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Negative regulation of NKG2D expression by IL-4 in memory CD8 T cells

Running title: IL-4 regulates Memory CD8 T cell associated functions

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Abstract

IL-4 is one of the main cytokines produced during Th2 inducing pathologies. This cytokine has been shown to affect a number of immune processes such as T-helper differentiation and innate immune responses. However, the impact of IL-4 on CD8 T cell responses remains unclear. Here, we analyzed the effects of IL-4 on global gene expression profiles of antigen-induced memory CD8 T cells in the mouse. Gene ontology analysis of this signature revealed that IL-4 regulated most importantly genes associated with immune responses. Moreover, this IL-4 signature overlapped with the set of genes preferentially expressed by memory CD8 T cells over naive CD8 T cells. In particular, IL-4 down regulated *in vitro* and *in vivo* in a STAT6-dependent manner the memory-specific expression of NKG2D, thereby increasing the activation threshold of memory CD8 T cells. Furthermore, IL-4 impaired activation of memory cells as well as their differentiation into effector cells. This phenomenon could have an important clinical relevance as patients affected by Th2 pathologies such as parasitic infections or atopic dermatitis often suffer from viral-induced complications possibly linked to inefficient CD8 T cell responses.

Introduction

Immunological memory, a hallmark of the adaptive immune system, is the basis for protection against previously encountered antigens and thus the ultimate goal of vaccination. Long-term CD8 T-cell immunity is provided by an increased number of Ag-specific precursors, which persist over time (1) and display stronger and faster effector functions (1-3). Indeed, following activation, the production of certain soluble effectors as well as the display of cytolytic activity is strongly accelerated in memory cells compared to naive cells. We have previously demonstrated that, unlike naive cells, resting memory CD8 T cells produce CCL5/RANTES protein immediately upon TCR triggering (4). This immediate CCL5 secretion correlates with the maintenance of high levels of stored CCL5 mRNA. These elevated CCL5 mRNA levels have been observed in memory CD8 T cells generated in a variety of systems (4, 5). They are maintained through constitutive transcription of the *Ccl5* gene and increased stabilization of the mRNA (6). The increased efficiency of memory CD8 responses also arise from their lower activation threshold compared to naive cells. This results from the selection into the memory subset of CD8 T cells expressing TCR with a higher affinity for their cognate MHC-peptide complex, but also from the constitutive expression of surface costimulatory receptors, such as NKG2D (7). This receptor was first described as an activating receptor expressed at the surface of NK cells (8), but is also expressed at the surface of activated and memory CD8 T cells, where it can act as a costimulatory receptor (9-11). Indeed, under limited TCR stimulation engagement of NKG2D increases the proliferation and effector functions of CD8 T cells (12). This costimulatory function of NKG2D contributes to the efficient recall CD8 response against certain viral infections (13) and hence is one of the molecules responsible for the increased responsiveness of memory CD8 T cells.

Numerous studies have focused attention on the role of common gamma chain (γ c) cytokines, such as IL-7 or IL-15 in memory CD8 T cell persistence (14, 15). Both cytokines are essential for memory CD8 T cell homeostasis. More recently, the injection of IL-2/anti-IL-2 Ab complex that increases the IL-2 biological activity was shown to induce the homeostatic proliferation of memory CD8 T cells in mice (16). Unexpectedly a similar approach using IL-4/anti-IL-4 ab complex also induced the proliferation of memory-phenotype CD8 T cells, suggesting a role for IL-4 in the maintenance of memory cells (16). Likewise, proliferation of memory-phenotype CD8 T cells was observed in response to the IL-4 produced following NKT activation (17). IL-4 acted directly on CD8 T cells as neither *Stat6*- nor *Il4ra*- deficient CD8 T cells did proliferate when NKT cells were stimulated to produce IL-4 (17). Th2 CD4 T cells induced by parasitic infection or allergen produce IL-4 that cause induce STAT6 phosphorylation and homeostatic proliferation of memory phenotype CD8 T cells (18, 19). Recently, it has also been shown that the IL-4 produced by NKT cells drives the differentiation of CD8 single-positive thymocytes into memory-like CD8 T cells that have recently been renamed innate CD8 T cells (20, 21). These cytokine-induced innate CD8 T cells display a surface phenotype similar to pathogen-induced memory CD8 T cells, and share certain memory traits such as surface expression of CXCR3 or extemporaneous production of IFN γ in response to TCR-triggering (20, 21).

IL-4 has been shown to regulate CD8 T cell immune functions as well. For instance, IL-4 has been shown to be essential for the development of a protective anti-malaria CD8 T cell response (22), while other studies show that the CD8 mediated protection against viruses or tumours was impaired by IL-4 (23, 24). However, the

nature of the genes and underlying effector functions regulated by IL-4 in CD8 T cells remain unknown.

To address this issue, we have performed whole genome expression microarray analysis to identify a gene signature that is specifically regulated by IL-4 in memory CD8 T cells. To decipher the potential impact of IL-4 on memory functions we have focused on genes that are differentially expressed by memory cells compared to naive cells and that are involved in the increased responsiveness associated with memory cells. We showed that IL-4 affects the expression of a number of genes associated with memory CD8 T cells among which *Ccl5* and *Nkg2d* and we demonstrated that IL-4 impairs NKG2D mediated costimulation of memory CD8 T cells. Moreover, we showed that IL-4 inhibits the activation of memory CD8 T cells and their differentiation into cytolytic effector cells.

Material and methods

Mice

F5-TCR transgenic mice were provided by Dr. Kioussis (NIMR, London, U.K.). *Stat6*^{-/-} mice were provided by S. Akira (Osaka University, Osaka, Japan). CD45.2⁺ C57Bl/6, CD45.1⁺ CD57Bl/6 and Balb/c mice were purchased from Charles River laboratories. CD45.1⁺ F5 and *Stat6*^{-/-} F5 on a C57Bl/6 background were generated by crossing. **GFP expressing transgenic mice under the control of a CAG promoter were generated by lentigenesis on a C57Bl/6 background and crossed with F5 mice to generate GFP⁺ F5 mice.** Mice were bred in our animal facility “Plateau de Biologie Expérimentale de la Souris” (Lyon, France) in specific pathogen free conditions. All Experimental procedures were approved by an institutional review board: le Comité d'Evaluations Commun au Centre Léon Bérard, à l'Animalerie transit de l'ENS, au PBES et au laboratoire P4.

Peptides

The full agonist NP68 (ASNENMDAM) was used to activate F5 CD8 T cells (ProImmune, Oxford, UK). In some experiments, cells were also activated with the partial agonist NP3R (ASRENMDAM) (Synt:em, France).

Memory cells generation

T_{IM} (Inflammatory Memory T cells) were generated by injecting twice 50 nmol of Influenza A nucleoprotein NP₃₆₆₋₃₇₄ in PBS, i.p., in naive thymectomized transgenic F5 mice, as previously described (2, 25). Antiviral Central Memory and Effector Memory CD8 T cells (T_{CM} and T_{EM}) were generated by immunizing naive GFP⁺ F5 CD8 T cells with a recombinant vaccinia virus including the NP68 epitope as previously described (26). Analyses of memory cells were performed at least 6 weeks after infection.

Cell culture and cell sorting

The medium used for CD8 T cell cultures was DMEM supplemented with 6% FCS, 50µg/mL gentamicin, 2mM L-Glutamin, 10mM HEPES, 50µg/mL Gentamicin and 50µM 2-ME (all from Invitrogen Life Technologies). To measure the effects of IL-4 *in vitro*, cells were cultured for 20 hours at a concentration of $3 \cdot 10^6$ cells/ml in presence or absence of mIL-4 (Peprotech) at a concentration of 10 ng/mL. In some experiments, cells were activated *in vitro* at a concentration of $1 \cdot 10^6$ cells/mL in 96-well plates with 2% IL-2 and the indicated concentrations of NP68 for 24 hours or 72 hours. For qPCR experiments, spleen and lymph node CD8 T cells were purified by magnetic beads depletion as previously described (27). Purified CD8 T cells were then stained for cell surface expression of CD44, CD122, CD8 and sorted by flow cytometry using a FACS Aria (BD biosciences): T_{IM} were CD8⁺/CD44^{intermediate}/CD122^{intermediate} and antiviral T_{CM}/T_{EM} were CD8⁺/CD44^{high}/GFP⁺. Purity was routinely above 99%.

In vitro production of BMDC

Briefly, BMDC were generated as preciously described (28). Bone marrow cells, obtained from C57Bl/6 mouse femora, were resuspended in RPMI 1640 medium supplemented with 10% FCS, 2mM L-Glutamin, 10mM HEPES, 50µg/mL Gentamicin and 50µM 2-ME (all from Invitrogen Life Technologies). Cells (2×10^6 /mL) were cultured for 7 days in the presence of 0,2µg/mL recombinant human Flt3-ligand (TEBU) at 37°C. Cells were further matured with LPS (250ng/mL; Sigma-Aldrich), and pulsed with 300nM NP68 during an extra 16 hours.

In vivo cytotoxic assay

In vivo cytotoxic assay was performed as previously described (29). Target cells were prepared from C57Bl/6 spleens. Suspension was divided into two populations, control cells were not pulsed and labelled with a low concentration of CFSE (2 μ M), target cells were pulsed with 1 μ M NP68 for 1 hour and 30 minutes at 37°C, washed extensively, and labeled with a high concentration of CFSE (10 μ M). Peptide-pulsed CFSE^{high} cells and unpulsed CFSE^{low} cells were mixed together in a 1 to 1 ratio. A total of 2×10^7 CFSE-labeled cells were injected (i.p.) into C57Bl/6 mice either previously transferred with T_{IM} cells and immunized with NP68-pulsed BMDC or left unprimed. After 20 hours, mice were killed and the percentage of target and control cells among splenocytes was measured by flow cytometry. The percentage-specific lysis that normalized cytolytic activity between primed and control mice was determined by the following formula: percentage-specific lysis = $[1 - (\text{ratio primed}/\text{ratio unprimed})] \times 100$.

