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Negative Regulation of NKG2D Expression by IL-4 in Memory CD8 T Cells

Erwan Ventre, Lilia Brinza,1 Stephane Schicklin,1 Julien Mafille, Charles-Antoine Coupet, Antoine Marçais, Sophia Djebali, Virginie Jubin, Thierry Walzer, and Jacqueline Marvel

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 Th differentiation and innate immune responses. However, the impact of IL-4 on CD8 T cell responses remains unclear. In this study, we analyzed the effects of IL-4 on global gene expression profiles of Ag-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 downregulated 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. The Journal of Immunology, 2012, 189: 3480–3489.

Immunological memory, a hallmark of the adaptive immune system, is the basis for protection against previously encountered Ags, and thus, the ultimate goal of vaccination. Long-term CD8 T cell immunity is provided by more 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 with 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 with 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 γ-chain (γc) 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 induces 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), whereas other studies show that the CD8-mediated protection against viruses or tumors was impaired by IL-4 (23, 24). However, the nature of the genes and underlying effectors 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 with naïve 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 cytophilic effectors cells.

Materials and Methods

Mice

F5-TCR transgenic mice were provided by Prof. D. Kioussis (National Institute of Medical Research, London, U.K.). Stat6−/− mice were provided by S. Akira (Osaka University, Osaka, Japan). CD45.2+ C57Bl/6, CD45.1+ C57Bl/6, and BALB/c mouse were purchased from Charles River Laboratories. CD45.1+ Fv and Stat6−/− Fv 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 Fv mice to generate GFP+ Fv 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 l’ENS, au PBES et au laboratoire P4.

Peptides

The full agonist NP68 (ASNE:NMDAM) was used to activate Fv CD8 T cells (Prolimmune, Oxford, U.K.). In some experiments, cells were also activated with the partial agonist NP3R (ASREN: MDAM) (Syntem, Lyon, France).

Memory cell generation

Inflammatory memory T cells (TEM) were generated by injecting twice 50 nmol Influenza A nucleoprotein NP66–74 in PBS, i.p., in naïve thymectomized transgenic Fv mice, as previously described (2, 25). Antiviral central memory and effector memory CD8 T cells (TCM and TEM) were generated by immunizing naïve GFP− Fv 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 wk postinfection.

Cell culture and cell sorting

The medium used for CD8 T cell cultures was DMEM supplemented with 6% FCS, 50 μg/ml L-glutamine, 10 mM HEPEs, and 50 μM 2-ME (all from Invitrogen Life Technologies). To measure the effects of IL-4 in vitro, cells were cultured for 20 h at a concentration of 3 × 10^4 cells/ml in presence or absence of murine IL-2 (PeproTech) at a concentration of 10 ng/ml. In some experiments, cells were activated in vitro at a concentration of 1 × 10^5 cells/ml in 96-well plates with 2% IL-2 and the indicated concentrations of NP68 for 24 or 72 h. For quantitative real-time PCR (qPCR) experiments, spleen and lymph node CD8 T cells were purified by magnetic bead depletion, as previously described (27). Purified CD8 T cells were then stained for cell surface expression of CD44, CD122, and CD8 and sorted by flow cytometry using a FACSAria (27). Purified CD8 T cells were then stained for cell surface expression of CD44, CD122, and CD8 and sorted by flow cytometry using a FACSAria (27). Purified CD8 T cells were then stained for cell surface expression of CD44, CD122, and CD8 and sorted by flow cytometry using a FACSAria (27). Purified CD8 T cells were then stained for cell surface expression of CD44, CD122, and CD8 and sorted by flow cytometry using a FACSAria (27).

In vitro production of bone marrow-derived dendritic cells

Briefly, bone marrow-derived dendritic cells (BMDC) were generated, as previously described (28). Bone marrow cells, obtained from C57Bl/6 mouse femora, were resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 10 mM HEPEs, 50 μg/ml gentamicin, and 50 μM 2-ME (all from Invitrogen Life Technologies). Cells (2 × 10^6/ml) were cultured for 7 d in the presence of 0.2 μg/ml recombinant human Flt3 ligand (TEBU) at 37°C. Cells were further matured with LPS (250 ng/ml; Sigma-Aldrich) and pulsed with 300 nM NP68 during an extra 16 h.

