

1 Supplementary Information

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4 **Steady-state memory-phenotype conventional CD4⁺ T cells exacerbate autoimmune**
5 **neuroinflammation in a bystander manner via the Bhlhe40/GM-CSF axis**

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14 **Supplementary Methods**

15 **Single-cell RNA sequencing sample preparation**

16 MP (TCR β ⁺CD4⁺CD25⁻CD62L^{low}CD44^{high}) CD4⁺ T cells from the spleens of 10-week-old mice were isolated
17 using a FACS Aria cell sorter II (BD Biosciences, Franklin Lakes, NJ, USA). FACS-sorted MP CD4⁺ T cells
18 were stimulated with IL-1 β (20 ng/mL, R&D Systems, Minneapolis, MN, USA), IL-23 (20 ng/mL, R&D
19 Systems), IL-12 (20 ng/mL, Peprotech, Rocky Hill, NJ, USA), IL-18 (20 ng/mL, R&D Systems), IL-33 (20
20 ng/mL, R&D Systems), IL-25 (20 ng/mL, R&D Systems), or IL-7 (10 ng/mL, Peprotech, Rocky Hill, NJ, USA)
21 for 5 days at 37 °C in an incubator.

22 **Single-cell RNA- and TCR-seq data processing**

23 Raw sequencing data were processed using a well-developed program, Cellranger (10X genomics), for quality
24 control (QC) and gene expression estimation. Low-quality cells were filtered using 4 QC metrics: the percentage
25 of mitochondrial gene unique molecular identifier (UMI) counts, the number of expressed genes (UMI count >
26 0), the sum of UMI counts per cell, and the doublet score estimated using Doublet Finder
27 [10.1016/j.cels.2019.03.003]. The outlier cells from each QC metric were excluded from further analyses. To
28 collect pure TCR α / β ⁺ CD4⁺ T cells, cells expressing PLZF (*Zbtb16*) and *Cd74*⁺-cells in which at least 1 UMI
29 was counted were removed. All TCR α / β ⁺ CD4⁺T cells were integrated by cytokine treatment (set 1: IL-7, IL-
30 7+ IL-1 β , and IL-7+ IL-1 β +IL-23; set 2: IL7, IL7+IL-25, IL-7+IL-33, and IL-7+IL-25+IL-33; set 3: IL-7, IL-
31 7+IL-12, IL-7+IL-18, and IL-7+IL-12+IL-18) using monocle3 [10.1038/nmeth.4402]. TCR-seq raw sequencing
32 data were processed using the Cellranger ‘adj’ program. The TCR clonotypes from previously filtered scRNA-
33 seq were used in the further analyses.

34 **Single-cell RNA-seq data analysis**

35 The preprocessed scRNA-seq data were aligned with the IL-7 treatment group using ‘align_cds’ from the
36 monocle3 program [https://doi.org/10.1038/s41586-019-0969-x], and data were integrated for each set. Each
37 cluster was characterized using the marker gene expression levels and referring to previous studies. The top
38 specifically expressed genes of each cluster were selected by the specificity of each gene, as estimated using
39 ‘FindAllMarkers’ in Seurat v4.0 [https://doi.org/10.1016/j.cell.2021.04.048], and then their expression level
40 was visualized using ‘DoHeatmap’ in the same program. We calculated the percentage of cells in each condition
41 for each cell type by dividing the number of cells in each condition by the number of clustered cells. A DEGs
42 analysis was performed for each cytokine treatment of each set using ‘FindMarkers’ in Seurat with the following
43 parameters: min.pct = 0, min.cells.feature = 1, min.cells.group = 1, logfc.threshold = 0. DEGs between cytokine
44 treatments and DEGs between conditions were defined at FDR <0.05 and average log2 fold change $\geq$ 0.1. A
45 functional analysis of DEGs and cluster specific gene sets was performed in MSigDB's C2 (KEGG) and C5
46 (GO). The functions were analyzed using the maximum and minimum size of the gene sets (KEGG with min 2
47 genes and max 500, GO with min 2 genes and max 150 genes). The gene set enrichment test was performed

48 using the 'enricher' function of the clusterProfile package [10.1089/omi.2011.0118]. The significance level was
49 set as the function that satisfied $FDR < 0.2$ and had at least 2 overlapping genes.

50 **Signature score of the MP CD4⁺ T cells**

51 The signature score of the CD4⁺ T cells was estimated to define the features of each cell type. The monocle3
52 object of each set of cytokine treatments was transformed into the Seurat object. They were normalized by
53 condition and then integrated. The signature score of each cell type was calculated using AddModuleScore in
54 the Seurat package.

55 **Trajectory inference of MP CD4⁺ T cells by cytokine stimulation**

56 Effector memory CD4⁺ T-cells have the plasticity to differentiate into various helper cells. We performed a
57 trajectory analysis on the subcluster composed of the initial cells that are likely to be differentiated according to
58 each cytokine stimulus and the cells that responded. First, we selected 2,000 highly variable genes by cell type,
59 and then we inferred the trajectory using reduceDimension with DDRTree in the monocle2 program
60 [10.1038/nbt.2859]. Pseudo-time was calculate using orderCells in the same program. DEGs following the
61 pseudo-time trajectory were defined at a q-value $< 1e-50$.