Administration of cytokines and antibodies in vivo

Age- and gender-matched mice received daily i.p. injections of a rat IgG1 or a mixture of 1.5 μ g rmIL-4 plus 50 μ g of the anti-mouse IL-4 mAb 11B11 (rat IgG1). After 4 or 7 days of treatment, spleens were analyzed by flow cytometry as described below.

MAbs and Flow cytometry

All surface staining were performed at 4°C in PBS (Invitrogen Life Technologies) supplemented with 1% FCS (Lonza, Köln, Germany) and 0.09% NaN₃ (Sigma-Aldrich). All antibodies were from BD-biosciences, eBioscience and R&D systems, except the anti-mCCL5 mouse monoclonal antibody that was produced in house (30). The F5-TCR was detected with NP68-conjugated H-2D^b-APC tetramers from ProImmune. Fixation and permeabilization for intracellular CCL5 detection was performed using

CytoFix/CytoPerm from BD Pharmingen. All analyses were performed using a LSRII (BD biosciences).

RT-qPCR experiments

RNA extraction from naive CD8 T cells and T_{IM} was performed from 3.10⁵ cells using RNA NOW reagent (Ozyme) and from 3.10⁴ antiviral T_{CM} cells using RNeasy Isolation kit (Qiagen). 100ng of RNA was retro-transcribed into cDNA using iScript cDNA Synthesis Kit (Biorad), and cDNA was analysed by real-time PCR using the Platinum Sybr Green qPCR SuperMix-UDG (Invitrogen). Relative gene expression was calculated using *ubiquitin* as the endogenous control housekeeping gene. Primers were as follow:
 UBIQUITIN forward: 5'-AAGAATTCAGATCGGATGACACACT-3' reverse: 5'-GCCACTTGGAGGTTGACACTTT-3'. NKG2D forward: 5'-CCTGGCAGTGGGAAGATGG-3' reverse: 5'-TTGAGCCATAGACAGCACAGG. CCL5 forward: 5'-GCACCTGCCTCACCATATGG-3' reverse: 5'-AGCACTTGCTGCTGGTGTAG-3'. Myb forward: 5'-CATTTGATGGGGTTTGGGCATG-3' reverse: 5'-GCTTCGGCGATGTGGTAATAGG-3'. Reck forward: 5'-CACTAGCTCATAGGCTGACTCC-3' reverse: 5'-GTGTGAGACAGGCACGAGTG-3'. Ccl5 forward: 5'-GCACCTGCCTCACCATATGG-3' reverse: 5'-AGCACTTGCTGCTGGTGTAG-3'. Bcl2 forward: 5'-CCATCTACCGAGCAAGTGG-3' reverse: 5'-ATATAGAGATCACATTGAAGCCTTCTT-3'. PCR experiments were performed using a ABI Prism 7000 (Applied Biosystems).

Microarrays processing and determination of the gene expression signatures

mRNAs were extracted from 10⁵ sorted naive or T_{IM} memory CD8 T Cells directly *ex vivo* or after 20 hours of *in vivo* culture in presence or absence of 10ng/mL of mIL-4 (Peprotech). Culture medium was supplemented with 10ng/mL of IL-7 (Peprotech) to

sustain cell viability. mRNAs were extracted using the RNeasy kit (Qiagen). 50ng of RNA was amplified using the MessageAmp II kit (Ambion) and labelled using the IVT labeling kit (Affymetrix). cRNA were fragmented and hybridized on GeneChip Mouse Genome 430 2.0 Array (Affymetrix). These data have been submitted to Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/> - accession number GSE32423). Data analysis was performed using the GeneSpring GX 10.0 software and Bioconductor/R packages. Probesets were filtered according to their expression value: retained probesets are those with at least one replicate with an expression value higher than the 20 percentile of the mean expression data of all the probesets of the chip. The differential gene expression analysis was carried out using Limma on RMA-summarized and quantile-normalized data. The p-values of the differential expression analysis were adjusted for multiple testing correction using the Benjamin-Hochberg false discovery rate (FDR) controlling procedure. Finally, a cut-off of 0.05 was used for the adjusted p-values. Two gene-expression signatures are defined in our paper: the IL-4 gene-expression signature of memory CD8 T cells (Supplementary table 1) and the T_{IM} memory cells gene-expression signature (Supplementary table 2). The IL-4 gene-expression signature of memory CD8 T cells was obtained using the microarrays performed with the T_{IM} memory CD8 T cells in *ex vivo*, control (medium supplemented with IL-7) and IL-4 conditions. The list of probesets differentially expressed between the IL-4 and the control condition (502 probesets) and the list of probesets differentially expressed between the IL-4 and the *ex vivo* conditions (5506 probesets) were intersected to obtain a list of 332 common probesets, having coherent transcriptional variations (up-regulated or down-regulated in both comparisons). This list of probesets was summarized to finally propose a list of 245 genes. For summarization we retained only one probeset per gene, the one having the highest absolute logFC. The T_{IM} memory

gene-expression signature, composed of 310 genes, is the summarized list of the 402 probesets differentially expressed between the ex vivo T_{IM} memory CD8 T cells and the ex vivo naive CD8 T cells.

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Results

Determination of the IL-4 gene-expression signature in T_{IM} memory CD8 T cells.

To evaluate the impact of IL-4 on memory CD8 T cell functions, we used T_{IM} memory cells that are generated by synchronously activating F5 CD8 T cells, *in vivo*, with NP68 peptide (2). Using pan-genomic microarrays, we compared gene expression patterns of sorted T_{IM} either freshly isolated (*ex vivo*) or incubated *in vitro* for 20 hours in presence or absence of IL-4. The IL4 gene-expression signature of memory CD8 T cells (thereafter called “IL-4 signature”), corresponding to genes that saw their expression level modified by IL-4 compared to *ex-vivo* and to control *in vitro* incubation, was defined using the analysis procedure described in the methods. Among the 245 genes defined using this approach, 81 were down regulated and 164 were up regulated. The strength of this signature was demonstrated by its capacity to segregate, by hierarchical clustering, an independent set of arrays performed with T_{IM} memory cells that were treated with IL-4 or not (figure 1A). To provide independent validation of the array analysis we measured, for a subset of genes, changes in gene or protein expression by quantitative real-time PCR or flow cytometry on purified T_{IM} memory CD8 T cells treated with IL-4 (figure 1B-C). In agreement with array results, *Myb*, *Reck* and *Ccl5* gene expressions were strongly inhibited by IL-4 while *Bcl2* gene expression was up-regulated. Likewise, IL-4R α , GP49a and Eomes protein expression were induced by IL-4 (Figure 1C). We used Fatigo (<http://babelomics.bioinfo.cipf.es/>) to find significant enrichment in biological process ontology terms associated with the genes in the IL-4 signature (31). IL-4 impacted on the expression of genes involved in multiple biological processes (supplementary table 3), the most significant one being “immune response” with in particular regulation of multiple cytokines/chemokines/cytokine receptors (*Lif*, *Lta*, *Ccl5*, *il2R α* , *il4R α* , *il18bp*). A

number of genes that are involved in CD8 T cells function or activation, such as *Ccl5*, *Klrk1*, *Cd55*, *Lyst* or *Trat1*, were also targeted by IL-4 suggesting that associated CD8 T cell functions might be modified by this cytokine.

Improved effector functions of memory CD8 cells are associated with differential expression of certain genes when compared with naive cells. Thus, to analyse the impact of IL-4 on the functionality of memory CD8 T cells we searched within the “IL-4 signature” for genes that are specifically associated with memory cells. To establish the T_{IM} memory cells associated gene signature (thereafter called “memory signature”), the gene expression profile of T_{IM} memory CD8 T cells and naive CD8 T cells were compared. 310 genes differed in their expression level between these two populations (supplementary table 2). We identified 12 genes shared between the IL-4 and the memory signatures (table 1). In agreement with previous results (6), we found *Ccl5* to be part of these genes. We also identified the *Klrk1* gene also known as *Nkg2d* that codes for an activating NK cell receptor.

IL-4 regulates NKG2D expression in memory CD8 T cells via a Jak/STAT6-dependent pathway.

NKG2D is expressed by activated and memory CD8 T cells in the mouse and can serve as a costimulatory receptor inducing increased proliferation, IFN γ secretion and cytotoxicity (32). Down-regulation of this receptor by IL-4 could thus impair memory CD8 T cell activation. We first sought to confirm the effects of IL-4, *in vitro*, on *Nkg2d* expression by T_{IM} memory CD8 T cells. These cells are generated under sterile inflammatory conditions and are arrested at an early stage of memory differentiation (25). In addition, to extend these results to pathogen-induced memory CD8 T cells, we also generated F5 memory CD8 T cells by immunising mice with a recombinant vaccinia

virus expressing the NP68 epitope recognised by the F5 TCR (26). Memory cells generated in response to vaccinia virus belong to the T_{CM} or T_{EM} subset and therefore will be designated hereafter as T_{CM}/T_{EM} . We measured the expression of NKG2D by these memory subsets using flow cytometry. As depicted in figure 2A, we observed that NKG2D is expressed by all T_{CM}/T_{EM} and by the majority of T_{IM} . We then measured the effects of IL-4 on *Nkg2d* expression by culturing purified CD8 T cells in presence or absence of IL-4. *Nkg2d* mRNA levels were strongly down-regulated in all memory subsets following a 20 hour incubation with IL-4. *Ccl5* and *Bcl2* mRNA that are respectively down and up regulated by IL-4 in T_{IM} were similarly modified in T_{CM}/T_{EM} (figure 2B). Moreover, at the protein level, NKG2D expression by T_{IM} was also reduced after the incubation with IL-4. However, there was no significant effect of IL-4 on the surface expression of NKG2D in T_{CM}/T_{EM} , suggesting that the half-life of the NKG2D protein in these subset might be longer than in T_{IM} (figure 2C).

