Encephalitogenicity of T_H17 cells is dependent on interleukin-1- and interleukin-23-induced GM-CSF cytokine production

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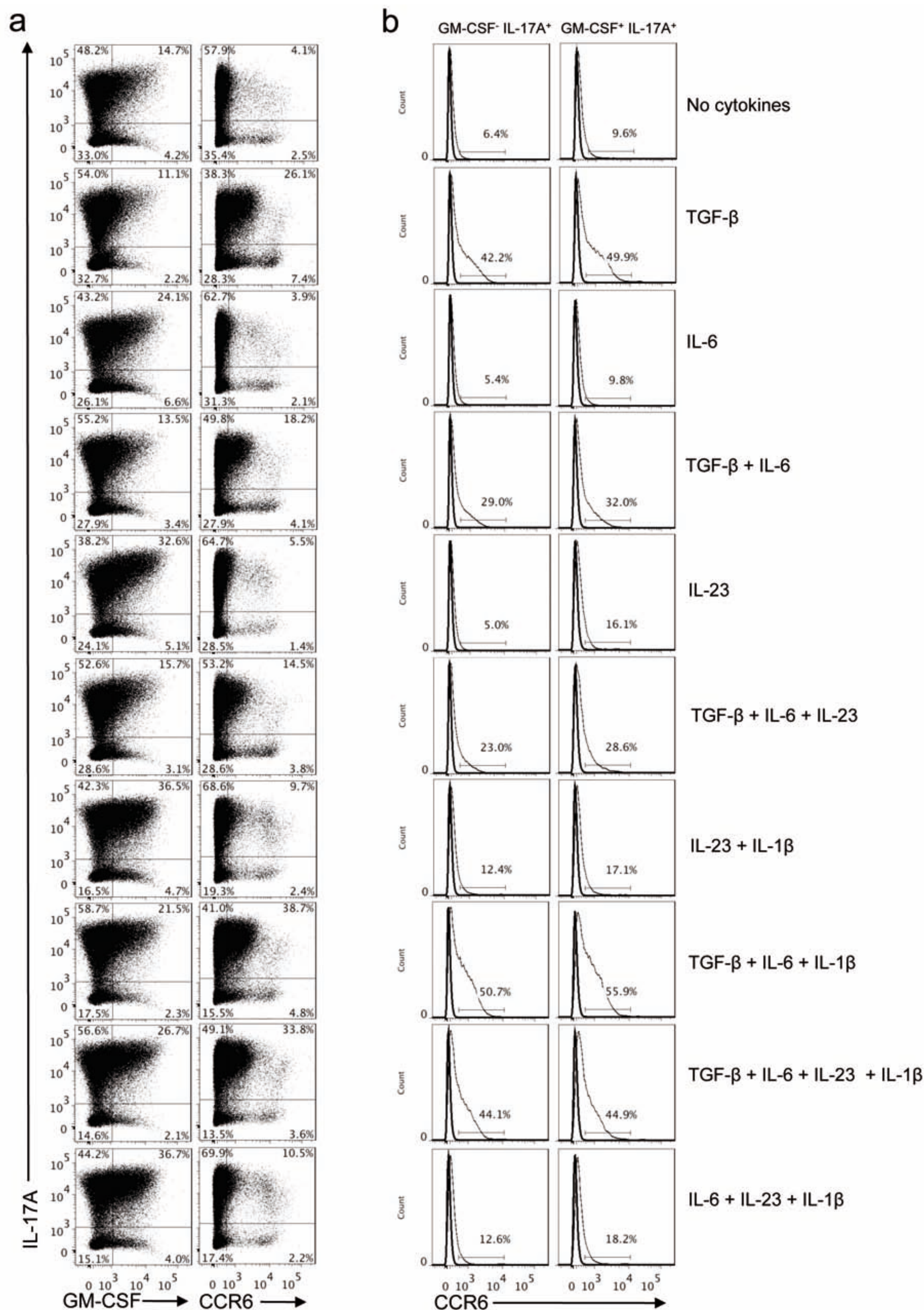

Supplementary Figure 1: Expression of GM-CSF and CCR6 by T_H17 cells that developed *in vitro* is not correlated

Splenocytes of 2D2 mice were activated with MOG_{35-55} in the presence of TGF-β plus IL-6, anti-IFN-γ and anti-IL-4 for 72 h. Cells were rested for 2 days in the presence of IL-2 and reactivated with MOG_{35-55} for 72 h in the presence of indicated cytokines. Cells were then stimulated with PMA and ionomycin in the presence of GolgiPlug, stained and analyzed by flow cytometry. (a) Expression of CCR6, IL-17A and GM-CSF by all $CD4^+$ cells. (b) Expression of CCR6 on $GM\text{-}CSF^-IL\text{-}17A^+$ and $GM\text{-}CSF^+IL\text{-}17A^+CD4^+$ cells. Histograms with heavy line represent isotype control. Data are representative of three independent experiments.