62 **Estimation of regulon activity score**

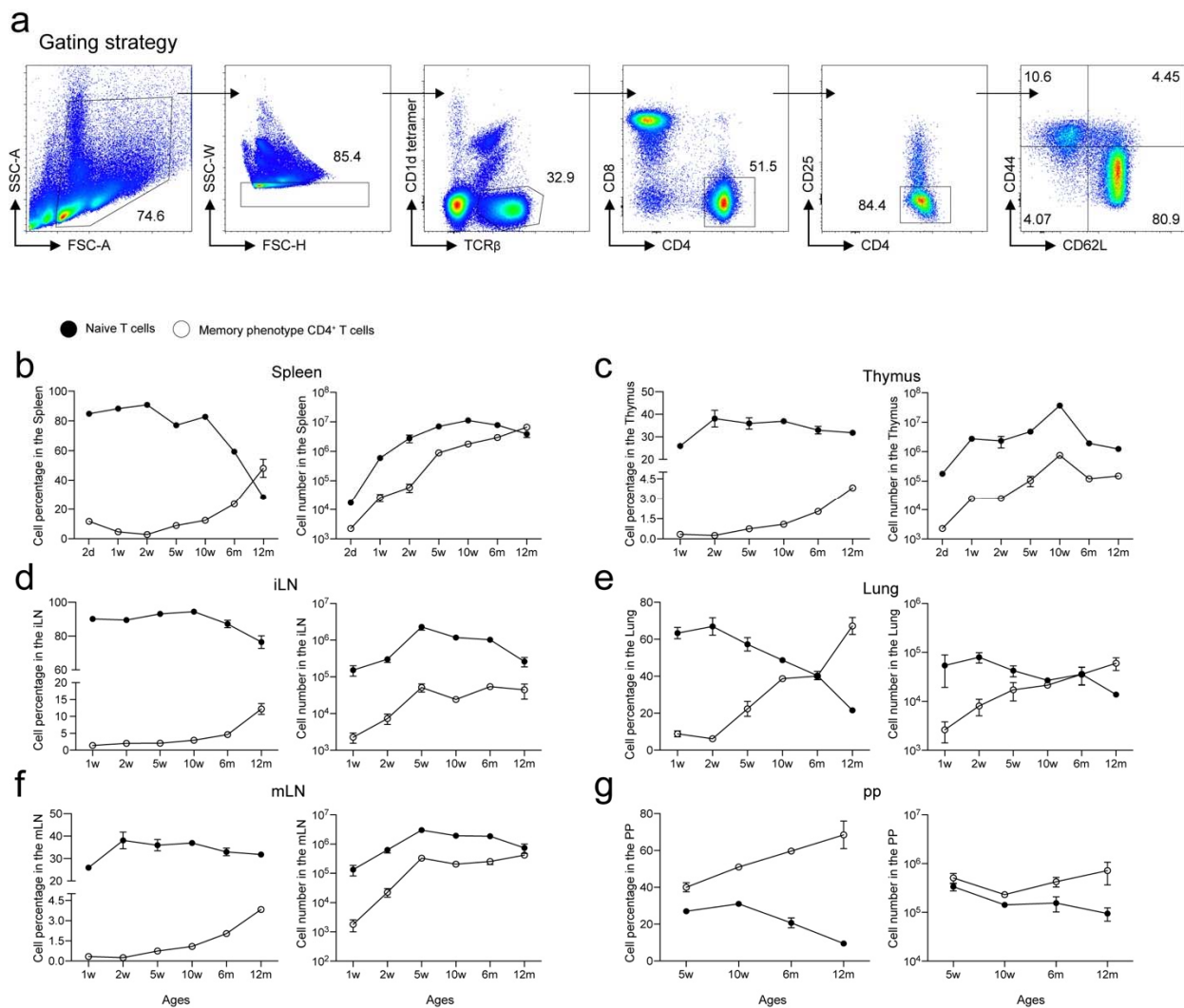
63 *Regulon* describes the relationship between a transcription factor and its regulatory target genes, and its activity
64 is calculated using the expression levels of the genes in the regulon. First, we constructed a correlation matrix
65 between genes, and then we calculated the activity of each regulon using GENIE3
66 [10.1371/journal.pone.0012776]. At that time, all the parameters were set to the default. The regulon
67 relationship was derived from mm10__refseq-r80__500bp_up_and_100bp_down_tss.mc9nr and
68 mm10__refseq-r80__10kb_up_and_down_tss.mc9nr in the 'cisTarget_databases' of the R package. All
69 processes were performed using the SCENIC program in R [10.1038/nmeth.4463]. Differentially activated
70 regulons were considered only when they contained at least 1% of expressing cells.