IL-4 signals through its receptor IL-4R α and common γ c chain and activates the JAK1 and JAK3 kinases leading to STAT6 activation. We have previously shown that in memory CD8 T cells *Ccl5* expression is down-regulated by IL-4 in a STAT6-dependent manner (6). Thus, we investigated if STAT6 was also involved in the regulation of *Nkg2d* expression by IL-4 in T_{IM} . T_{IM} memory cells were generated in F5 WT or F5 *Stat6*^{-/-} mice and the impact of IL-4 on the level of *Nkg2d* mRNA was measured. Results in figure 2D show that IL-4 was unable to induce the down regulation of *Nkg2d* mRNA in *Stat6*-invalidated cells, indicating that *Nkg2d* regulation by IL-4 is dependent on the JAK/STAT6 pathway. This seems to be the case for a large fraction of genes in the IL-4 gene expression signature. Indeed, we found that the IL-4 signature that we identified here was highly enriched in STAT6-regulated genes identified by Wei and colleagues

using deep sequencing of promoters bound to immuno-precipitated STAT6 in CD4 T cells (supplementary table 4) (33).

IL-4 induced innate CD8 T cells do not express NKG2D or CCL5.

To test the impact of IL-4 on CD8 T cells *in vivo*, we injected mice with IL-4/anti-IL-4 mAb complexes (IL-4C). This has been shown to induce memory CD8 T cells proliferation *in vivo* (16) and to mimic the impact of IL-4 on CD8 T cells that is observed during a Th2 response induced by parasites or allergens (19). These complexes are used because recombinant IL-4 has a very short half-life *in vivo* and it has been shown that its association with the anti-IL-4 mAb 11b11 increases both bioavailability and half-life of IL-4 in the mouse (34). We first monitored the impact of IL-4 on endogenous naive CD8 T cells. In accordance with previous reports, we found that **treating C57Bl/6 mice with IL-4C every day for 4 or 7 days** induced CD8 T cell proliferation as well as an accumulation of CXCR3⁺/CD44^{Int} innate CD8 T cells (figure 3A,B). This was associated with a strong up-regulation of Eomes expression (figure 3C), a transcription factor involved in the generation of these innate CD8 T cells in response to IL-4 (21, 35). IL-4 acted directly on CD8 T cells as IL-4 had no effect on CXCR3/CD44/Eomes expression in STAT6 deficient CD8 T cells adoptively transferred into WT recipients (figure 3D,E).

Using adoptive transfers, we showed that IL-4 was also able to drive the differentiation of naive F5 CD8 T cells into memory like CD8 T cells that expressed increased levels of CXCR3 and Eomes (figure 4A). As previously shown by others, this was associated with the acquisition of the capacity to produce IFN γ in response to TCR engagement (figure 4B) (20). However, NKG2D and CCL5, two other hallmarks of T_{CM}/T_{EM} memory cells were not up-regulated by innate CD8 T cells (figure 4C), indicating that IL-4 induced Eomes up-regulation leads only to a partial acquisition of CD8 memory properties.

Next, we wanted to determine whether a physiological source of IL-4 could also have an effect of NKG2D/CCL5 expression by memory CD8 T cells *in vivo*. For this, we took advantage of Balb/c mice in which a previous study clearly established that the majority of naturally occurring memory CD8 T cells were induced by constitutive IL-4 production by PLZF-expressing NKT cells and thus are innate CD8 T cells (20). The majority of CXCR3⁺CD44^{int} memory-like cells found in Balb/c mice did not express NKG2D or CCL5 in contrast to naturally occurring memory cells found in C57bl/6 mice (figure 5B-C). Thus, IL-4C complexes or NKT cell-produced IL-4 induce a restricted memory CD8 T cell differentiation program characterized by the lack of NKG2D and CCL5 expression. Moreover, NKG2D-positive Balb/c CD8 memory cells expressed significantly lower levels of NKG2D than their C57Bl/6 counterparts (figure 5D), suggesting that the increased levels of IL-4 found in Balb/c mice also acts on NKG2D expression by these memory cells.

Decreased expression of NKG2D, in vivo, in response to IL-4 is associated with the inhibition of NKG2D-dependent CD8 T cell activation.

Next, we tested the impact of IL-4 on memory CD8 T cells subsets. F5 T_{1M} memory cells were transferred in C57Bl/6 mice or naive F5 CD8 T cells transferred in C57Bl/6 mice were challenged with vaccinia virus to generated pathogen-specific F5 T_{CM}/T_{EM}. Mice containing F5 memory CD8 T cells were treated with IL-4C for 4 days, the expression of NKG2D and Eomes was then analysed. As shown in figure 6A Eomes expression was strongly up-regulated and the MFI of NKG2D was significantly reduced in both subsets of memory cells (figure 6B). NKG2D has been shown to deliver a costimulatory signal to memory CD8 T cells. This can be best observed when T cells are stimulated in suboptimal conditions. To test if IL-4 could have an impact on memory CD8 T cell

functions and NKG2D-dependant CD8 T cell activation, we first analysed CD8 T cell activation and cytotoxic functions after a treatment with IL-4C *in vivo*. IL-4C treated and control T_{IM} memory cells were activated *in vitro* with the antigenic peptide NP68: as shown in figure 7A, the expression of the early activation marker CD69 by IL-4 treated cells was strongly inhibited 24 hours after activation. Similarly, we found that the expression of CD25, which is upregulated at a later stage, was inhibited in IL-4 treated memory CD8 T cells 72 hours after activation (figure 7B), indicating that IL-4 treatment globally impact memory CD8 T cells activation. We then tested if IL-4 could affect the cytotoxic capacities of memory CD8 T cells *in vivo*. IL-4C treated or control TIM memory cells were transferred into recipient mice and subsequently stimulated with NP68 peptide-loaded dendritic cells. Two days after activation, we performed a 20 hours *in vivo* cytotoxicity assay with NP68 peptide-loaded targets. As shown in figure 7C, we found that the CD8 T cells-mediated cytotoxicity observed with the IL-4C treated T_{IM} cells was strongly reduced compared with control cells (figure 7C). Thus, our results show that IL-4 reduces the cytotoxic capacities of memory CD8 T cells. As NKG2D engagement delivers a costimulatory signal to memory CD8 T cells, we used an *in vitro* assay to test the impact of IL-4-induced NKG2D reduced expression on the cytotoxic capacities of memory CD8 T cells. T_{IM} that had been exposed to IL-4C for 4 days *in vivo* were stimulated *in vitro* with the F5-TCR full agonist NP68 (figure 7A) or the partial agonist NP3R (figure 7B) peptide and the fraction of activated cells was quantified by measuring CD107a exposure, a phenomenon previously found to correlate with activation and the cytotoxic activity (36). To mimic NKG2D engagement, the anti-NKG2D activating mAb A10 was added to the assay. As shown in figure 7 all T_{IM} memory cells exposed CD107 after 4 hours stimulation with the NP68 full agonist in contrast to the NP3R stimulated cells where only a fraction of cells became CD107 positive. Addition of

the NKG2D activating antibody restored full activation of NP3R stimulated control Tim, but was unable to induce NKG2D costimulation of IL-4C treated memory cells (figure 7), thus indicating that *in vivo* IL-4 treatment impaired NKG2D-dependent memory CD8 T cell activation.

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Discussion

In the present study, we have evaluated the impact of IL-4 on the gene expression profile of memory CD8 T cells. To evaluate the functional impact of IL-4 on one memory CD8 function, we focused on a well-described receptor: NKG2D that is up-regulated by memory CD8 T cells as compared to naive cells. Originally described as an activating receptor for NK cells, it has been shown that NKG2D acts as a costimulatory receptor for CD8 T cells. A feature of this receptor is that it can be engaged by multiple ligands that are not expressed by healthy tissues but are rapidly induced in stressed cells such as tumour cells (37) or cells infected by viruses or bacteria (38, 39). We show that, *in vitro*, IL-4 inhibits NKG2D mRNA levels in two subsets of Ag-induced memory CD8 T cells, T_{IM} induced by peptide priming and T_{CM}/T_{EM} induced in response to a viral infection. This mRNA down-regulation, led to a concomitant decrease in the surface expression of NKG2D by T_{IM} but not T_{CM}/T_{EM} . The lack of NKG2D surface expression decrease by T_{CM}/T_{EM} memory cells compared to T_{IM} could result from their higher NKG2D expression levels, although an increased half-life of the protein in these cells, that are more differentiated cells than T_{IM} memory cells, cannot be excluded. This hypothesis is supported by our *in vivo* results showing that surface expression of NKG2D is significantly decreased at the surface of T_{CM}/T_{EM} after a 4 days treatment with IL-4C (figure 6). Using T_{IM} memory cells we show that *in vivo* IL-4 contact inhibits memory CD8 T cells activation and differentiation into cytolytic effector cells. Moreover, we demonstrate that the reduction in NKG2D expression is sufficient to inhibit NKG2D-dependent costimulation of memory CD8 T cells. This could impact the efficiency of memory CD8 T cells responses given that a contribution of NKG2D expression in CD8 T cell mediated preprotection has been documented in several pathological contexts such as bacterial or viral infections (12, 13, 40). A diversity of signals can inhibit NKG2D

expression such as CD4 T cells-derived soluble NKG2D ligands (9) or cytokines: indeed a number of γ c cytokines have been shown to regulate NKG2D expression in several contexts, but these cytokines come in two flavours, inhibitors or inducers. IL-4, like IL-21 (41) decreases NKG2D surface expression by CD8 T cells, in contrast to IL-2 and IL-15 that sustain NKG2D expression by these cells. The fine-tuning of NKG2D expression by a diversity of signals could be essential to avoid CD8 T cell activation in a context of non-pathological cellular-stress as NKG2D engagement by its ligands leads to a decreased CD8 activation-threshold that could favour activation of autoreactive T cells. Indeed, NKG2D expression has been associated with the development autoimmune diseases such as celiac disease (42) or diabetes (43).

Innate-memory cells have been shown to develop in the thymus in response to IL-4 (20). These cells share several features with pathogen-induced memory cells, such as the capacity for immediate IFN γ secretion in response to TCR engagement or the expression of CXCR3. We show that innate-memory cells can also develop from naive CD8 T cell precursors upon exposure to IL-4 for 4 days *in vivo*. However, these memory-like cells lack certain hallmarks of the Ag-induced memory cells such as the presence of CCL5 intracellular expression and NKG2D surface expression. The function of innate CD8 T cells is not fully understood. It has been proposed that they could act as early sensors of an inflammatory environment during primary infection. Recently, Eomes expressing “memory-like” cells rapidly secreting IFN γ upon TCR triggering have been described in the foetal spleen and thymus at 18 weeks of gestation (44). These cells could play an important role in the newborn immune response, before the maturation of their immune system.