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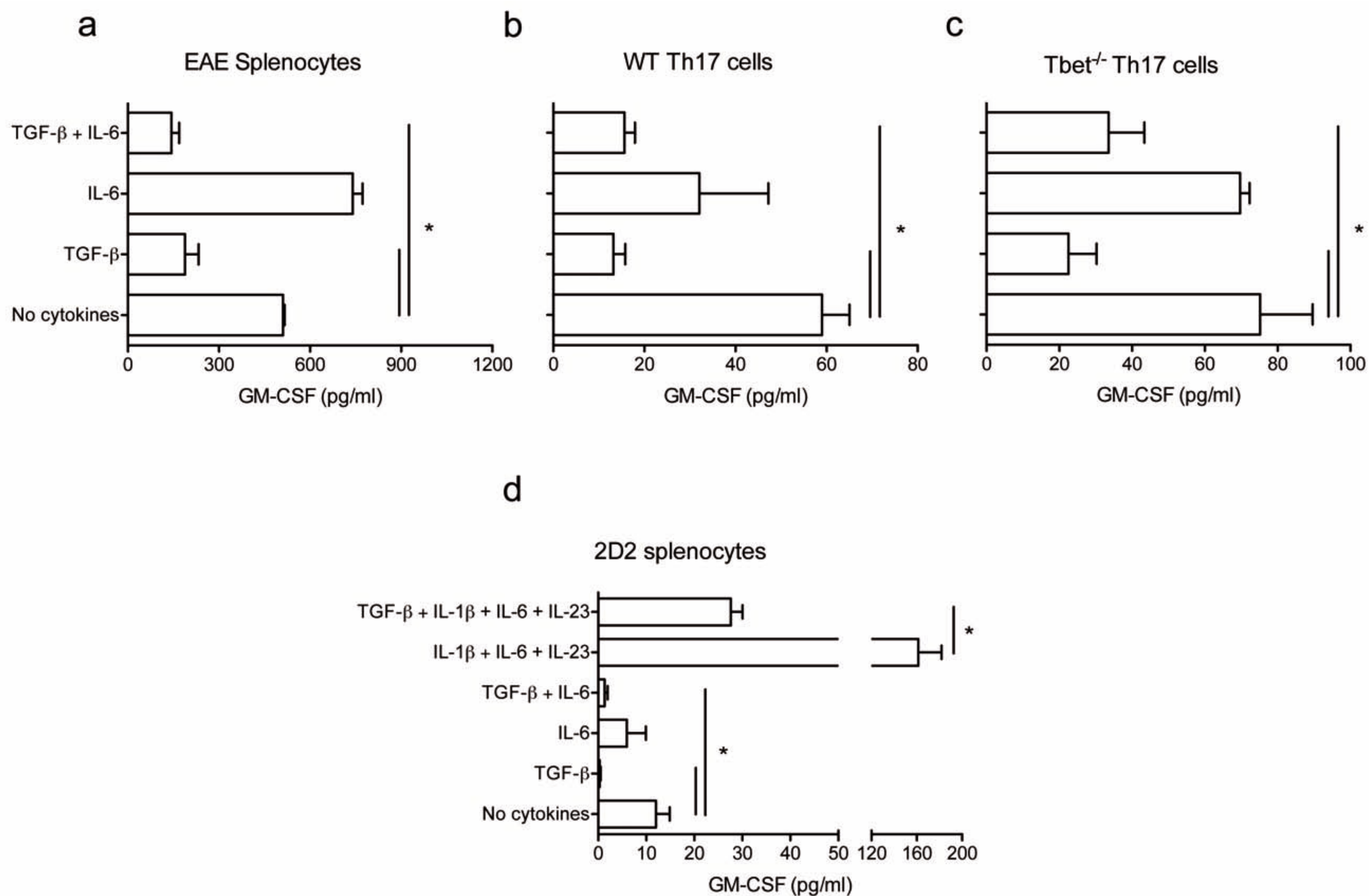
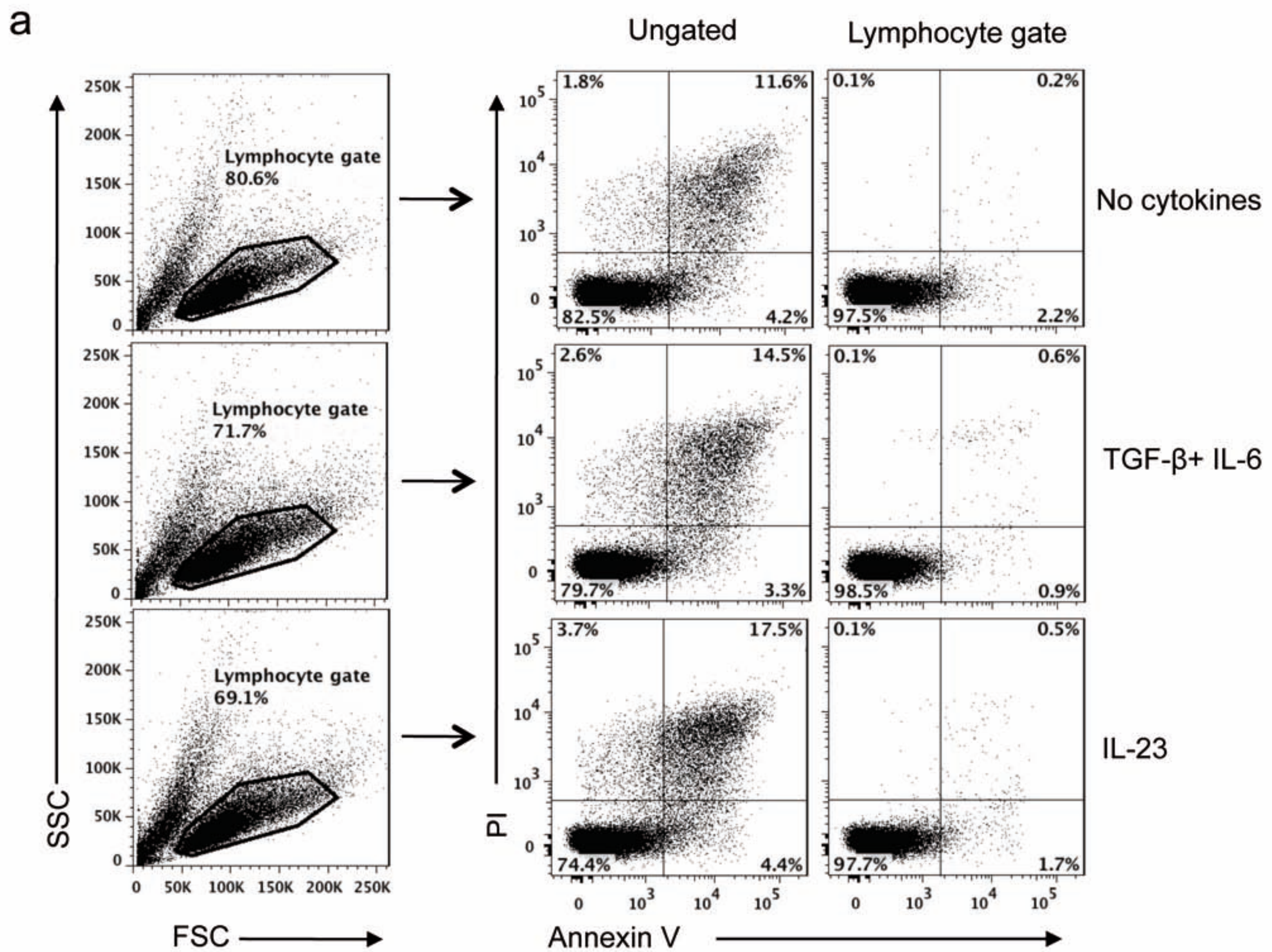
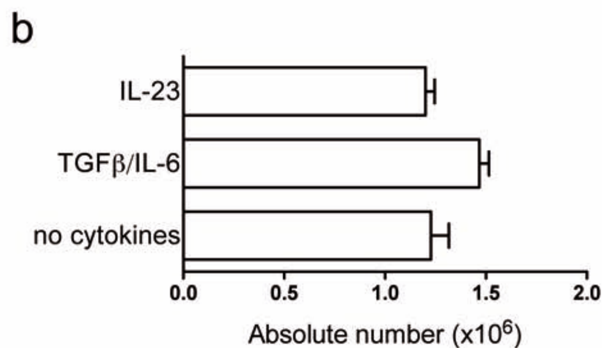


Supplementary Figure 2: TGF-β suppresses GM-CSF expression

Splenocytes from $MOG_{35\text{-}55}$ immunized mice (7 days p.i.) were cultivated with $MOG_{35\text{-}55}$ in the presence of indicated cytokines. Wild-type (b), or *Tbx21*$^{-/-}$ (c) T_H17 cells differentiated *in vitro* with anti-CD3 and anti-CD28 in the presence of TGF-β and IL-6 were restimulated for 3 days in the indicated cytokine conditions. (d) 2D2 T_H17 cells differentiated *in vitro* with $MOG_{35\text{-}55}$ and TGF-β plus IL-6 were restimulated for 3 days in the presence of indicated cytokines. After 3 days of stimulation, supernatants were harvested and analyzed for GM-CSF by ELISA. Data are representative of results from three individually analyzed mice with EAE (a and b) or two independent experiments (c and d). *$p<0.01$ (error bars, s.e.m).





Supplementary Figure 3. Apoptosis and necrosis are similar in T_H17 cells stimulated in different cytokine conditions

(a) Naive $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ T cells were differentiated into T_H17 cells during the first stimulation and then reactivated with anti-CD3 and anti-CD28 for 72 h in the presence of TGF-β and IL-6, IL-23 or without added cytokines. $CD4^+$ cells stained for Annexin V and propidium iodide (PI) are shown. (b) Absolute numbers of live cells counted by trypan blue exclusion of dead cells from 0.7×10^6 differentiated T_H17 cells, restimulated during 72 h in the presence of TGF-β plus IL-6, IL-23 or without added cytokines. Data are representative of three independent experiments (error bars, s.e.m of triplicate wells).