In vivo cytotoxic assay

In vivo cytotoxic assay was performed, as previously described (29). Target cells were prepared from C57Bl/6 spleens. Suspensed was divided into two populations, as follows: control cells were not pulsed and labeled with a low concentration of CFSE (2 μM); target cells were pulsed with 1 μM NP68 for 1 h and 30 min at 37°C, washed extensively, and labeled with a high concentration of CFSE (10 μM). Peptide-pulsed CFSEhigh cells and unlabeled CFSElow cells were mixed together in a 1:1 ratio. A total of 2 × 10^7 CFSE-labeled cells was injected (i.p.) into C57Bl/6 mice either previously transfected with TCM cells and immunized with NP68-pulsed BMDM or left untreated. After 20 h, 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 − [ratio primed/ratio unprimed]) × 100.

Administration of cytokines and Abs in vivo

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

mAbs and flow cytometry

All surface stainings were performed at 4°C in PBS (Invitrogen Life Technologies) supplemented with 1% FCS (Lona, Köln, Germany) and 0.05% NaN3 (Sigma-Aldrich). All Abs were from BD Biosciences, eBioscience, and RD Systems, except the anti-mICL5 mouse mAb that was produced in house (30). The F5-TCR was detected with NP68-conjugated H-2Dd allophycocyanin tetramers from Prolimmune. Fixation and permeabilization for intracellular CCL5 detection were performed using CytoFix/CytoPerm from BD Pharmingen. All analyses were performed using a LSRII (BD Biosciences).

RT-qPCR experiments

RNA extraction from naïve CD8 T cells and TEM was performed from 3 × 10^6 cells using RNA NOW reagent (Ozyme) and from 3 × 10^6 antiviral TEM cells using RNasefree Isolation Kit (Qiagen). A total of 100 ng RNA was retrotranscribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad), and cDNA was analyzed 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 follows: Ubiquitin forward, 5′-AACATCATCGACGTGAG-3′ and reverse, 5′-GACCAGTGATGGTTGACACTTT-3′; forward, 5′-CTCTGGCAATGGGAAGTGG-3′ and reverse, 5′-TTGCGCCTGACCAACAGG-3′; Myb forward, 5′-CACTTGATGCTGTGAAGGGGTGTTGACGAT-3′ and reverse, 5′-GCTTCCGGCATGTGGTAAATGGG-3′; and reverse, 5′-CCTACACGACTGACTCTCCC-3′ and reverse, 5′-GGTGGACAGACGACGGAGTT-3′; Ccl5 forward, 5′-AGAACATCGTGAATGTCGAG-3′ and reverse, 5′-AGCATTGTGGCTGTCGTCGAG-3′; and Bcl2 forward, 5′-AAATTACATCGAAGACCCCTT-3′

Microarray processing and determination of the gene expression signatures

mRNAs were extracted from 10^5 sorted naïve or TEM memory CD8 T cells directly ex vivo or after 20 h in vivo culture in presence or absence of 10 ng/ml IL-4 (PeproTech). Culture medium was supplemented with 10 ng/ml IL-7 (PeproTech) to sustain cell viability, mRNAs were extracted using the RNasefree kit (Qiagen). A total of 50 ng RNA was amplified using the MessageAmp II kit (Ambion) and labeled using the IVT labeling kit (Affymetrix). cRNA was 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. Probe sets were filtered according to their expression value; retained probe sets are those with at least one replicate with an expression value >20 percentile of the mean expression data of all the probe sets of the chip. The differential gene expression analysis was carried out using limma on RMA-summated
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 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 (Supplemental Table 1) and the TIM memory cells gene expression signature (Supplemental Table 2). The IL-4 gene expression signature of memory CD8 T cells was obtained using the microarrays performed with the TIM memory CD8 T cells in ex vivo, control (medium signature of memory CD8 T cells was obtained using the microarrays of naive CD8 T cells.