71 **T-cell receptor repertoire analysis**

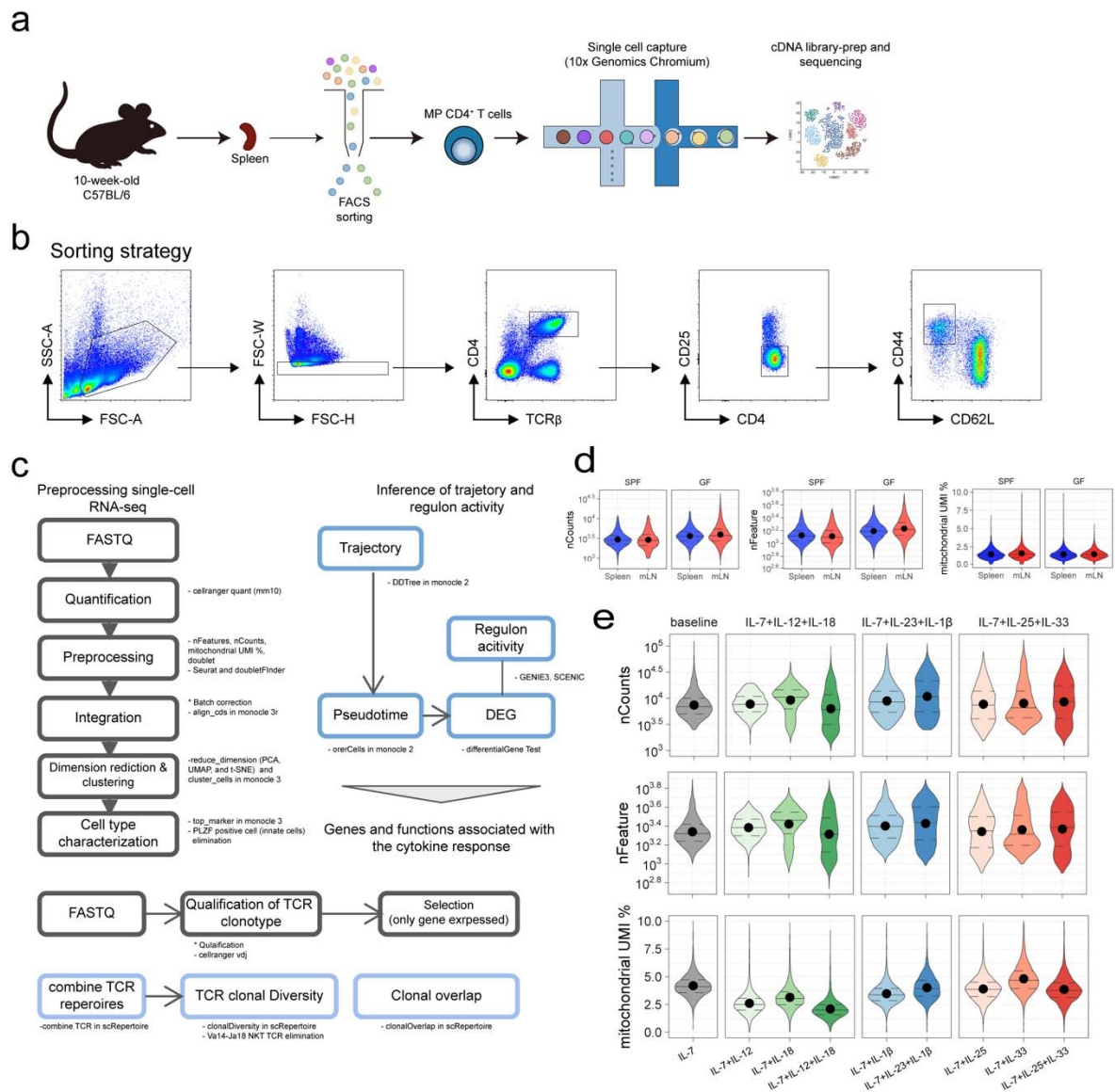
72 The TCR repertoire of the cells was considered only from the scRNA-seq data. The degree of TCR expansion
73 was categorized as hyperexpanded ($100 < X \leq 500$), large ($20 < X \leq 100$), medium ($5 < X \leq 20$), small (1
74 $< X \leq 5$), and single ($0 < X \leq 1$). The TCR diversity of each sample was calculated using Shannon's index,
75 which considers non-uniformity in the frequency of the clonotype (gene and nucleotide sequences). All
76 procedures were performed using scRepertoire [10.12688/f1000research.22139.2] in R.

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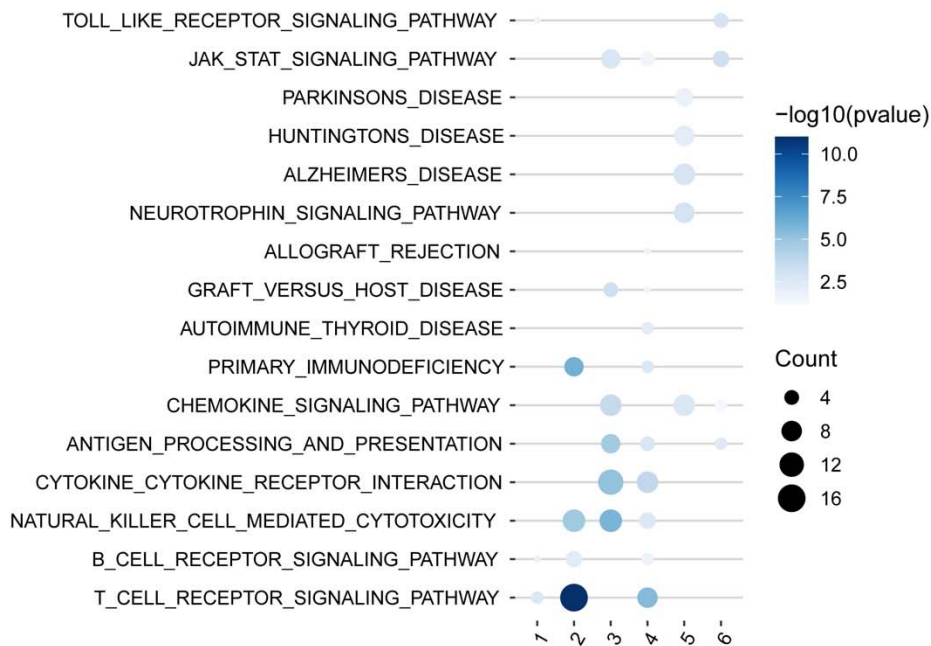


Supplementary Fig. 1 Steady state CD44^{high} memory phenotype (MP) CD4⁺ T cells increase with age in mice. (a) Gating strategy of Splenic MP CD4⁺ T cells (TCRβ⁺CD1d tetramer⁻CD8⁻CD4⁺CD25⁻CD62L^{low}CD44^{high}). Analysis of cell percentage and number of naïve (TCRβ⁺CD1d tetramer⁻CD8⁻CD4⁺CD25⁻CD62L^{high}CD44^{low}) and MP CD4⁺ T cells (TCRβ⁺CD1d tetramer⁻CD8⁻CD4⁺CD25⁻CD62L^{low}CD44^{high}) by age from (b) the spleen, (c) thymus, (d) iLN, (e) lung, (f) mLN, and (g) PP.