Finally, we showed that IL-4 modulates the expression of genes that are directly involved in memory CD8 T cell functions. Thus several genes involved in the control of

cell proliferation such as *Cdk6*, *Tob1* or *Klf6*, are regulated by IL-4. Expression of several genes, *Cdh1*, *Itgb7*, *Reck* and *Gelsolin*, coding for proteins controlling cell migration are also affected suggesting that IL-4 could impact this capacity of memory CD8 T cells. Cytokine responsiveness could also be modified: interestingly, the gene coding for IL18bp, an inhibitor of IL-18 signalling, is induced by IL-4 in memory CD8 T cells. Recently, Balb/c memory-phenotype CD8 T cells have been shown to produce lower levels of IFN γ compared with C57Bl/6 mice in response to a combination of IL-12/IL-18 (45). This reduced responsiveness of Balb/c memory phenotype CD8 T cells could result from an increased IL-18bp expression in response to the higher basal levels of IL-4 observed in these mice. Finally, we identified several genes coding for proteins that could play a role in leukocyte activation, such as *Klrk1*, *Klrd1*, *Cd55*, *Cd84*, *Trat1*, *Ccl5* or *Trem12*, suggesting that IL-4 could modify the activation threshold of memory CD8 T cells and we demonstrate that IL-4-induced down-regulation of NKG2D expression on memory CD8 T cells impairs the costimulatory function of this receptor.

About 1 billion people in the world are chronically infected with parasites (46) that elicit a strong Th2 immune response. The levels of IL-4 associated with these responses are sufficient to affect a number of immune processes, impairing or deviating the immune response of the host. Thus, an impaired CD8 T cells effector response to vaccinia virus has been observed in a *Schistosoma mansoni* infected host (47). More recently, it has been shown that IL-4 produced during a parasitic infection with *Nippostrongylus brasiliensis* impairs the innate immune response to a bystander infection with *Mycobacterium tuberculosis* (48). The IL-4 induced decreased antimicrobial peptide secretion by keratinocytes would be one of the potential mechanisms responsible for this increased susceptibility (49). Finally, it has also been recently described that, in a breast cancer, TSLP produced by epithelial cancer cells can induce the development of

Th2 cells that promote tumour growth (50). Thus, our results suggest that Th2 derived IL-4 could also impact on the function of pre-existing memory CD8 T cells and contribute to the pleiotropic regulation of the immune response associated with Th2 inducing pathologies.

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Footnotes

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Abbreviations used in this paper: qPCR, quantitative real-time PCR; T_{CM}, Central Memory T cells; T_{EM}, Effector Memory T cells; T_{IM}, Inflammatory Memory T cells.

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Figures legend

Figure 1: Regulation of gene expression by IL-4 in Tim memory CD8 T cells

T_{IM} memory cells were generated as described in the methods

(A) *T_{IM}* memory cells were sorted (Ex-vivo) and treated 20h in presence (IL-4) or absence (medium) of IL-4. RNA was isolated and subjected to Affymetrix Gene Chip expression analysis as described in the methods. The IL-4 gene-expression signature (supplementary table 1) was used to perform a Hierarchical clustering using euclidian centred algorithm of probesets and samples on an independent set of arrays performed with sorted *T_{IM}* CD8 memory cells, not treated (ex-vivo), incubated 20h in medium in absence (Medium) or presence of IL-4 (IL-4). Results are presented as a heatmap. (B,C) *T_{IM}* memory cells were isolated and incubated 20h in presence or absence of IL-4. Total RNA was isolated and mRNA coding for *c-myb*, *Reck*, *Ccl5* and *Bcl2* was quantified by RT-qPCR (B), or cells were stained for IL-4R α , GP49a and *Eomes* expression and analysed by flow cytometry (C). The expression fold change observed in the microarray experiment between IL-4-treated and control memory cells is indicated for each gene (Array FC). (B) mean \pm SEM of 3 independent experiments. (C) data representative of at least 5 experiments.

Figure 2 : IL-4 inhibits NKG2D expression by memory CD8 T cells in vitro

(A,B,C) *T_{IM}* memory cells and *T_{CM}/T_{EM}* memory cells were generated as described in the methods and NKG2D expression was assessed by flow cytometry (A). *T_{IM}* and *T_{CM}/T_{EM}* were isolated and incubated in presence or absence of IL-4 for 20h; Total RNA was isolated and mRNA coding for *Nkg2d*, *Ccl5* and *Bcl2* were quantified using RT-qPCR (B), or NKG2D surface expression was measured by flow cytometry (C).

(D) T_{IM} memory cells were generated by peptide immunization of thymectomized F5 or F5 x $stat6^{-/-}$ mice. T_{IM} were then sorted and incubated 20h in presence or absence of IL-4. RNA was isolated and *Nkg2d* mRNA levels were measured using RT-qPCR. (A,C) data are representative of at least 5 experiments (mean \pm SEM). (B,D) mean \pm SEM of 3 independent experiments. (* p <0.05; ** p <0.01; paired student t-test)

Figure 3: IL-4C treatment induces innate CD8 T Cells generation in the spleen

C57Bl/6 mice were daily injected IP either with a control IgG1 or a combination of rmlL-4 and anti-IL-4 11B11. After 4 to 7 days of treatment, spleen was removed and CD8 T cells were analysed by flow cytometry. (A) CD8 T cells number in the spleen of control or IL-4C treated mice after 4 or 7 days. (B) CD44 and CXCR3 expression by CD8 T cells from control or IL-4C treated mice after 4 days. (a) and (b) represent respectively the $CXCR3^+/CD44^{int}$ and the $CXCR3^+/CD44^{high}$ populations. (C) *Eomes* expression by CD8 T cells from control or IL-4C treated mice after 4 days. (D,E) 3.10^6 CFSE-labelled $Stat6^{-/-}$ CD45.2 CD8 T cells and 3.10^6 CFSE-labelled WT CD45.1 CD8 T cells were co-transferred into C57Bl/6 CD45.1/CD45.2 recipient mice. After 4 days of IL-4C treatment, spleen was removed and CFSE-positive CD45.2 ($Stat6^{-/-}$) and CFSE-positive CD45.1 (WT) CD8 T cells were analyzed for CXCR3 and CD44 expression (D) or *Eomes* expression (E). (a) and (b) are as in B. (A,B,C) data representative of at least 5 independent experiments with $n = 5$ mice (mean \pm SEM). (D,E) mean \pm SEM of 3 independent experiments ($n = 4$ mice). (* p <0.05; ** p <0.01; unpaired student t-test)

Figure 4: IL-4-induced innate CD8 T cells do not express NKG2D and CCL5

3.10⁶ F5 Naive CD45.2 CD8 T cells were transferred into recipient CD45.1/CD45.2 mice that were subsequently daily injected IP either with a control IgG1 or a combination of rmlL-4 and anti-IL-4 11B11. After 4 days of treatment, spleen was removed and CD8 T cells were analyzed by flow cytometry. (A) CXCR3 and Eomes expression by transferred CD8 T cells from control or IL-4C treated mice. (B) Intracytoplasmic expression of IFN γ in transferred CD8 T cells isolated from control or IL-4C treated mice and stimulated 4 hours with NP68. (C) NKG2D surface- and CCL5 intracellular-expression by naive F5 CD8 T cells from control or IL-4C treated mice. (A,B,C) mean \pm SEM of 3 independent experiments with n = 4 mice. (**p<0.01; unpaired student t-test)

Figure 5: Balb/c mice contain high frequency of memory-phenotype CD8 T cells expressing low levels of NKG2D and CCL5

(A) Frequency of naturally occurring memory CD8 T cells in 6 months Balb/c and C57Bl/6 mice. (B,C) NKG2D expression by naturally occurring memory CD8 T cells from Balb/c or C57Bl/6 mice of indicated age. (D) MFI of NKG2D expression by naturally occurring memory CD8 T cells from Balb/c or C57Bl/6 mice. (A,D) mean \pm SEM of 3 independent experiments with n = 3 mice. (B,C) representative example of 2 independent experiments with n = 3 mice (mean \pm SEM). (*p<0.05; **p<0.01; unpaired student t-test)

Figure 6: IL-4 inhibits NKG2D expression by memory CD8 T cells in vivo

T_{IM} memory cells were generated as described in the method and $3 \cdot 10^6$ sorted T_{IM} memory cells were injected into recipient mice. T_{CM}/T_{EM} were generated as described in the methods. Recipient mice were then daily injected IP either with a control IgG1 or a combination of rmlL-4 and anti-IL-4 11B11. After 4 days of treatment, spleen was removed and CD8 T cells were analyzed by flow cytometry. (A) Eomes expression by T_{IM} memory cells or T_{CM}/T_{EM} cells from control or IL-4C treated mice after 4 days. (B) NKG2D expression by T_{IM} memory cells or T_{CM}/T_{EM} cells from control or IL-4C treated mice after 4 days. (A,B) mean \pm SEM of 4 independent experiments with $n = 5$ mice (* $p < 0.05$; ** $p < 0.01$; unpaired student t-test)

Figure 7: IL-4 inhibits NKG2D-dependant costimulation of memory CD8 T cells

T_{IM} memory cells were generated as described in the method. $3 \cdot 10^6$ sorted T_{IM} memory cells were then sorted and injected into recipient mice that were treated daily with a control IgG1 or a combination of rmlL-4 and anti-IL-4 mAb for 4 days. (A,B) T_{IM} were recovered and stimulated in vitro with the F5 TCR full agonist NP68 at the indicated concentration. Concentration 0,3 nM is shown. CD69 expression was assessed after 24h of simulation (A) and CD25 expression was assessed after 72h of stimulation (B). (C) IL-4C treated or control T_{IM} were transferred into naive recipient mice and were subsequently challenged with NP68-pulsed BMDCs. Two days later, NP68-pulsed or non-pulsed target cells were injected into the recipient mice as described in Material and Methods. Twenty hours later, the frequency of target cells killed was evaluated in the spleen. (D) IL-4C treated or control T_{IM} were recovered and stimulated in vitro with the F5 TCR full agonist NP68 or the partial agonist NP3R in presence or absence of the NKG2D activating antibody A10 (@NKG2D) for 4