To perform a hierarchical clustering using Euclidean-centered algorithm of probe sets and samples on an independent set of arrays performed with sorted TIM memory CD8 T cells in ex vivo, control (medium) and naive CD8 T cells, the list of probe sets differentially expressed between the IL-4 and the control condition (502 probe sets) and the list of probe sets differentially expressed between the IL-4 and the ex vivo conditions (5506 probe sets) were intersected to obtain a list of 332 common probe sets, having coherent transcriptional variations (upregulated or downregulated in both comparisons). This list of probe sets was summarized to finally propose a list of 245 genes. For summarization, we retained only one probe set per gene, the one having the highest absolute logFC. The TIM memory gene expression signature, composed of 310 genes, is the summarized list of the 402 probe sets differentially expressed between the ex vivo TIM memory CD8 T cells and the ex vivo naive CD8 T cells.

Results

Determination of the IL-4 gene expression signature in TIM memory CD8 T cells

To evaluate the impact of IL-4 on memory CD8 T cell functions, we used TIM 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 TIM either freshly isolated (ex vivo) or incubated in vitro for 20 h in presence or absence of IL-4. The IL-4 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 with ex vivo and with control in vitro incubation, was defined using the analysis procedure described in Materials and Methods. Among the 245 genes defined using this approach, 81 were downregulated and 164 were upregulated. The strength of this signature was demonstrated by its capacity to segregate, by hierarchical clustering, an independent set of arrays performed with TIM memory cells that were treated with IL-4 or not (Fig. 1A). To provide independent validation of the array analysis, we measured, for a subset of genes, changes in gene or protein expression by qPCR or flow cytometry on purified TIM memory CD8 T cells treated with IL-4 (Fig. 1B, 1C). In agreement with array results, Myb, Reck, and Ccl5 gene expressions were strongly inhibited by IL-4, whereas Bcl2 gene expression was upregulated. Likewise, IL-4Rxα, GP49a, and Eomes protein expression were induced by IL-4 (Fig. 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 (Supplemental Table 3), the most significant one being immune response, in particular regulation of multiple cytokines/chemokines/cytokine receptors (Lif, Ltu, Ccl5, il2Rα, il4Rα, il18bp). A number of genes that are involved in CD8 T cell 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 analyze 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 TIM memory cell-associated gene signature (thereafter called memory signature), the gene expression profile of TIM memory CD8 T cells and naive CD8 T cells was compared. A total of 310 genes differed in their expression level between these...
two populations (Supplemental Table 2). We identified 12 genes shared between the IL-4 and the memory signatures (Table I). 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). Downregulation 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 TIM 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 immunizing mice with a recombinant vaccinia virus expressing the NP68 epitope recognized by the F5 TCR (26). Memory cells generated in response to vaccinia virus belong to the TCM or TEM subset, and therefore will be designated hereafter as TCM/TEM. We measured the expression of NKG2D by these memory subsets using flow cytometry. As depicted in Fig. 2A, we observed that NKG2D is expressed by all TCM/TEM and by the majority of TIM.

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 downregulated in all memory subsets following a 20-h incubation with IL-4. Ccl5 and Bcl2 mRNA that are, respectively, downregulated and upregulated by IL-4 in TIM were similarly modified in TCM/TEM (Fig. 2B). Moreover, at the protein level, NKG2D expression by TIM 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 TCM/TEM, suggesting that the 1/2 of the NKG2D protein in these subsets might be longer than in TIM (Fig. 2C).

IL-4 signals through its receptor IL-4Rα and common γc 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 downregulated by IL-4 in a STAT6-dependent manner (6). Thus, we investigated whether STAT6 was also involved in the regulation of Nkg2d expression by IL-4 in TIM. TIM

TABLE I. TIM memory cell-associated genes modulated by IL-4

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene ID</th>
<th>FC IL-4/Control</th>
<th>FC TIM/Naive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Map3k8</td>
<td>26410</td>
<td>6.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Ifit1</td>
<td>15957</td>
<td>5.7</td>
<td>-4.0</td>
</tr>
<tr>
<td>Trat1</td>
<td>77647</td>
<td>5.5</td>
<td>-9.3</td>
</tr>
<tr>
<td>Snip13b</td>
<td>100340</td>
<td>3.3</td>
<td>2.7</td>
</tr>
<tr>
<td>Gsn</td>
<td>227753</td>
<td>3.3</td>
<td>-4.8</td>
</tr>
<tr>
<td>Cdh1</td>
<td>12550</td>
<td>3.1</td>
<td>11.6</td>
</tr>
<tr>
<td>C230098O21Rik</td>
<td>102153</td>
<td>2.0</td>
<td>-2.5</td>
</tr>
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<td>Ars2</td>
<td>121881</td>
<td>-2.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Frat2</td>
<td>212398</td>
<td>-2.2</td>
<td>-2.0</td>
</tr>
<tr>
<td>Ccl5</td>
<td>20304</td>
<td>-3.1</td>
<td>69.8</td>
</tr>
<tr>
<td>Atp1b1</td>
<td>11931</td>
<td>-3.2</td>
<td>-2.5</td>
</tr>
<tr>
<td>Klrk1</td>
<td>27007</td>
<td>-3.5</td>
<td>7.8</td>
</tr>
</tbody>
</table>