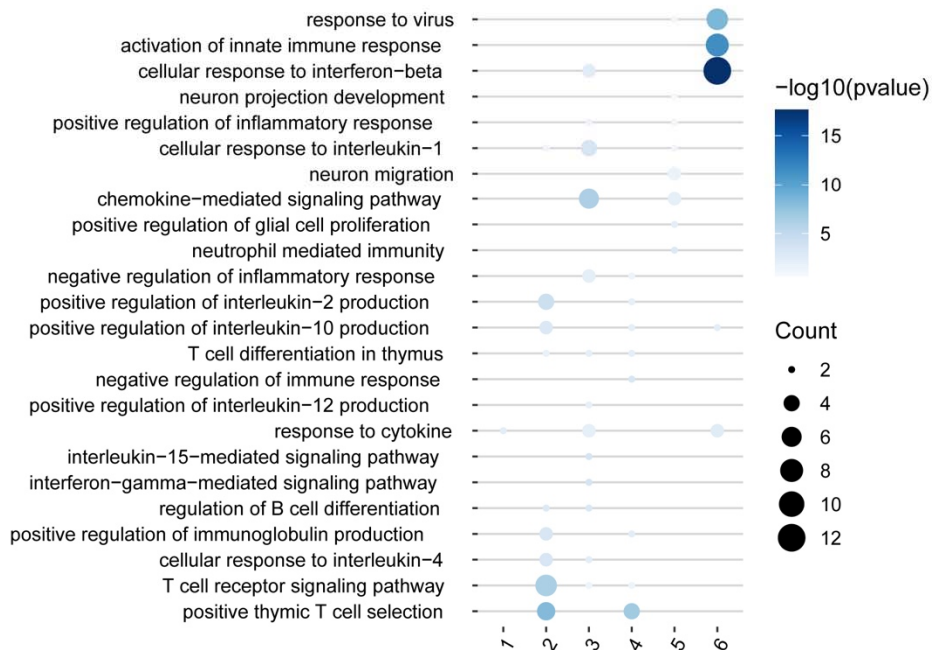


Supplementary Fig. 2 Strategy and flow of single cell RNA-sequencing analysis. (a) Visual concept of scRNA-seq for MP CD4⁺ T cells. (b) Gating strategy of splenic MP (TCRβ⁺ CD4⁺ CD25⁻ CD62L^{low} CD44^{high}) CD4⁺ T cells. (c) Workflow of scRNA-seq and TCR-seq of PLZF and TCR V_α14-J_α18 (TRAV11-TRAJ18) negative MP CD4⁺ T cells. (d) Quality measures of splenic and mLN MP CD4⁺ T cells from SPF- and GF-housed mice. (e) QC metrics for each condition of MP CD4⁺ T cells. (nFeature: the number of expressed genes per cell, nCounts: the number of total UMI counts per cell, mitochondrial UMI %: the percentage of UMI counts of mitochondrial genes per cell)

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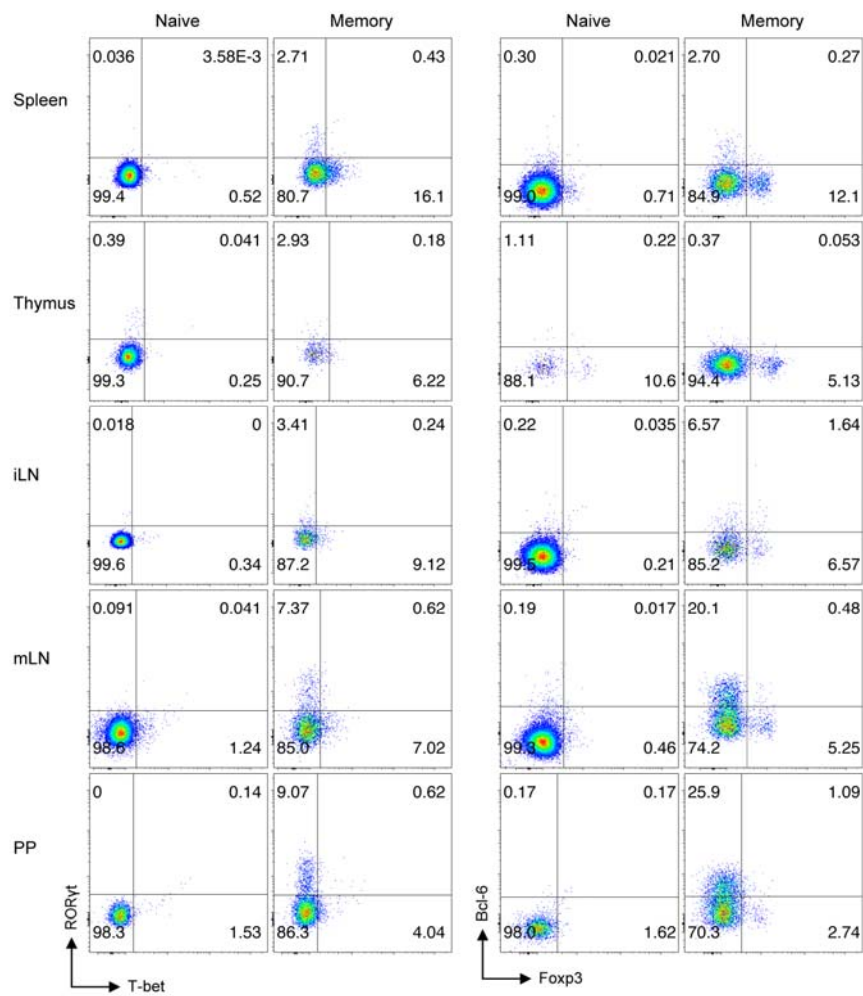


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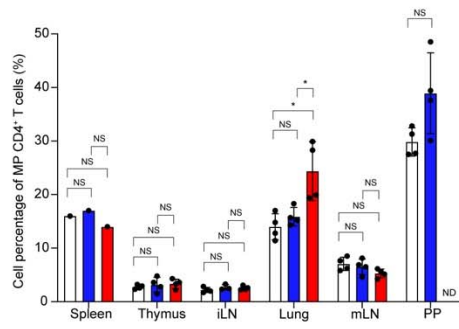
Supplementary Fig. 3 KEGG/GO analysis of steady-state MP CD4⁺ T cells. Selected (a) KEGG and (b) GO terms in each subpopulation of splenic MP CD4⁺ T cells.

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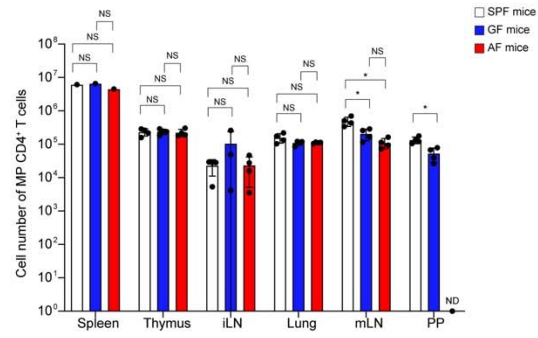


Supplementary Fig. 4 Effector T cell lineage-specific marker expression in MP CD4⁺ T cells. Transcription factor expression levels of T-bet, RORγt, Bcl-6, and Foxp3 in naïve and MP CD4⁺ T cells from the spleen, thymus, iLN, mLN, and PP.