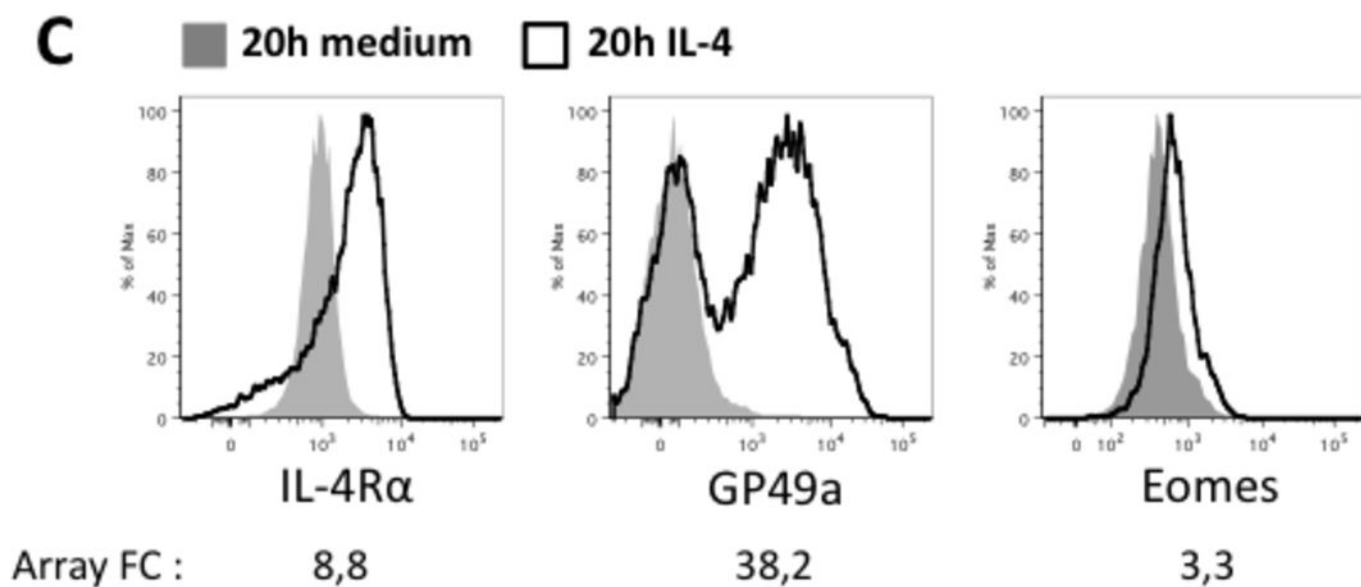
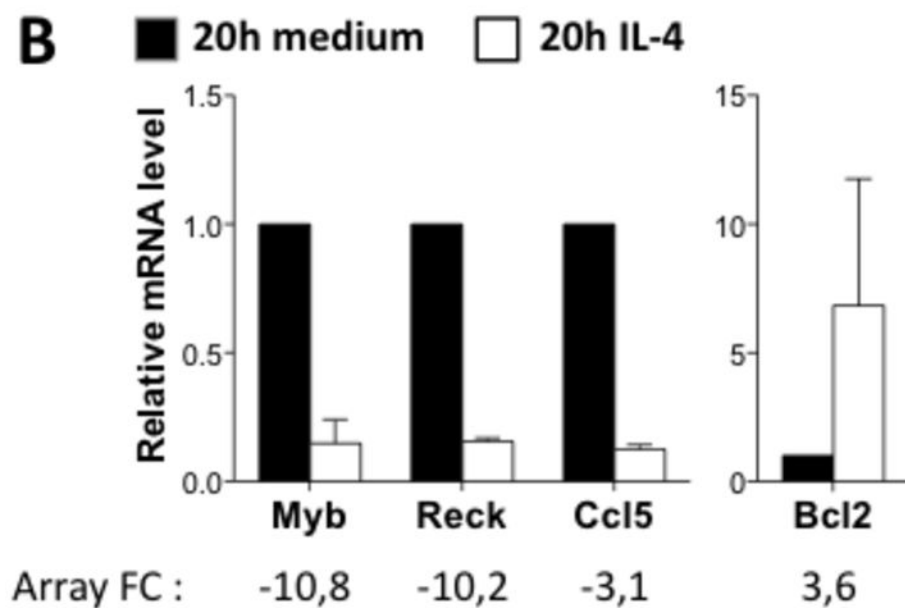
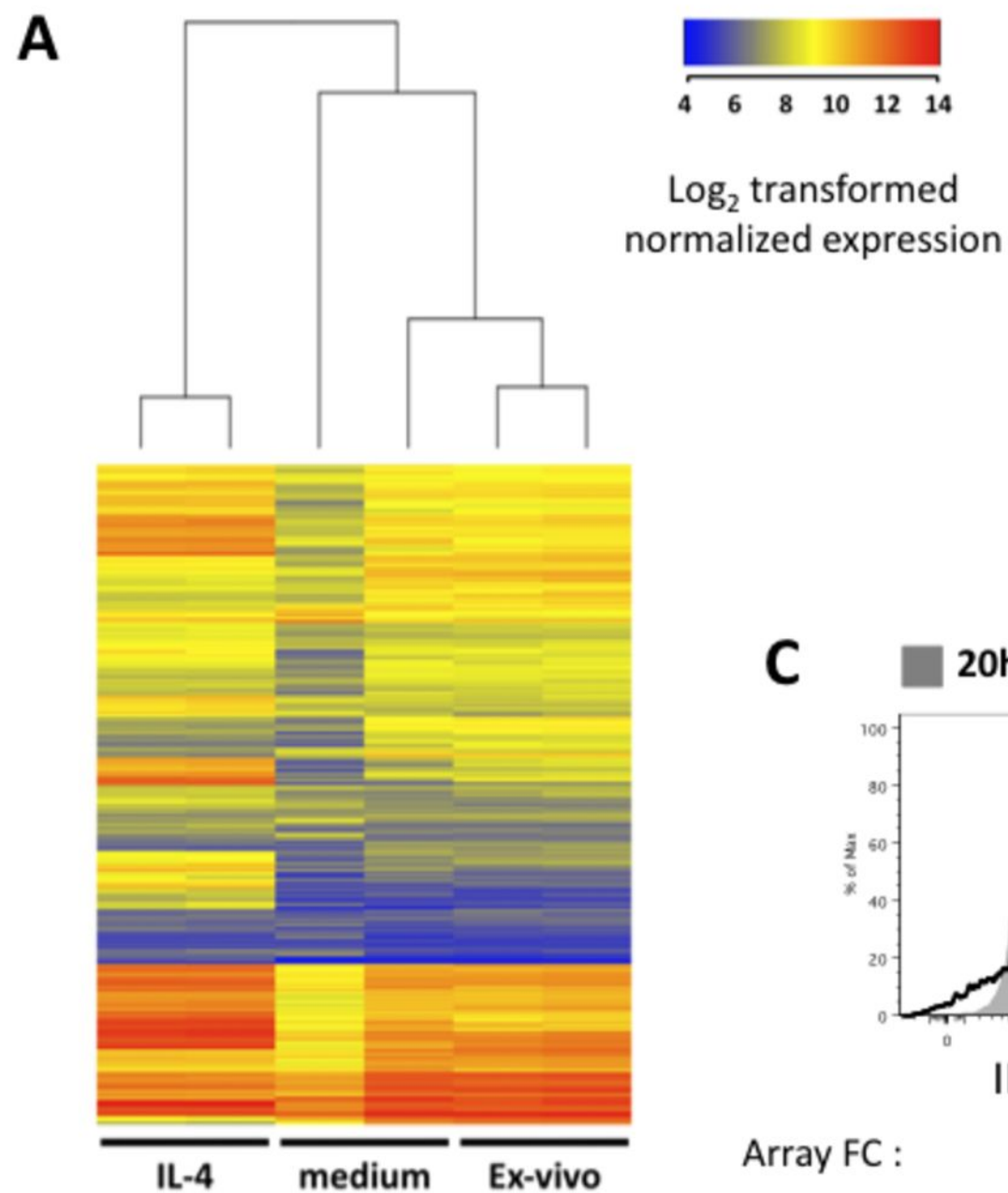
hours. CD107a expression by stimulated T_{IM} memory cells was then assessed using flow cytometry. (A,B,D) data representative of 3 independent experiments (mean \pm SEM). (C) mean \pm SEM of 2 independent experiments with at least $n = 3$ mice (* $p < 0.05$; ** $p < 0.01$; unpaired student t-test)

