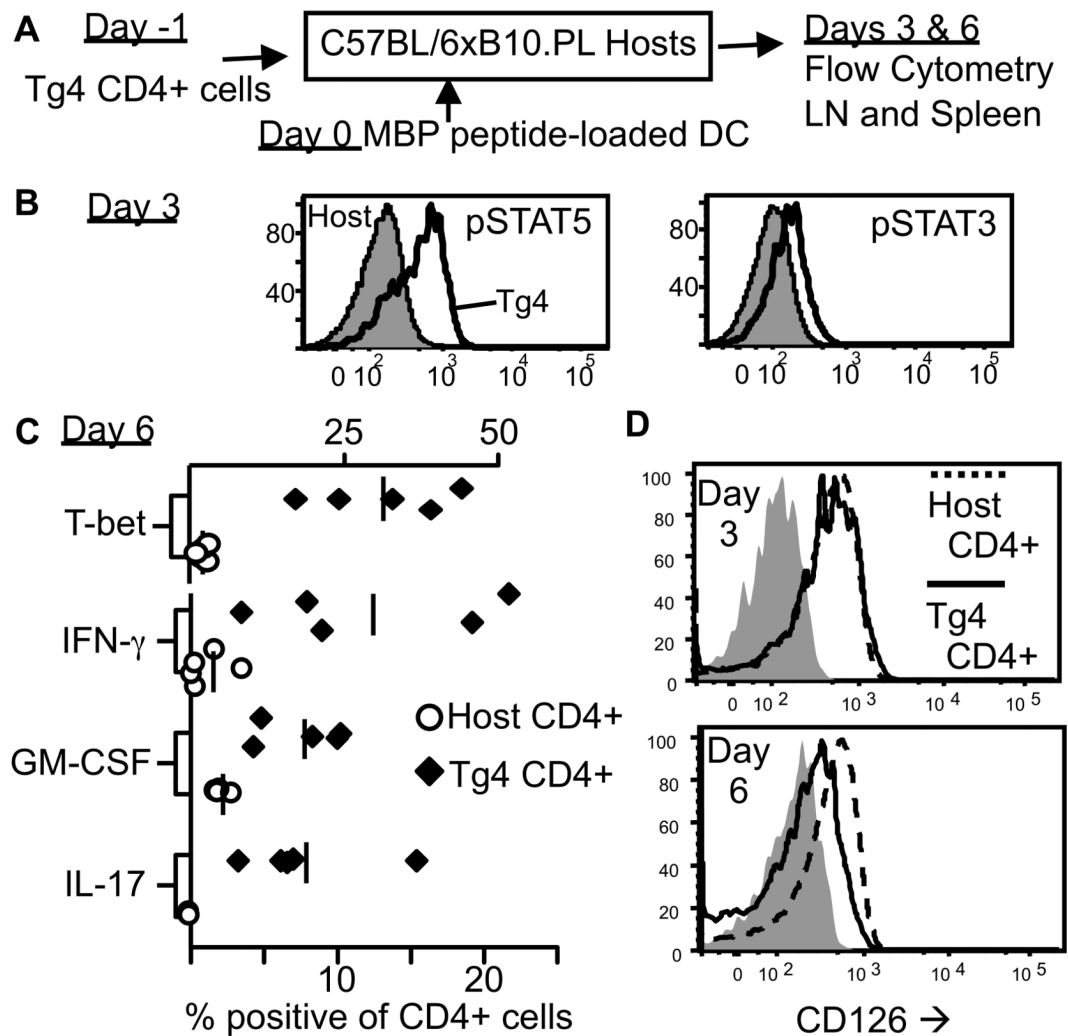
Figure 5. Loss of CD126 expression on autoreactive T cells following in vivo activation
(A) C57BL/6×B10.PL mice received naïve Tg4.WT CD4+ T cells prior to MBP peptide-loaded syngeneic BMDC. Lymphoid populations were sampled three and six days after BMDC transfer for flow cytometric comparison, identifying donor Tg4 cells by expression of CD45.1. (B) Phosphorylation of STAT3 and STAT5 in LN samples on day three. (C) Ex vivo T-bet expression and intracellular cytokine staining of day 6 splenocytes after overnight culture with MBP peptide (gated on CD4+ cells). Frequencies of cytokine/T-bet expressing cells were different between Tg4 and host cells (p<0.01 in each case, Mann Whitney test). (D) Ex vivo CD126 expression by splenic CD4+ cells on days three and six. Samples were processed individually (4-5 mice per group). Shaded histograms show staining with isotype control antibody. FACS-plots from representative mice are shown. Data are from one of two experiments giving consistent results.