To identify the genes shared between the IL-4 gene expression signature and the TIM memory cell-associated gene signature, we performed a Venn diagram between the two signatures. The 14 genes belonging to the TIM memory cell-associated gene expression signature that are modulated by IL-4 are shown with the Entrez gene identification (ID) number and the fold changes observed in the two comparisons performed (a minus symbol is added to indicate a downregulation).

FIGURE 2. IL-4 inhibits NKG2D expression by memory CD8 T cells in vitro. (A–C) TIM memory cells and TCM/TEM memory cells were generated, as described in Materials and Methods, and NKG2D expression was assessed by flow cytometry (A). TIM and TCM/TEM were isolated and incubated in presence or absence of IL-4 for 20 h; total RNA was isolated, and mRNA coding for Nkg2d, Ccl5, and Bcl2 was quantified using RT-qPCR (B), or NKG2D surface expression was measured by flow cytometry (C). (D) TIM memory cells were generated by peptide immunization of thymectomized F5 or F5 × stats−/− mice. TIM were then sorted and incubated 20 h in presence or absence of IL-4. RNA was isolated, and Nkg2d mRNA levels were measured using RT-qPCR. (A and C) Data are representative of at least five experiments (mean ± SEM). (B and D) Mean ± SEM of three independent experiments (*p < 0.05, **p < 0.01, paired Student t test).
memory cells were generated in F5 wild-type (WT) or F5 Stat6−/− mice, and the impact of IL-4 on the level of Nkg2d mRNA was measured. Results in Fig. 2D show that IL-4 was unable to induce the downregulation of Nkg2d mRNA in Stat6−/− 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 in this work was highly enriched in STAT6-regulated genes identified by Wei and colleagues (33) using deep sequencing of promoters bound to immunoprecipitated STAT6 in CD4 T cells (Supplemental Table 4).

![Figure 3](image1.png)

**FIGURE 3.** IL-4C treatment induces innate CD8 T cell generation in the spleen. C57BL/6 mice were daily injected i.p. either with a control IgG1 or a combination of rmIL-4 and anti-IL-4 11B11. After 4–7 d of treatment, spleen was removed and CD8 T cells were analyzed by flow cytometry. (A) CD8 T cell number in the spleen of control or IL-4C-treated mice after 4 or 7 d. (B) CD44 and CXCR3 expression by CD8 T cells from control or IL-4C-treated mice after 4 d. (a) and (b) represent, respectively, the CXCR3+C/CD44hi and the CXCR3−/CD44int populations. (C) Eomes expression by CD8 T cells from control or IL-4C-treated mice after 4 d. (D and E) A total of 3 × 10^6 CFSE-labeled Stat6−/− CD45.2 CD8 T cells and 3 × 10^6 CFSE-labeled WT CD45.1 CD8 T cells was cotransferred into C57BL/6 CD45.1/CD45.2 recipient mice. After 4 d 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–C) Data representative of at least five independent experiments with n = 5 mice (mean ± SEM). (D and E) Mean ± SEM of three independent experiments (n = 4 mice) (**p < 0.01, unpaired Student t test).