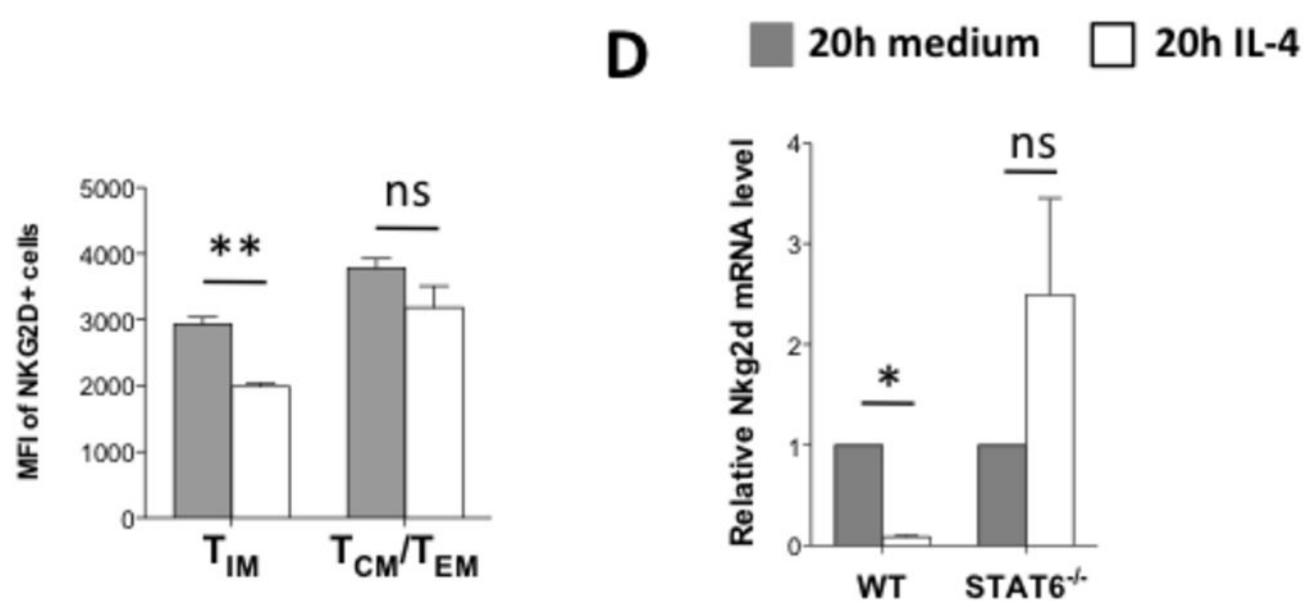
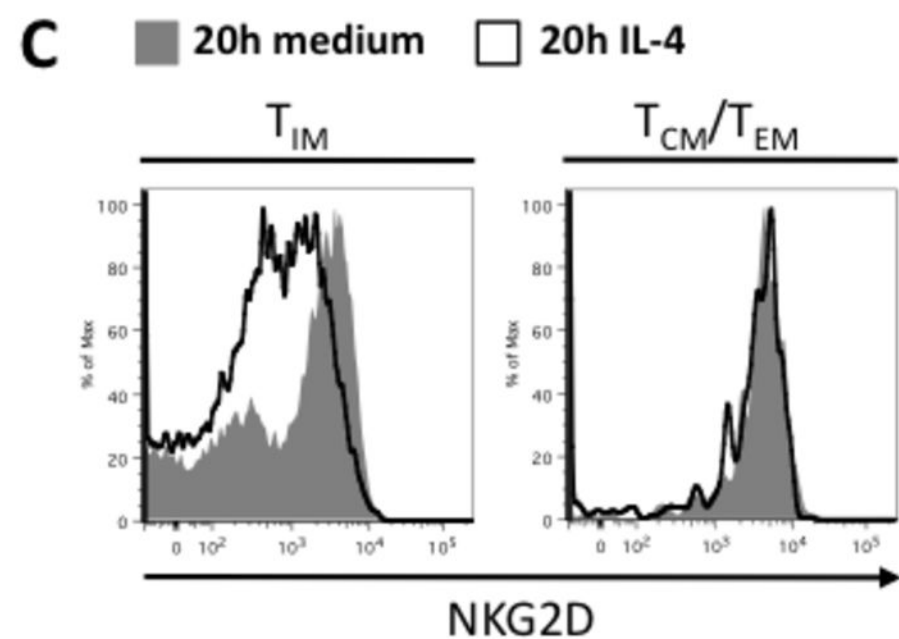
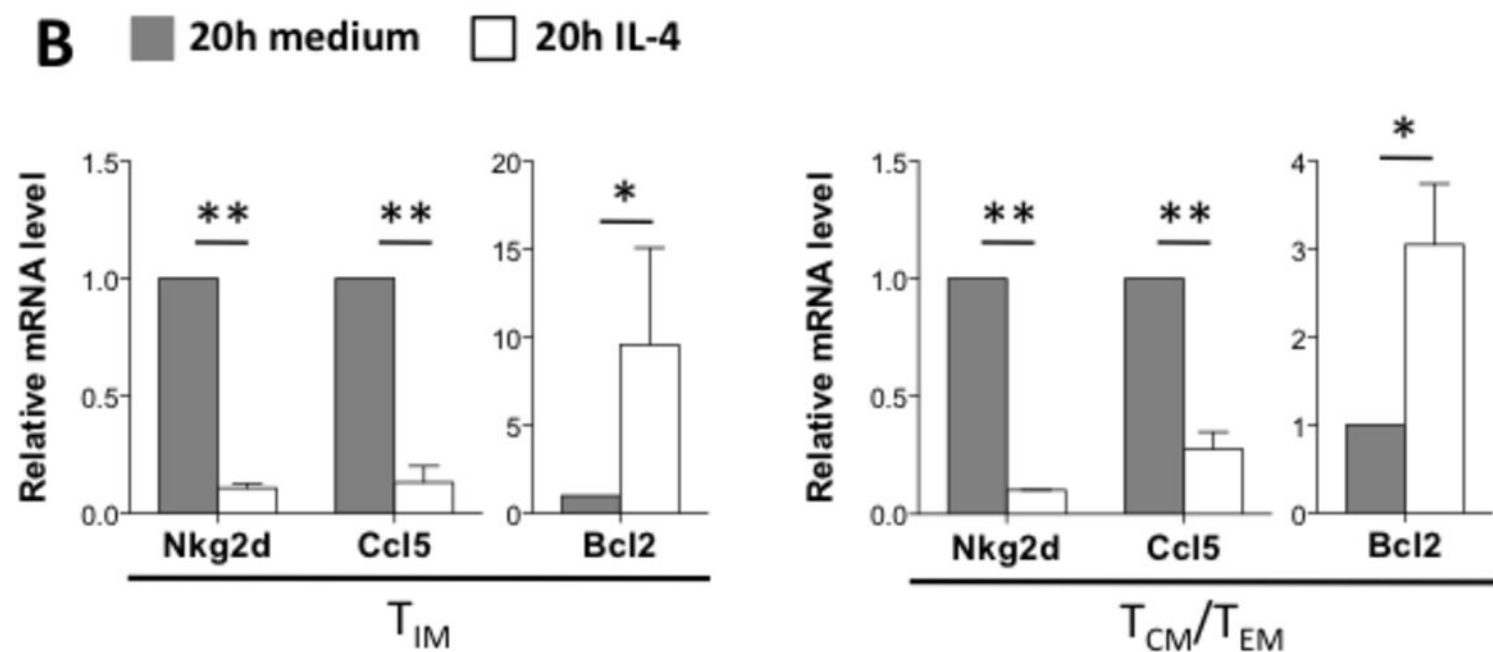
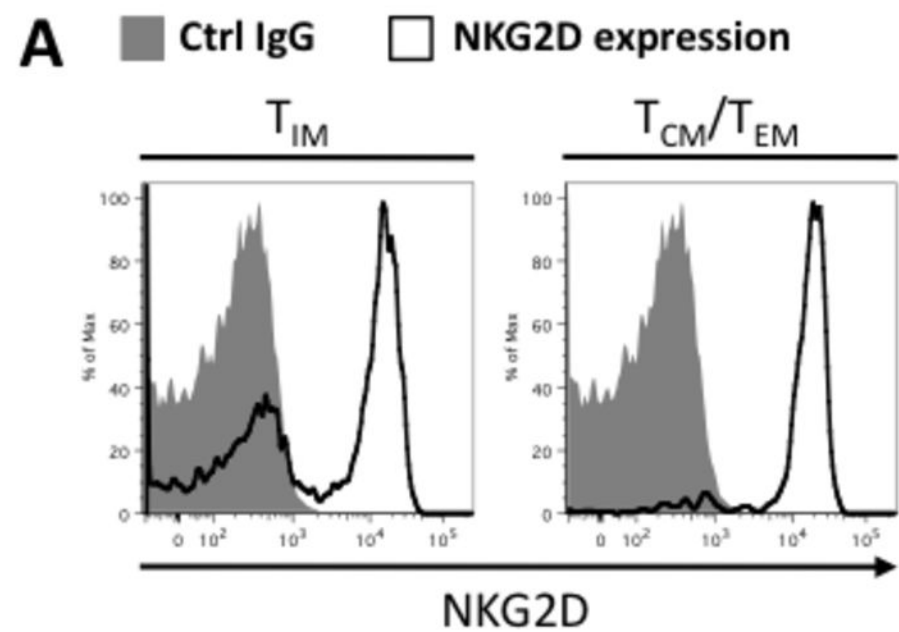
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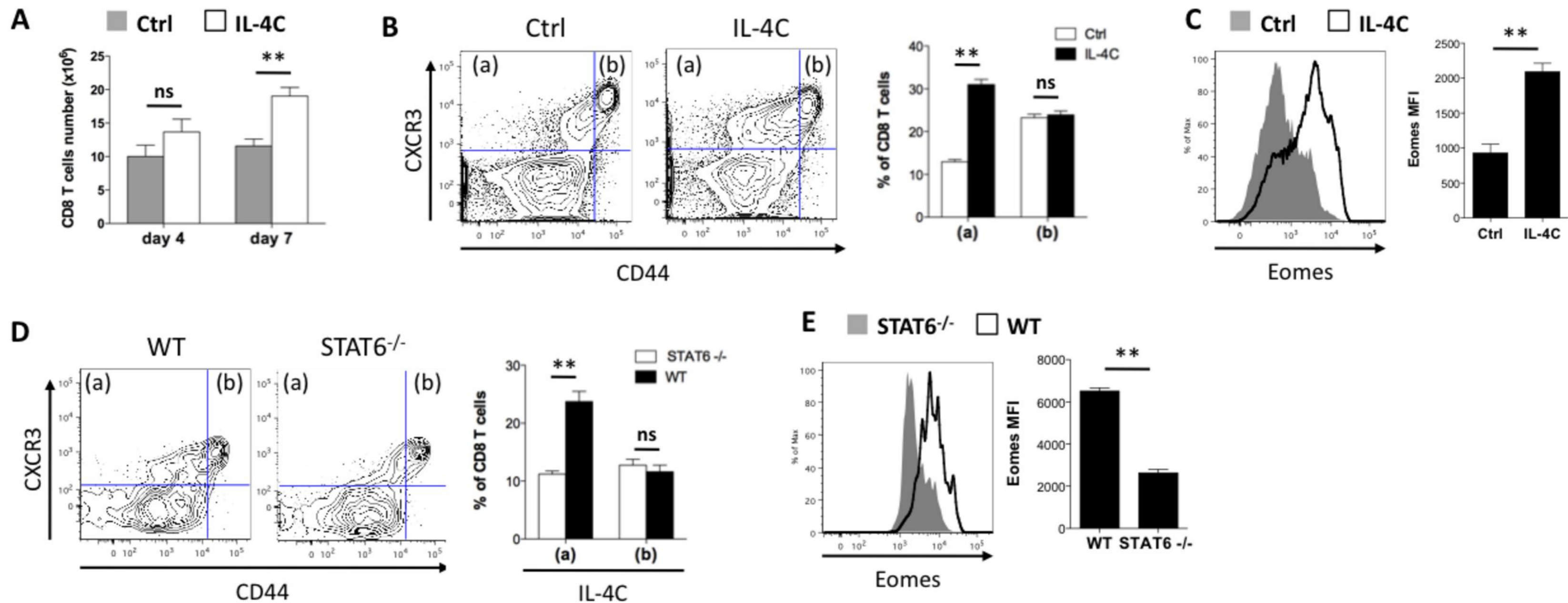
Gene Symbol	Gene ID	FC IL-4 / Control	FC T_{IM} / Naive
Map3k8	26410	6,0	3,2
Ifit1	15957	5,7	-4,0
Trat1	77647	5,5	-9,3
Smpd13b	100340	3,3	2,7
Gsn	227753	3,3	-4,8
Cdh1	12550	3,1	11,6
C230098021Rik	102153	2,0	-2,5
Arsb	11881	-2,2	2,1
Frat2	212398	-2,2	-2,0
Ccl5	20304	-3,1	69,8
Atp1b1	11931	-3,2	-2,5
Klrk1	27007	-3,5	7,8