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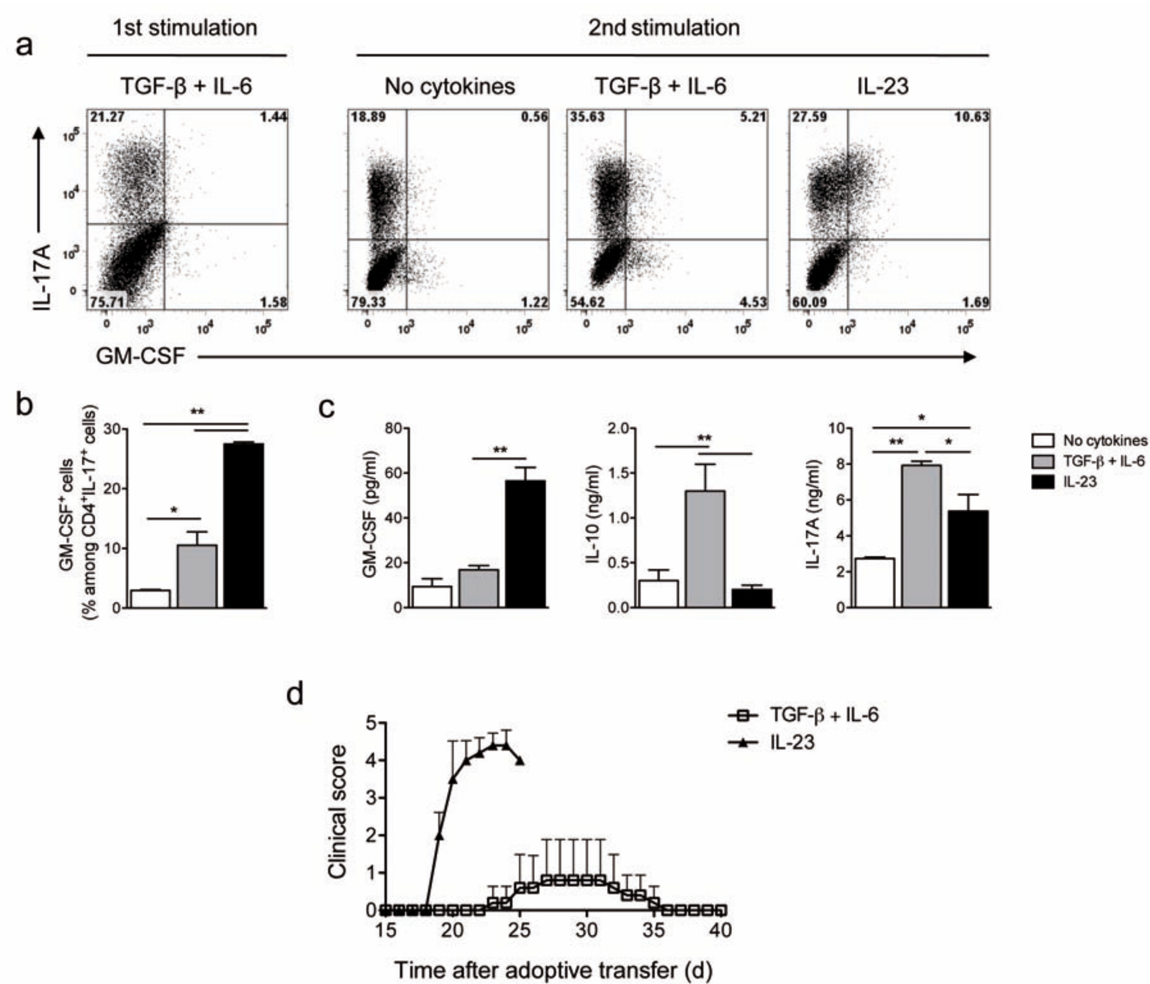
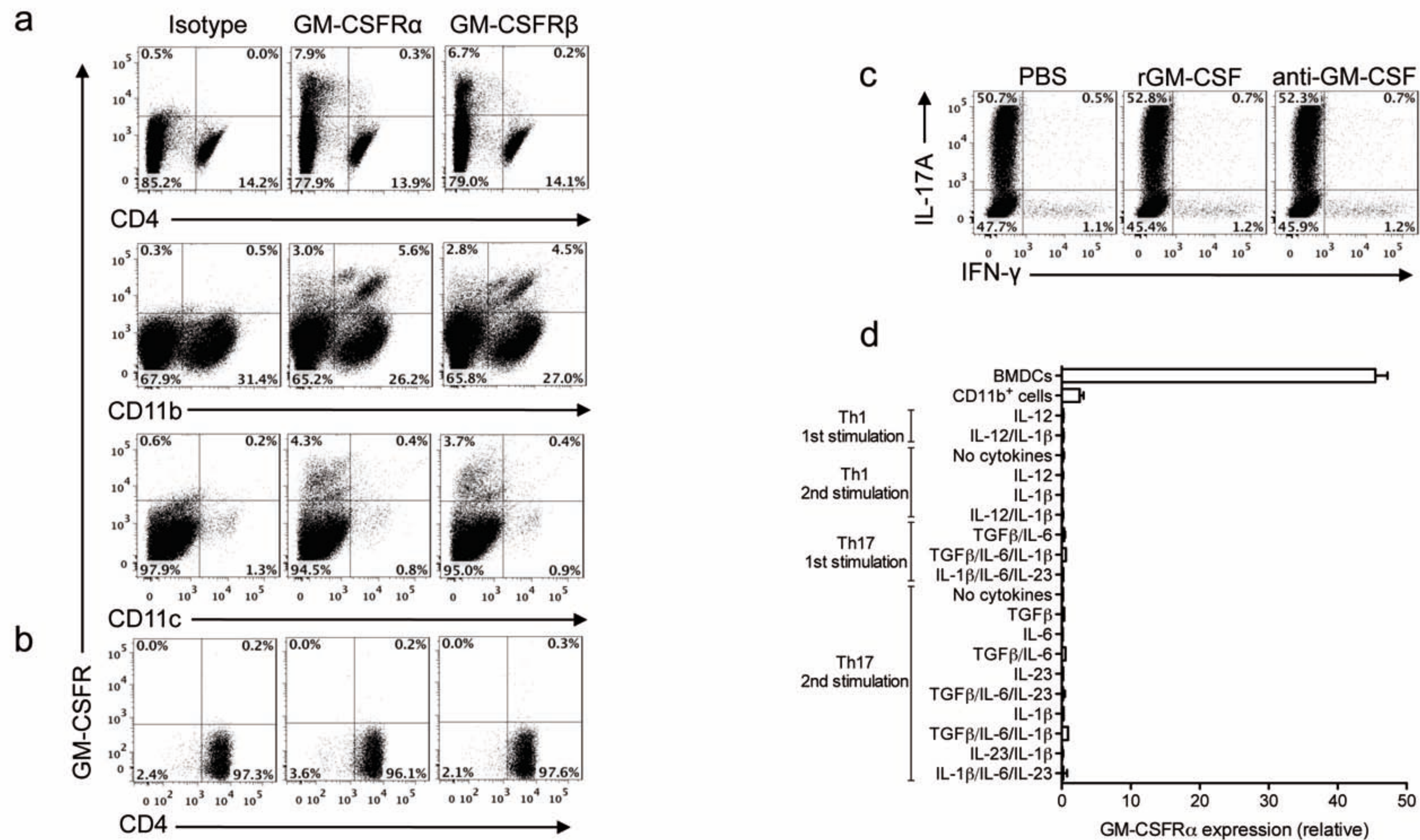