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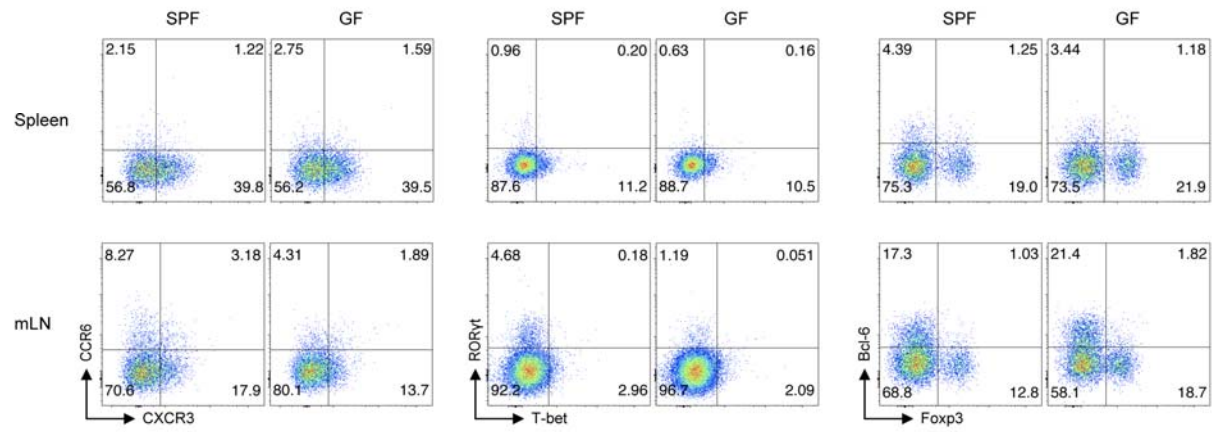


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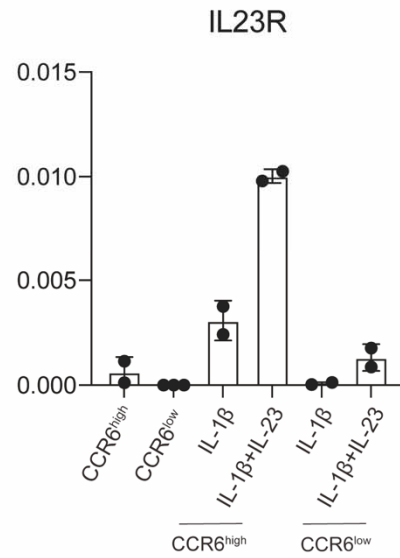
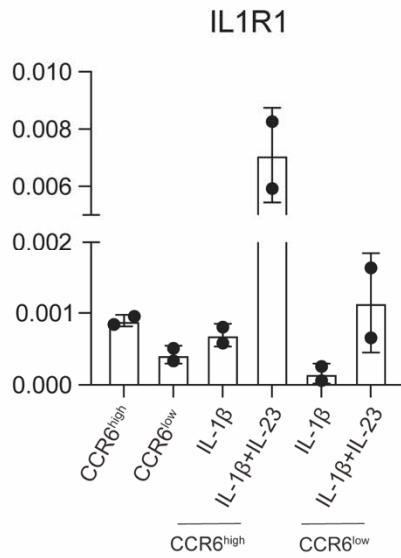
Supplementary Fig. 5 Comparison of MP CD4⁺ T cells in tissues from SPF, GF, and AF mice. (a) Proportion and (b) number of MP CD4⁺ T cells in the spleen, thymus, iLN, lung, mLN, and PP in SPF, GF, and AF mice (n=4). Data are presented as the mean $\pm$ S.D. P values were calculated using Mann-Whitney U-test (ND, not detected; NS, not significant; *p < 0.05, **p < 0.01, ***p < 0.001).

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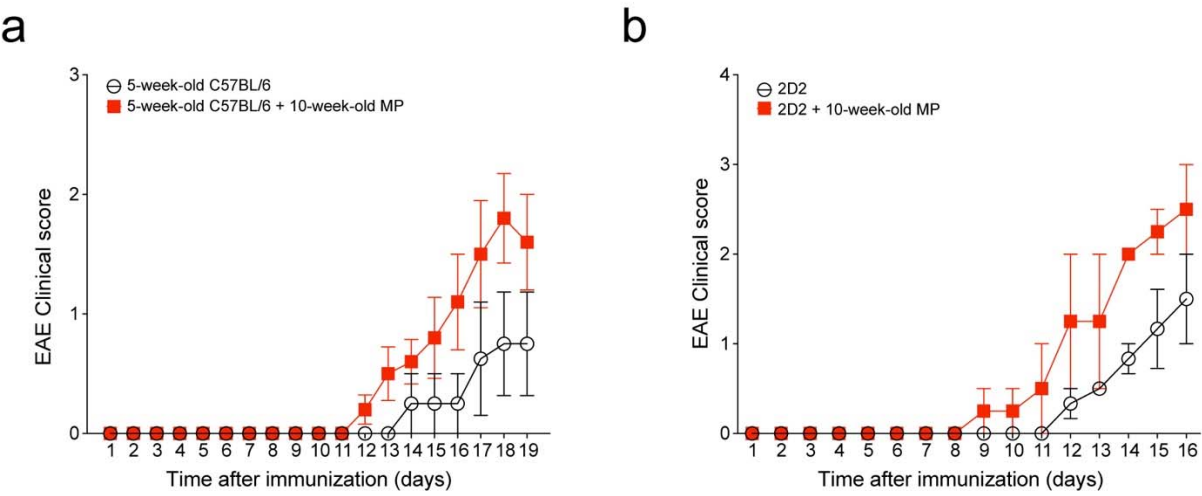
Supplementary Fig. 6 Effector T cell lineage-specific chemokine receptors and transcription factors in MP CD4⁺ T cells from SPF and GF mice. Expression of chemokine receptor of CCR6, CXCR3 and transcription factors of T-bet, RORγt, Bcl-6, and Foxp3 in spleen- and mLN-derived MP CD4⁺ T cells from SPF and GF mice.