Table I: T_{IM} memory cells associated genes modulated by IL-4

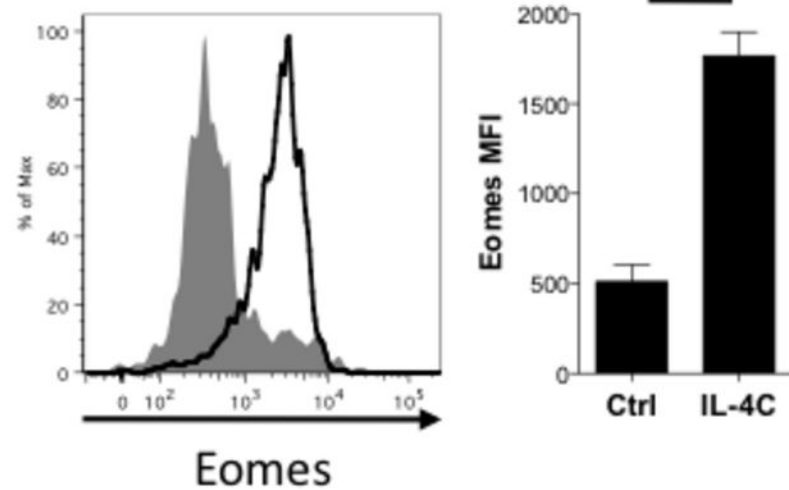
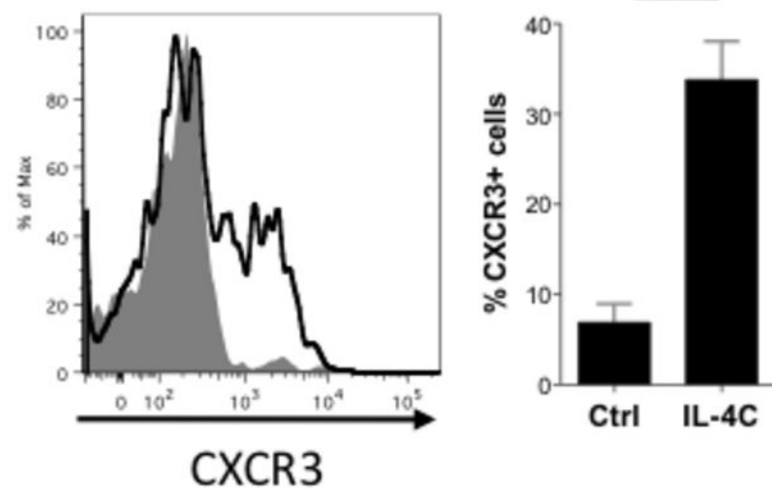
To identify the Genes shared between the IL-4-gene expression signature and the T_{IM} memory cells associated-gene signature we performed a Venn diagram between the two signatures. The 14 genes belonging to the T_{IM} memory cells associated gene expression signature that are modulated by IL-4 are shown, with additional information the Entrez Id and the fold change observed in the two comparisons performed (a minus symbol is added to indicate a down regulation).



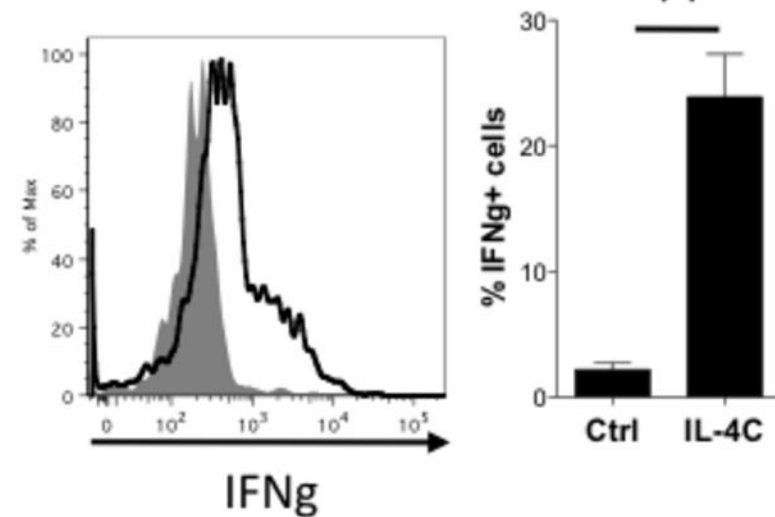




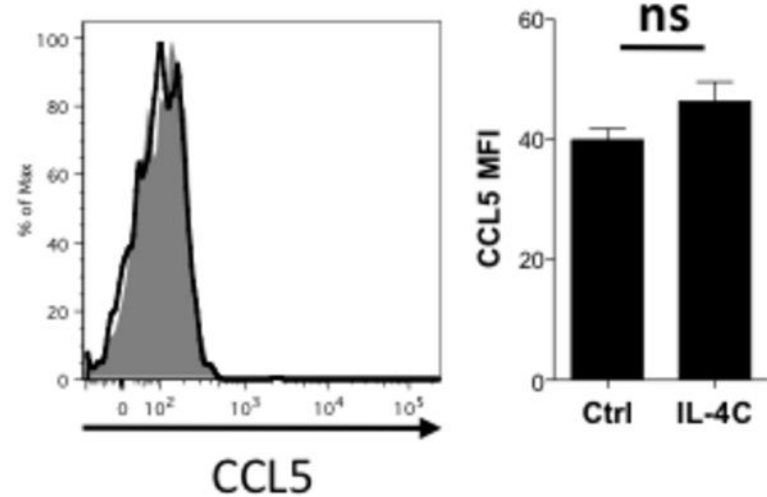
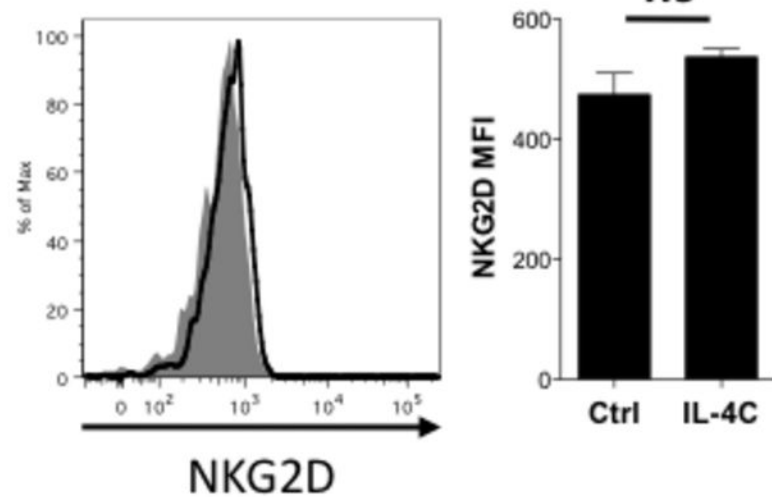
A ■ Ctrl □ IL-4C