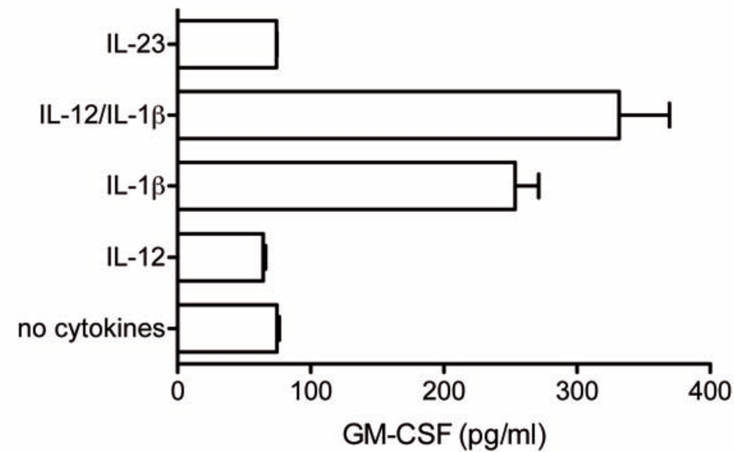
Supplementary Figure 4. IL-23 upregulates GM-CSF expression by 2D2 T_H17 cells

(a) Splenocytes of 2D2 mice were activated with MOG_{35-55} in the presence of TGF-β and IL-6, anti-IFN-γ and anti-IL-4 for 72 h (first stimulation). Cells were rested 2 days in the presence of IL-2 and then reactivated with MOG_{35-55} (second stimulation) for 72 h in the presence of TGF-β plus IL-6, IL-23 or without added cytokines. Cells were then stimulated with PMA and ionomycin in the presence of GolgiPlug, stained and analyzed by flow cytometry. $CD4^+$ cells are shown. (b) Percentage of GM-CSF$^+$ cells among $CD4^+$IL-17A$^+$ cells after the second stimulation. (c) GM-CSF, IL-10, and IL-17A concentrations in cell culture supernatants after the second stimulation measured by ELISA. (d) Clinical scores of sublethally irradiated wild-type recipient mice that received 20×10^6 2D2 T_H17 cells restimulated in the presence of TGF-β plus IL-6 or IL-23. *p< 0.01; **p < 0.001. Data are representative of two independent experiments (error bars, s.e.m).



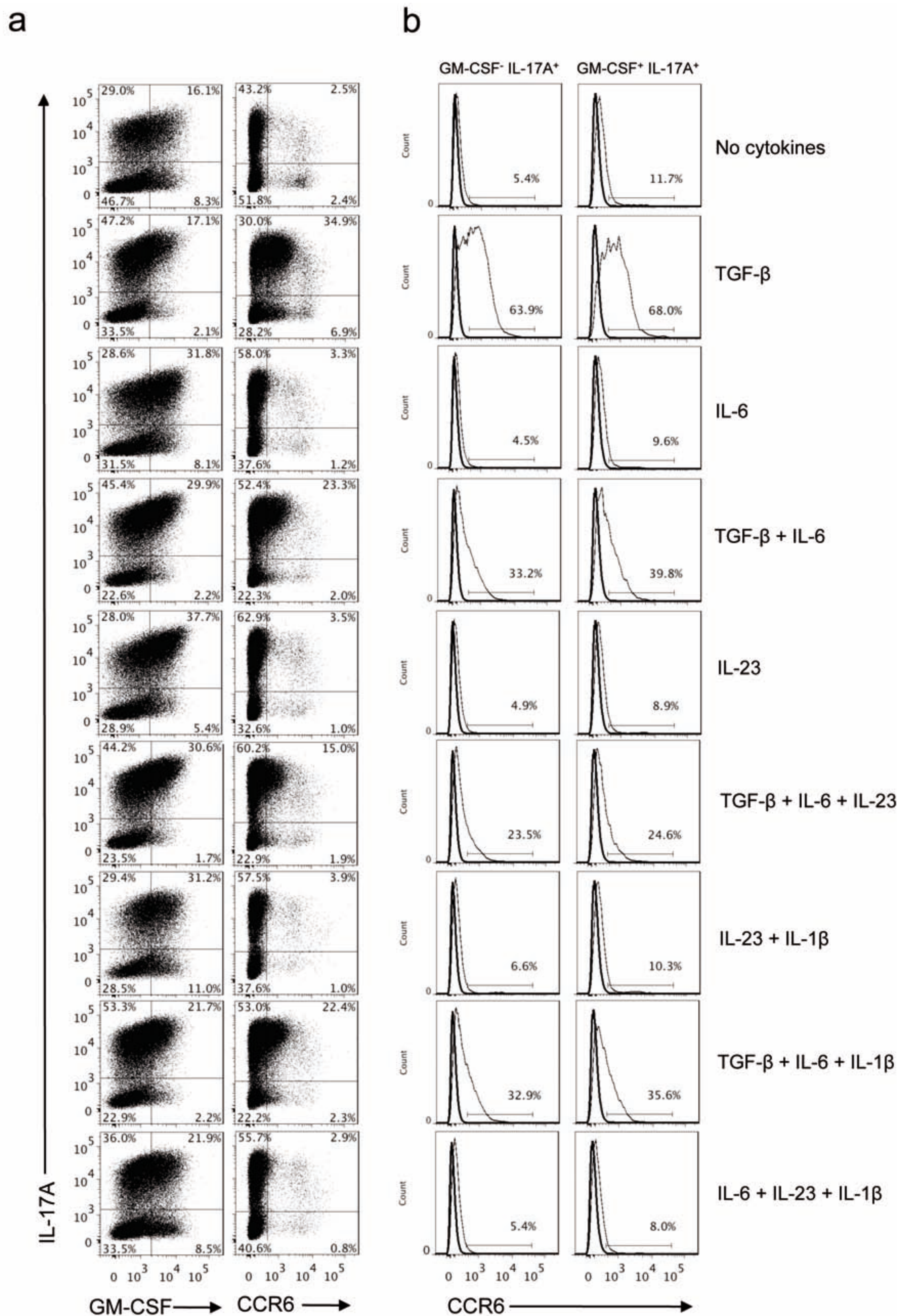
Supplementary Figure 5: CD4+ T cells do not express GM-CSFRα or GM-CSFRβ

(a) Flow cytometric analysis of GM-CSFRα and GM-CSFRβ expression on splenocytes of MOG_{35-55} -immunized mice, 7 days p.i. (b) Flow cytometric analysis of GM-CSFRα and GM-CSFRβ expression on T_H17 cells differentiated *in vitro* with anti-CD3 and anti-CD28 in the presence of TGF-β plus IL-6 and restimulated for 3 days in different cytokines conditions, as described in (d). Only a sample restimulated without added cytokines is shown. (c) Flow cytometric analysis of IL-17A and IFN-γ expression on naïve CD4+ T cells activated with anti-CD3 and anti-CD28, and TGF-β plus IL-6 in the presence of either rGM-CSF, anti-GM-CSF or PBS. (d) Real time PCR analysis of GM-CSFRα expression by BMDCs, splenic CD11b+ cells isolated from naïve C57BL/6 mice, T_H1 and T_H17 cells polarized *in vitro* (first stimulation) and reactivated under indicated cytokine conditions (second stimulation). Data are representative of two (a, c and d) or three (b) independent experiments (error bars, s.e.m).