Supplementary Fig. 7 IL1R1 and IL23R RNA expression level of CCR6^{high} and CCR6^{low} MP CD4⁺ T cells. mRNA expression of IL-1 β and IL-23 receptors in steady-state CCR6^{high} and CCR6^{low} MP CD4⁺ T cells and in the cells after activation with single or combination treatment of IL-1 β /IL-23 (n=2).

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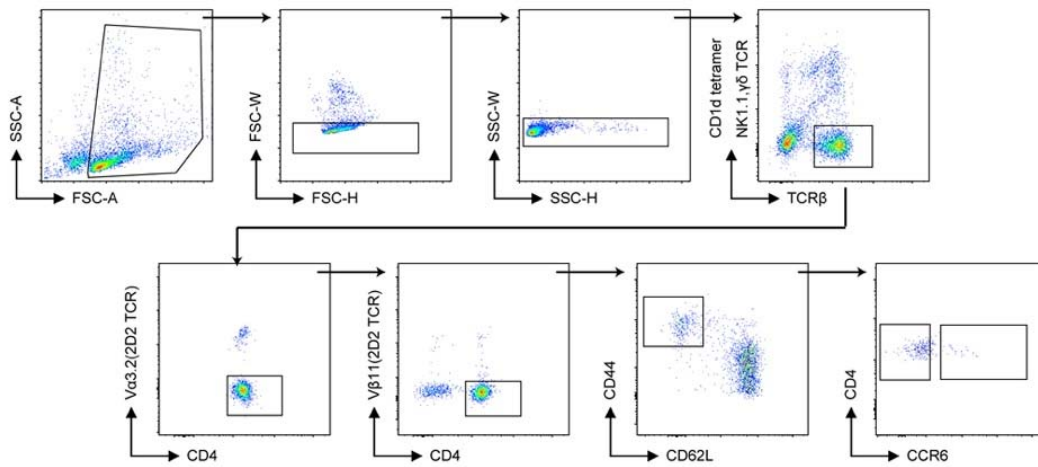


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Supplementary Fig. 8 Addition of MP CD4⁺ T cells in mice exacerbate autoimmune encephalomyelitis. (a) 5-week-old C57BL/6 mice with or without adoptively transferred 5×10^5 Treg-deleted MP CD4⁺ T cells (TCR β^+ CD1d tetramer⁻CD4⁺ Foxp3⁻CD62L^{low} CD44^{high}) from 10-week-old Foxp3-GFP mice were induced active EAE by MOG₃₅₋₅₅ in CFA (n=4). (b) 5×10^4 naïve CD4⁺ T cells (TCR β^+ CD4⁺ V β 11⁺ CD25⁻CD62L^{high} CD44^{low}) from 2D2 transgenic mice were adoptively transferred, with or without 5×10^5 splenic Treg-deleted MP CD4⁺ T cells (CD45.1⁺ $\gamma\delta$ TCR⁻ NK1.1⁻ V β 11⁻ TCR β^+ CD4⁺ CD1d tetramer⁻CD25⁻CD62L^{low} CD44^{high}) sorted from Foxp3-GFP mice, into Rag^{-/-} mice who were immunized with MOG₃₅₋₅₅ in CFA (n = 3).

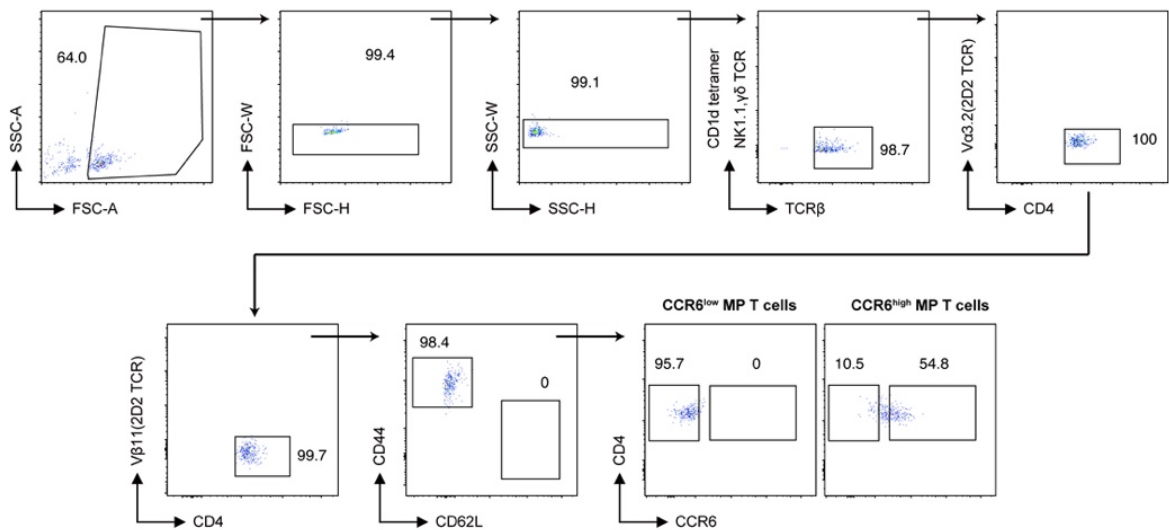
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Gating strategy