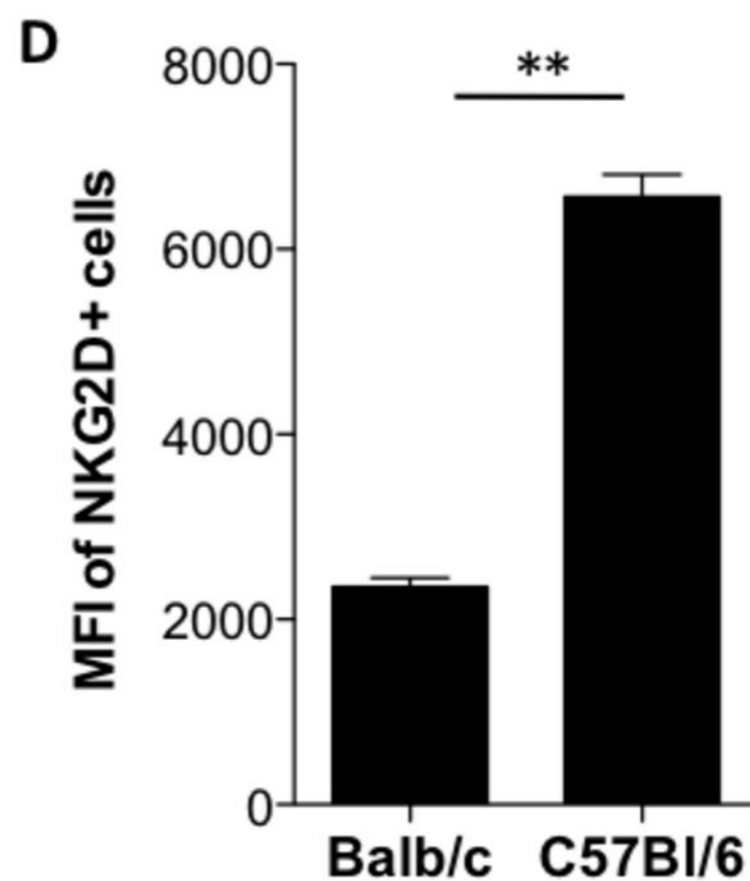
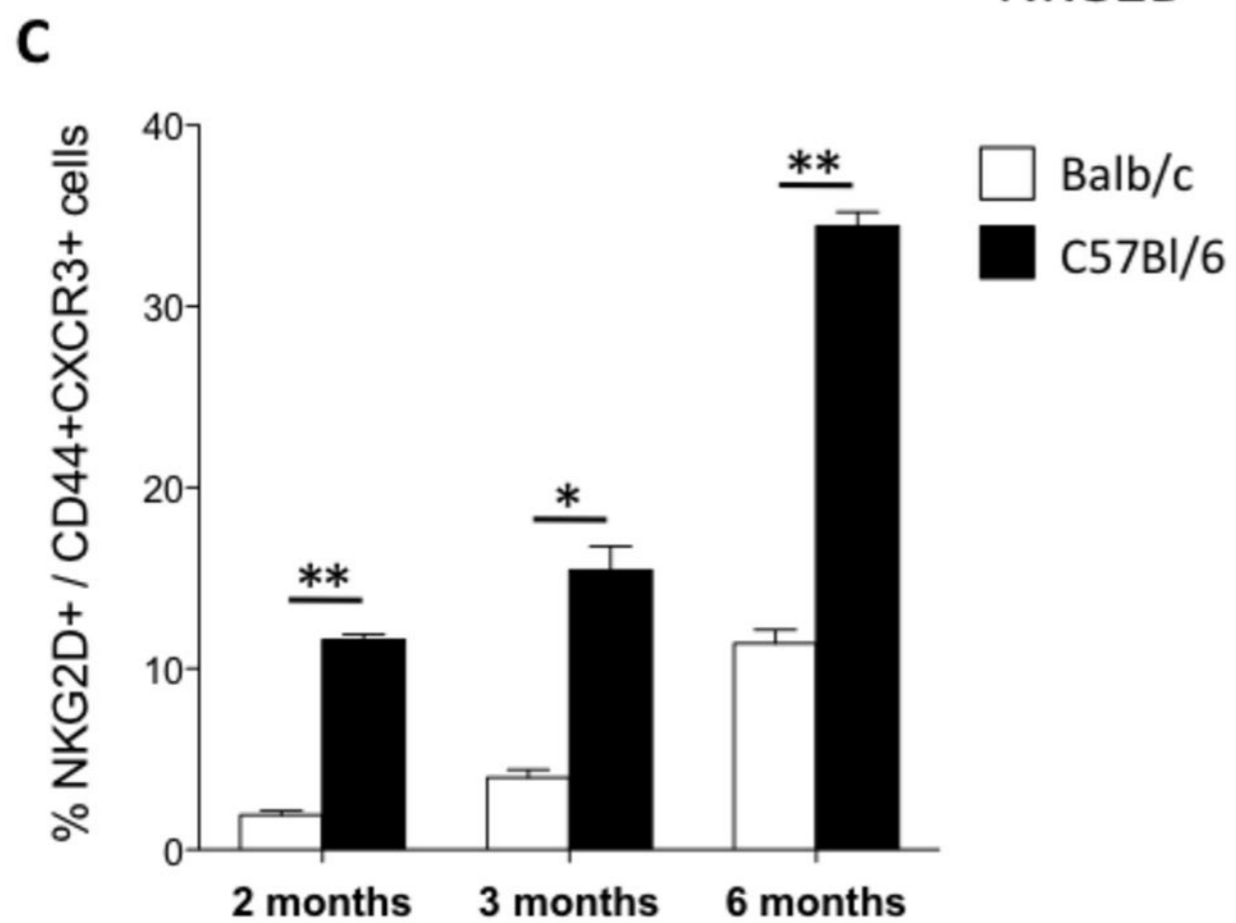
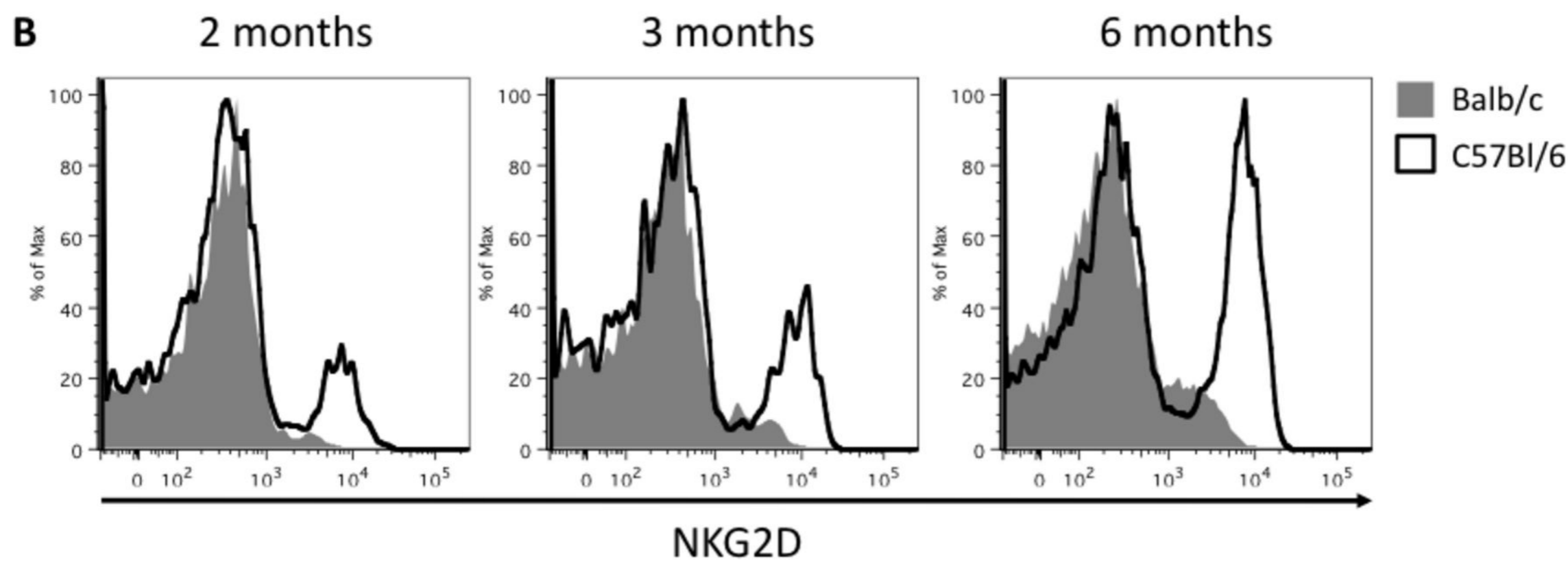
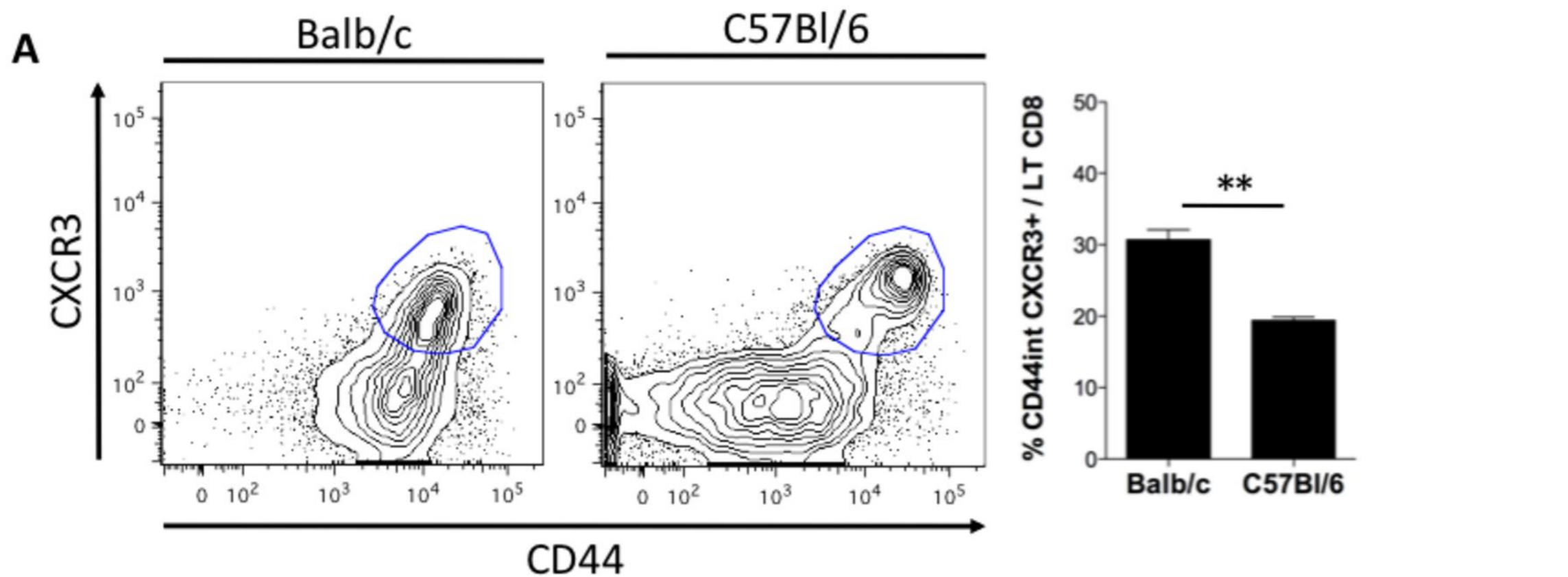


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