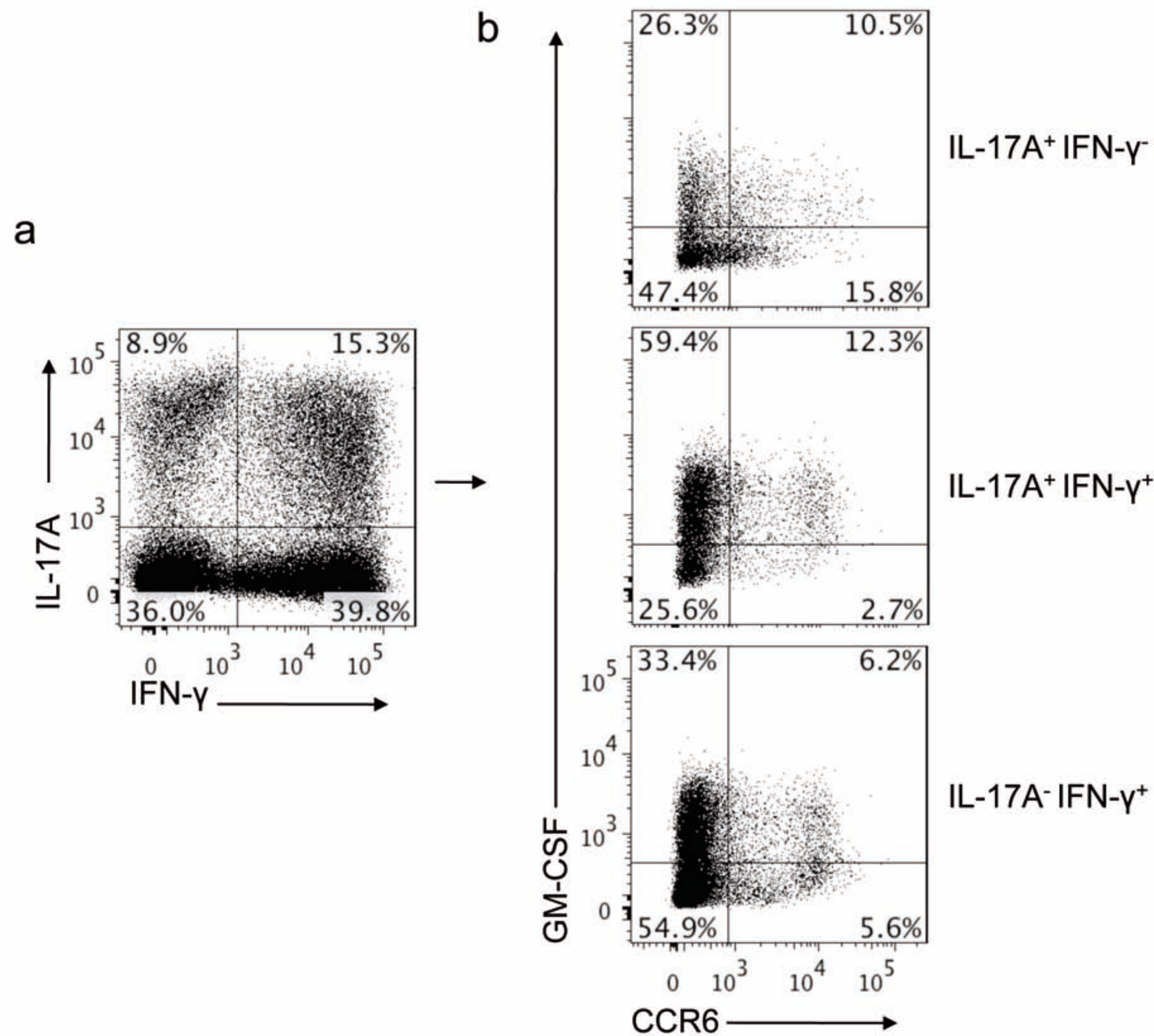
Supplementary Figure 6: IL-1β stimulates GM-CSF production by T_H1 cells, while IL-12 and IL-23 do not

Naive $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ T cells were differentiated into T_H1 cells during the first stimulation and then reactivated with anti-CD3 and anti-CD28 for 72 h in the presence of indicated cytokines. GM-CSF concentrations in cell culture supernatants after the second stimulation measured by ELISA are shown. Data are representative of two independent experiments (error bars, s.e.m).



Supplementary Figure 7: T-bet has no role in GM-CSF and CCR6 expression by T_H17 cells

Naïve $CD4^+$ T cells isolated from *Tbx21*$^{-/-}$ mice were stimulated with anti-CD3 and anti-CD28 in the presence of TGF-β and IL-6, anti-IFN-γ and anti-IL-4 for 72 h. Cells were rested for 2 days in the presence of IL-2 and reactivated with anti-CD3 and anti-CD28 for 72 h in the presence of indicated cytokines. Cells were then stimulated with PMA and ionomycin in the presence of GolgiPlug, stained and analyzed by flow cytometry. (a) Expression of CCR6, IL-17A and GM-CSF by all $CD4^+$ cells. (b) Expression of CCR6 on GM-CSF^-IL-$17A^+$ and GM-CSF^+IL-$17A^+$$CD4^+$ cells. Histograms with heavy line represent isotype control. Data are representative of two independent experiments.



Supplementary Figure 8: Expression of GM-CSF and CCR6 by T_H17 cells that developed *in vivo* is not correlated

C57BL/6 mice were immunized with $MOG_{35\text{-}55}$ and their spinal cords were harvested on day 14 p.i. (peak of EAE). Mononuclear cells were isolated, stimulated with PMA, ionomycin, and GolgiPlug stained and analyzed by flow cytometry. (a) Expression of IL-17A and IFN-γ on $CD4^+$ cells is shown. (b) Expression of CCR6 and GM-CSF on $IL\text{-}17A^+IFN\text{-}\gamma^-$, $IL\text{-}17A^+IFN\text{-}\gamma^+$ and $IL\text{-}17A^-IFN\text{-}\gamma^-$ $CD4^+$ cells is shown. Data are representative of two independent experiments.