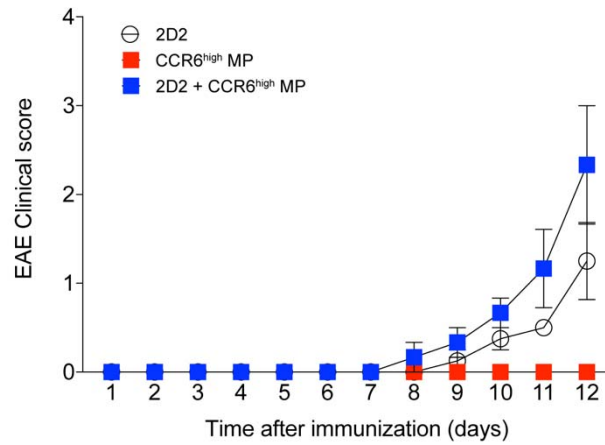


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Sorting purity

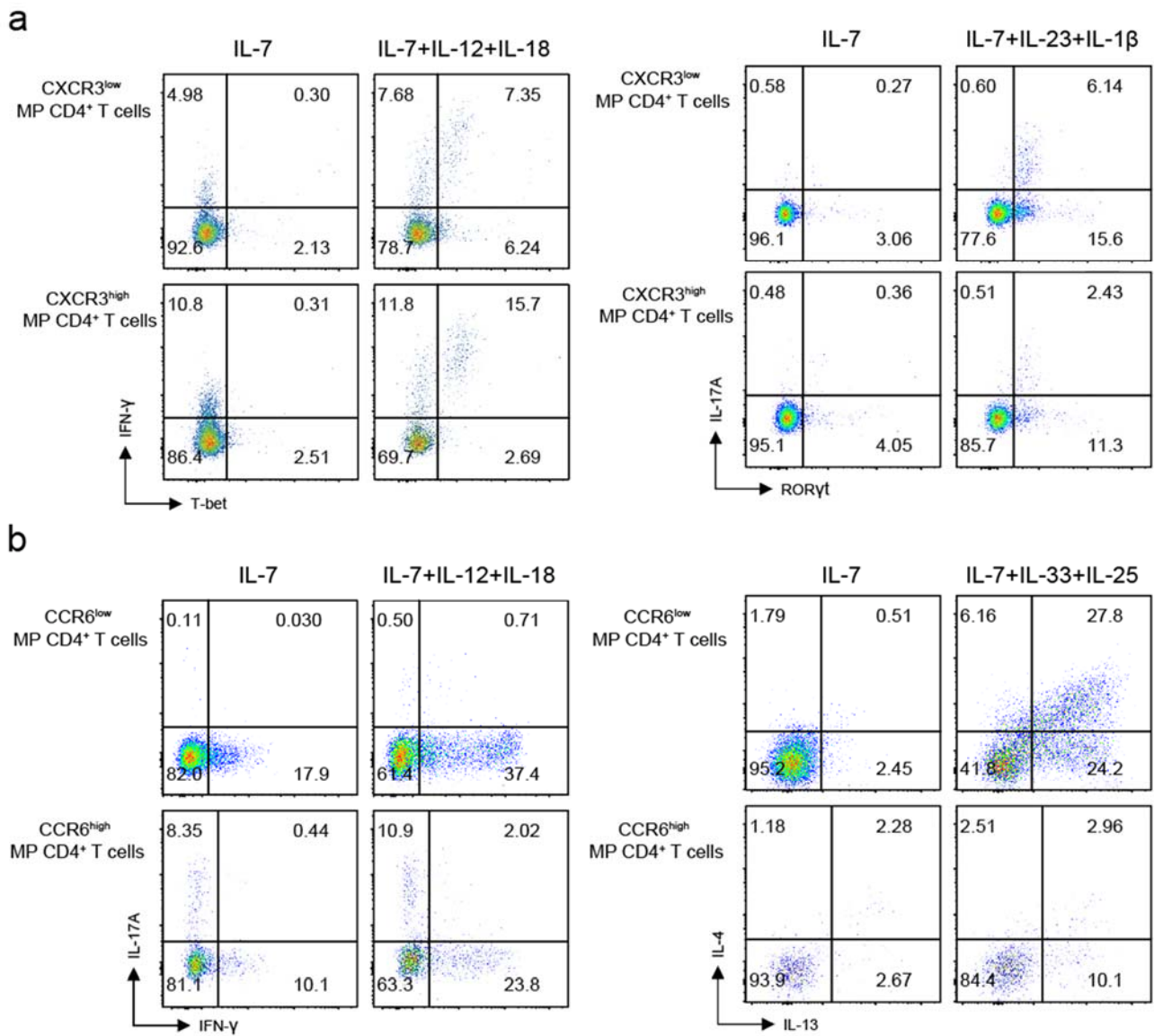


Supplementary Fig. 9 Gating strategy and sorting purity of CCR6^{high} and CCR6^{low} MP CD4⁺ T cells. (a) Gating strategy of splenic CCR6^{high} and CCR6^{low} MP CD4⁺ T cells. (b) Sorting purity of splenic CCR6^{high} and CCR6^{low} MP CD4⁺ T cells.