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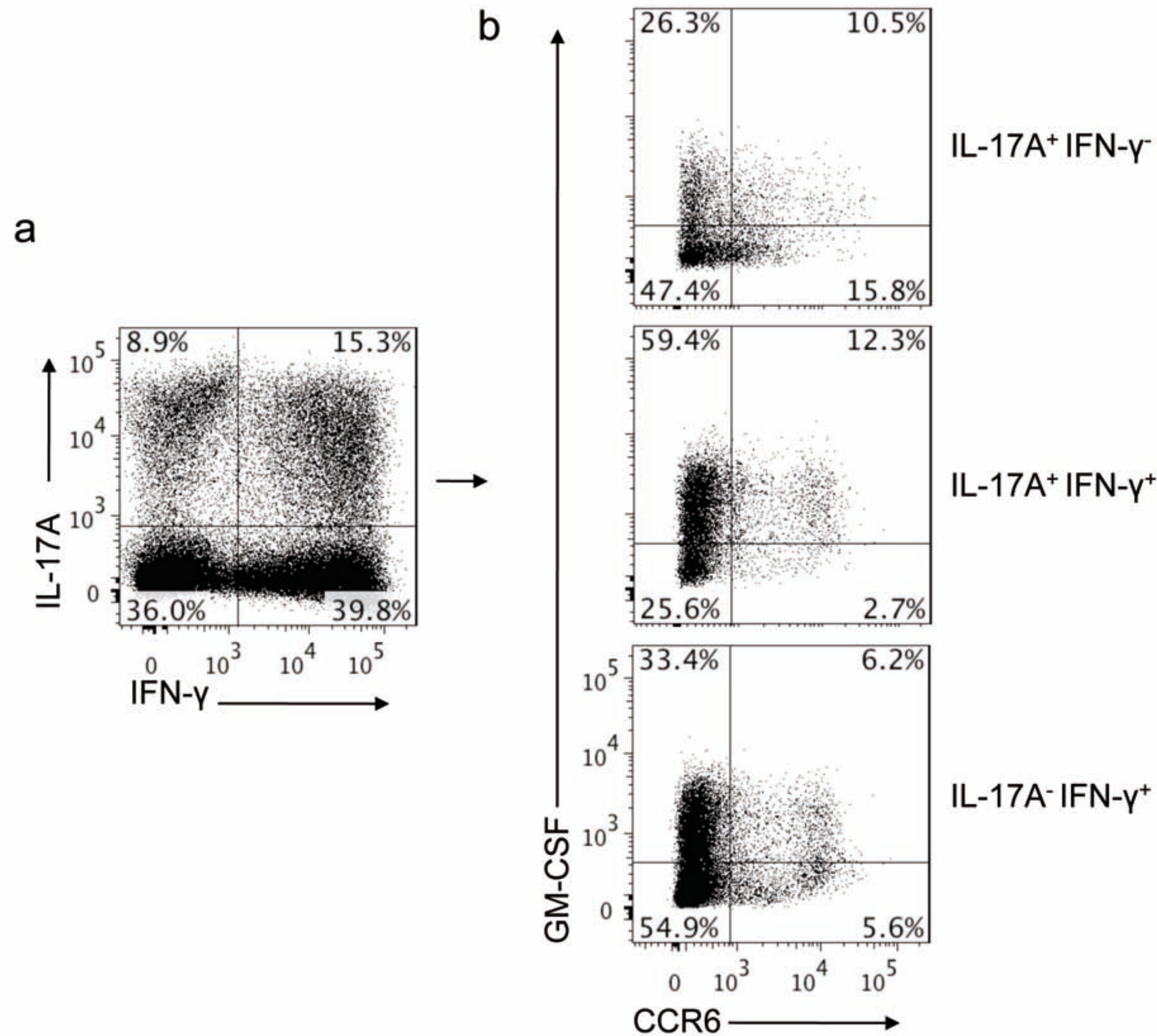
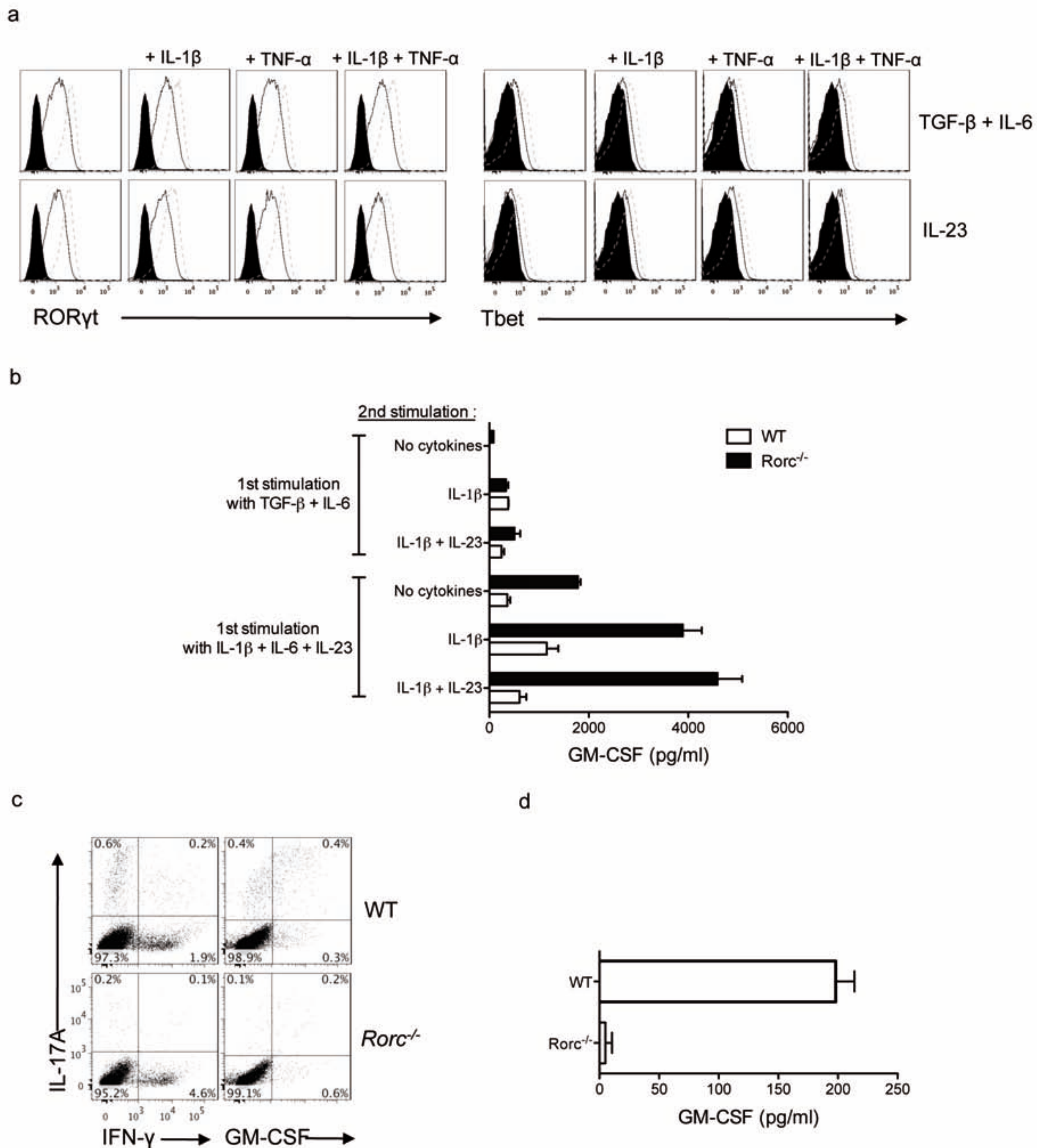


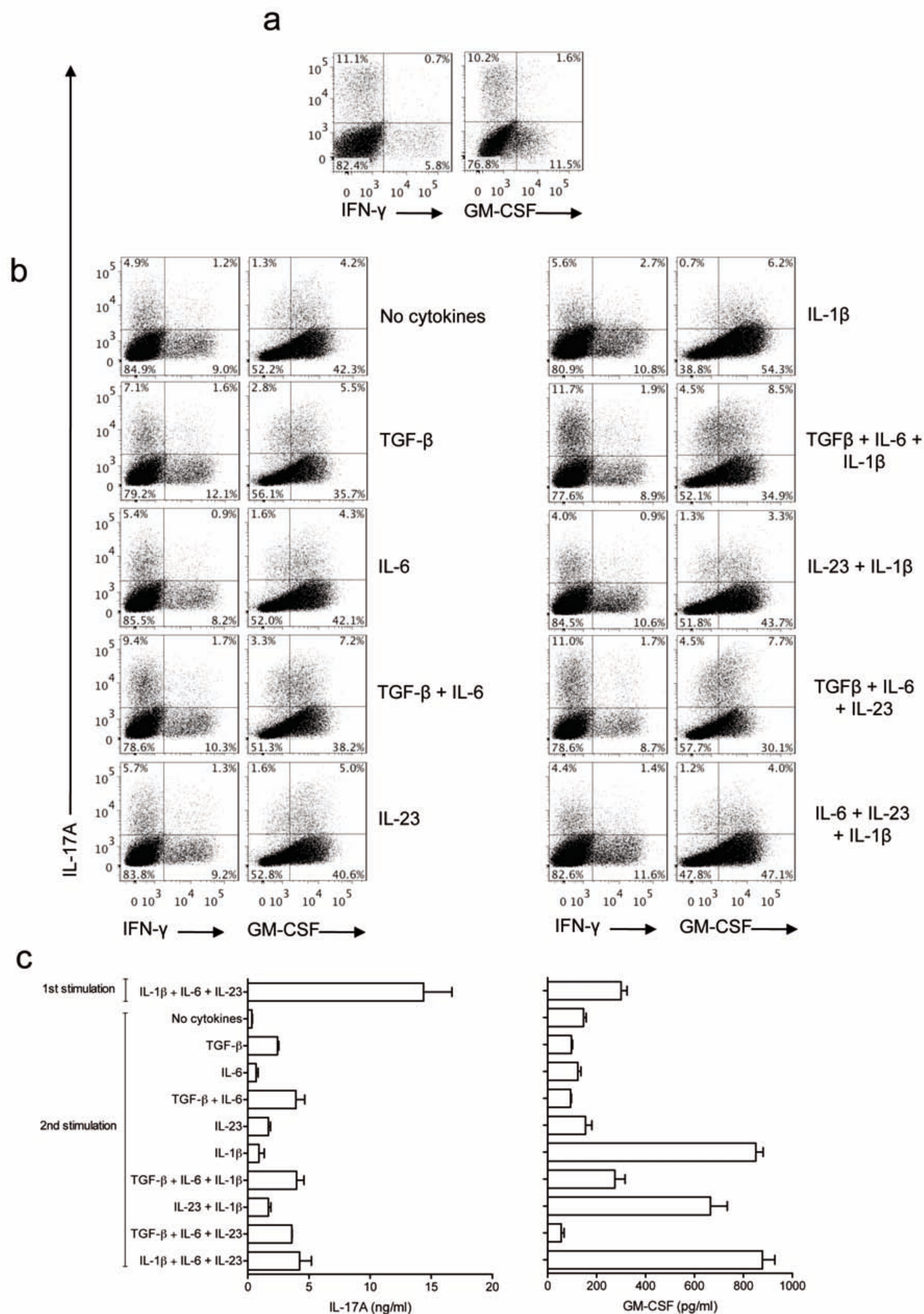
Supplementary Figure 9: IL-1R is expressed on a small percentage of CNS-infiltrating T_H1 and T_H17 cells of mice with EAE

C57BL/6 mice were immunized with MOG_{35-55} and their spinal cords were harvested on day 14 p.i. (peak of EAE). Mononuclear cells were isolated and stimulated with PMA, ionomycin, and GolgiPlug. Cells were then stained and analyzed by flow cytometry. (a) Expression of IL-1R on all mononuclear cells. (b) Expression of IL-17A and IFN-γ by $CD4^+$ cells. (c) Expression of IL-1R and GM-CSF on IL-17A$^+$IFN-γ$^-$, IL-17A$^+$IFN-γ$^+$ and IL-17A$^-$IFN-γ$^+$ $CD4^+$ cells is shown. Data are representative of two independent experiments.



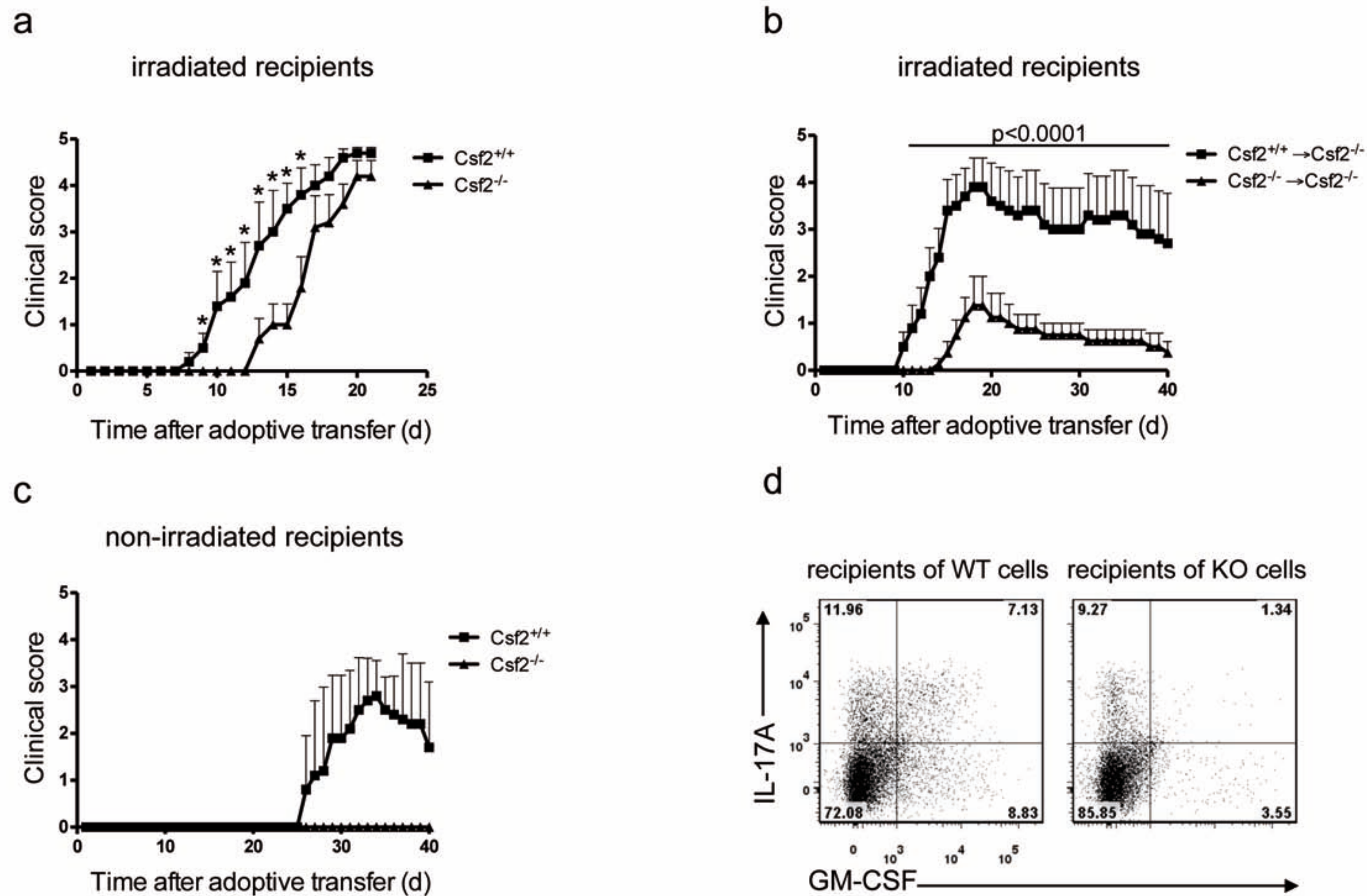
Supplementary Figure 10: T-bet and RORγt are not required for GM-CSF expression

(a) Naive $CD4^{+}CD25^{-}CD62L^{hi}CD44^{lo}$ T cells from spleens of C57BL/6 mice were polarized into $T_{H}17$ cells during the first stimulation. Cells were reactivated with anti-CD3 and anti-CD28 for 72 h in the presence of IL-23 or TGF-β plus IL-6, in combination with IL-1β and/or TNF. Cells were then stimulated with PMA and ionomycin in the presence of GolgiPlug, stained and analyzed by flow cytometry. (a) RORγt and T-bet expression in gated $CD4^{+}IL\text{-}17A^{+}GM\text{-}CSF^{-}$ (solid line) or $CD4^{+}IL\text{-}17A^{+}GM\text{-}CSF^{+}$ (dashed line). (b) Naive $CD4^{+}CD25^{-}CD62L^{hi}CD44^{lo}$ T cells from spleens of *Rorc*$^{-/-}$ and wild-type mice were purified by flow cytometry and differentiated into $T_{H}17$ cells during the first stimulation either with TGF-β and IL-6 or IL-1β, IL-6 and IL-23. Cells were then reactivated with anti-CD3 and anti-CD28 for 72 h in the presence of the indicated cytokines (second stimulation). GM-CSF concentration in cell culture supernatants after second stimulation measured by ELISA is shown. (c) Wild-type and *Rorc*$^{-/-}$mice were immunized with $MOG_{35\text{-}55}$ and their spleens were harvested on day 7 p.i. Splenocytes were then activated with $MOG_{35\text{-}55}$ and after 48 h stimulated with PMA and ionomycin in the presence of GolgiPlug, stained and analyzed by flow cytometry. $CD4^{+}$ cells are shown. (d) GM-CSF concentration in supernatants of cell cultures stimulated as in (c). Data are representative of two independent experiments (a and b), or one experiment with three mice of each genotype that were individually analyzed (c and d) (error bars, s.e.m).