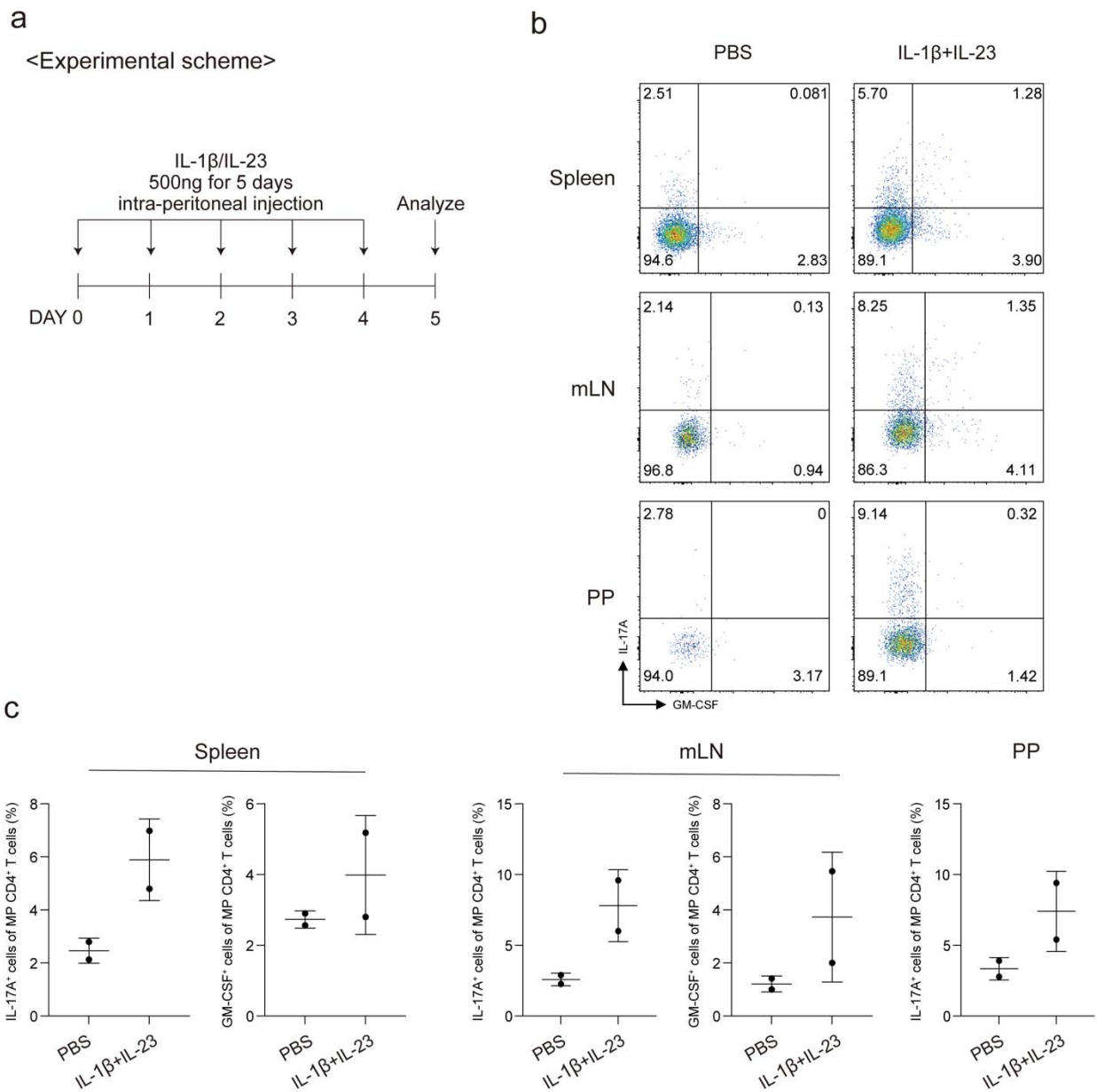


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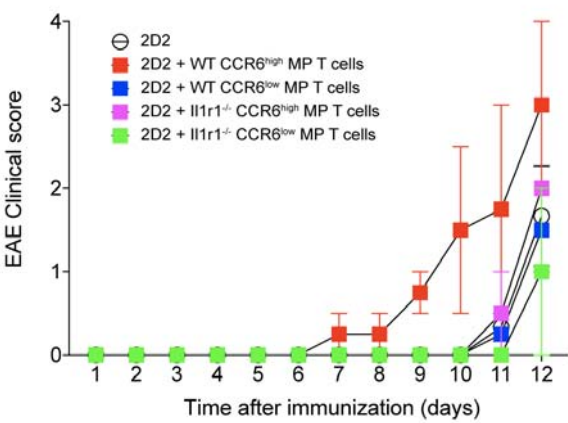
Supplementary Fig. 10 CCR6^{high} MP CD4⁺ T cells alone could not induce EAE disease. Naïve CD4⁺ T cells (5×10^4) from 2D2 transgenic mice were adoptively transferred, with or without CCR6^{high} or CCR6^{high} MP CD4⁺ T cells alone (1×10^5 , CD45.1⁺γδTCR⁻NK1.1⁻Vβ11⁻TCRβ⁺CD4⁺CD1d tetramer⁻CD25⁻CD62L^{low}CD44^{high}) of WT mice, into Rag^{-/-} mice who were immunized with MOG₃₅₋₅₅ in CFA. EAE clinical score was monitored daily (n=3).



Supplementary Fig. 11 Specific response pattern by MP CD4⁺ T cell subsets to the cytokines. (a) FACS-sorted CXCR3^{high} and CXCR3^{low} MP CD4⁺ T cells were stimulated with IL-12/IL-18 and IL-1β/IL-23 in the presence of IL-7 for 5 days. (b) FACS-sorted CCR6^{high} and CCR6^{low} MP CD4⁺ T cells were stimulated with IL-12/IL-18 and IL-25/IL-33 in the presence of IL-7 for 5 days.



Supplementary Fig. 12 In vivo production of IL-17A and GM-CSF by MP CD4⁺ T cells upon exogenous IL-1 β and IL-23 treatment. (a) Graphical experimental scheme. (b) Representative dot plots showing the population of IL-17A and GM-CSF producing memory-phenotype (MP) CD4⁺ T cells (TCR β ⁺CD1d tetramer⁻CD8⁺CD4⁺CD25⁻CD62L^{low}CD44^{high}) in murine spleens, mesenteric lymph nodes(mLNs), and Payer's patches (PP). (c) The proportion of IL-17A⁺ and GM-CSF⁺ cells in MP CD4⁺ T cells (n=2).



Supporting Fig. 13 IL-1R is required for CCR6^{high} MP CD4⁺ T cells to exacerbate autoimmune encephalomyelitis. Naïve CD4⁺ T cells (5×10^4) from 2D2 transgenic mice were adoptively transferred, with or without CCR6^{high} or CCR6^{low} MP CD4⁺ T cells (1×10^5 , CD45.1⁺γδTCR⁻NK1.1⁻Vβ11⁻TCRβ⁺CD4⁺CD1d tetramer⁻CD25⁻CD62L^{low}CD44^{high}) of WT or Il1r1^{-/-} mice, into Rag^{-/-} mice who were immunized with MOG₃₅₋₅₅ in CFA. EAE clinical score was monitored daily (n=4).