Supplementary Figure 11: Higher production of GM-CSF by T_H17 cells differentiated in the absence of TGF-β

(a) Naive $CD4^+$ T cells from spleens of C57BL/6 mice were differentiated into T_H17 cells during the first stimulation with anti-CD3 plus anti-CD28 in the presence of IL-1β, IL-6 and IL-23, and anti-IFN-γ and anti-IL-4. (b) Cells were then reactivated with anti-CD3 and anti-CD28 for 72 h in the presence of indicated cytokines. $CD4^+$ cells stained for IL-17A, IFN-γ and GM-CSF are shown. (c) GM-CSF and IL-17A concentrations in cell culture supernatants after the first and second stimulations measured by ELISA. Data are representative of two independent experiments (error bars, s.e.m).



Supplementary Figure 12: GM-CSF produced by cells of recipient mice contributes to development of adoptive EAE

(a) Clinical scores of irradiated wild-type recipient mice that received $5x10^6$ of either wild-type or *Csf2*$^{-/-}$ MBP_{Ac1-11}-specific T_H17 cells activated in the presence of IL-23. (b) Clinical scores of irradiated *Csf2*$^{-/-}$ recipient mice that received $5x10^6$ of either wild-type or *Csf2*$^{-/-}$ MBP_{Ac1-11}-specific T_H17 cells activated in the presence of IL-23. (c) Clinical scores of non-irradiated wild-type recipient mice that received $5x10^6$ of either wild-type or *Csf2*$^{-/-}$ MBP_{Ac1-11}-specific T_H17 cells activated in the presence of IL-23. Pertussis toxin was injected i.p. on days 0 and 2 post transfer. (d) Adoptive EAE was induced as described in (a). Brains and spinal cords of recipient mice were harvested at day 20 post cell transfer; mononuclear cells were isolated and stimulated with PMA, ionomycin, and GolgiPlug. $CD4^+$ cells are shown. *$p< 0.01$; Data are representative of two (b, c and d) or three independent experiments (a). (error bars, s.e.m).

Cells transferred		Incidence[a]	Mortality	Mean day of onset	Mean maximum clinical score
Th1	$Csf2^{+/+}$	88% (8/9)	0%	13.5 ± 0.75	1.81 ± 0.18[b]
	$Csf2^{-/-}$	0% (0/9)	0%	na	na
Th17	$Csf2^{+/+}$	100% (9/9)	62%	13.75 ± 1.1	3.69 ± 0.64
	$Csf2^{-/-}$	22% (2/9)	0%	16 ± 4.35	0.83 ± 0.16[c]

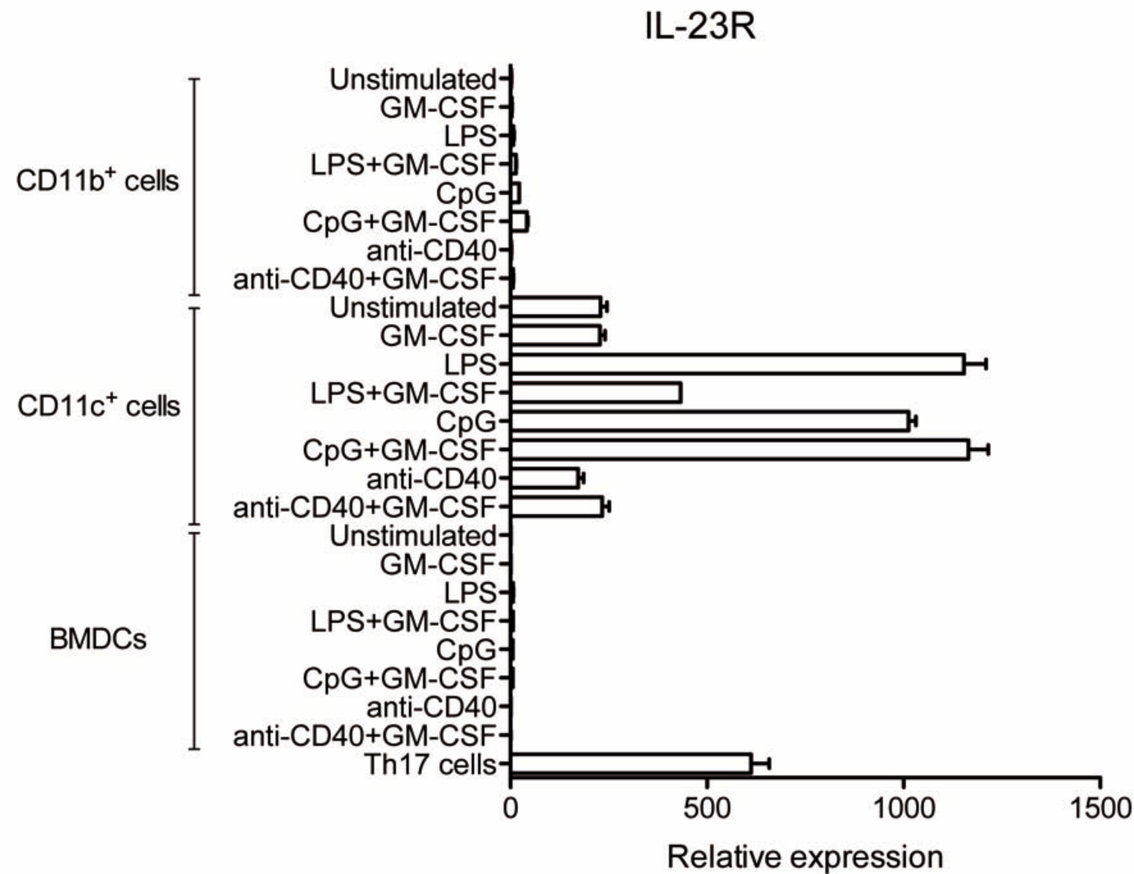
Supplementary Figure 13: Summary of EAE parameters induced by the adoptive transfer of WT or $Csf2^{-/-}$ Th1 and Th17 cells

[a] Represents the percentage of mice that developed a clinical score of at least 1

[b] p =0.014 compared to Th17 $Csf2^{+/+}$

[c] p =0,027 compared to Th17 $Csf2^{+/+}$

na : non applicable



Supplementary Figure 14: GM-CSF does not influence IL-23R expression in different subsets of APCs

$CD11b^+$ and $CD11c^+$ cells were isolated from splenocytes of naïve C57BL/6 mice. Bone marrow–derived DCs (BMDCs) were generated in 7-day culture with complete RPMI supplemented by rGM-CSF. Cells were stimulated with LPS, CpG, anti-CD40, rGM-CSF of left untreated (unstimulated) for 24 h. RNA was then extracted, reverse-transcribed and analyzed by real-time PCR for IL-23R expression. Levels of IL-23R mRNA were shown relative to mRNA level of unstimulated $CD11b^+$ cells (numerical value 1). Data are representative of two independent experiments. (error bars, s.e.m).