![Figure 4](image2.png)

**FIGURE 4.** IL-4–induced innate CD8 T cells do not express NKG2D and CCL5. A total of 3 × 10^6 F5 naive CD45.2 CD8 T cells was transferred into recipient CD45.1/CD45.2 mice that were subsequently daily injected i.p. either with a control IgG1 or a combination of rmIL-4 and anti–IL-4 11B11. After 4 d 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 h with NP68. (C) NKG2D surface and CCL5 intracellular expression by naive F5 CD8 T cells from control or IL-4C–treated mice. (A–C) Mean ± SEM of three independent experiments with n = 4 mice (**p < 0.01, unpaired Student t test).
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 cell 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 rIL-4 has a very short $t_{1/2}$ in vivo, and it has been shown that its association with the anti–IL-4 mAb 11b11 increases both biodisponibility and $t_{1/2}$ 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 d induced CD8 T cell proliferation as well as an accumulation of CXCR3+/CD44int innate CD8 T cells (Fig. 3A, 3B). This was associated with a strong upregulation of Eomes expression (Fig. 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 (Fig. 3D, 3E).

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 (Fig. 4A). As previously shown by others, this was associated with the acquisition of the capacity to produce IFN-γ in response to TCR engagement (Fig. 4B) (20). However, NKG2D and CCL5, two other hallmarks of TCM/TEM memory cells, were not upregulated by innate CD8 T cells (Fig. 4C), indicating that IL-4–induced Eomes upregulation 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

**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-mo BALB/c and C57BL/6 mice. (B and C) NKG2D expression by naturally occurring memory CD8 T cells from BALB/c or C57BL/6 mice of indicated age. (D) Mean fluorescence intensity of NKG2D expression by naturally occurring memory CD8 T cells from BALB/c or C57BL/6 mice. (A and D) Mean ± SEM of three independent experiments with $n = 3$ mice. (B and C) Representative example of two independent experiments with $n = 3$ mice (mean ± SEM) (*$p < 0.05$, **$p < 0.01$, unpaired Student $t$ test).
by constitutive IL-4 production by PLZF-expressing NKT cells and thus are innate CD8 T cells (20). The majority of CXCR3+ CD44int memory-like cells found in BALB/c mice did not express NKG2D and CCL5 in contrast to naturally occurring memory cells found in C57BL/6 mice (Fig. 5B, 5C). 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 (Fig. 5D), suggesting that the increased levels of IL-4 found in BALB/c mice also act 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 cell subsets. F5 TIM 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 TCM/TEM. Mice containing F5 memory CD8 T cells were treated with IL-4C for 4 d; the expression of NKG2D and Eomes was then analyzed. As shown in Fig. 6A, Eomes expression was strongly upregulated, and the mean fluorescence intensity of NKG2D was significantly reduced in both subsets of memory cells (Fig. 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 whether IL-4 could have an impact on memory CD8 T cell functions and NKG2D-dependent CD8 T cell activation, we first analyzed CD8 T cell activation and cytotoxic functions after a treatment with IL-4C in vivo. IL-4C–treated and control TIM memory cells were activated in vitro with the antigenic peptide NP68: as shown in Fig. 7A, the expression of the early activation marker CD69 by IL-4–treated cells was strongly inhibited 24 h 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 h after activation (Fig. 7B), indicating that IL-4 treatment globally impacts memory CD8 T cell activation. We then tested whether 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-h in vivo cytotoxicity assay with NP68 peptide-loaded targets. As shown in Fig. 7C, we found that the CD8 T cell-mediated cytotoxicity observed with the IL-4C–treated TIM cells was strongly reduced compared with control cells (Fig. 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. TIM that had been exposed to IL-4C for 4 d in vivo were stimulated in vitro with the F5-TCR full agonist NP68 or the partial agonist NP3R (Fig. 7D) 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 Fig. 7, all TIM memory cells exposed CD107 after 4-h 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 Ab restored full activation of NP3R-stimulated control TIM, but was unable to induce NKG2D costimulation of IL-4C-treated memory cells (Fig. 7), thus indicating that in vivo IL-4 treatment impaired NKG2D-dependent memory CD8 T cell activation.

Discussion

In the current study, we have evaluated the impact of IL-4 on the gene expression profile of memory CD8 T cells. To evaluate the
The functional impact of IL-4 on one memory CD8 function, we focused on a well-described receptor: NKG2D, which is upregulated by memory CD8 T cells as compared with 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 tumor 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 memory cells were generated, as described in Materials and Methods. A total of 3 × 10⁶ 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 rmIL-4 and anti–IL-4 mAb for 4 d. (A and 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 24 h of stimulation (A), and CD25 expression was assessed after 72 h 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 nonpulsed target cells were injected into the recipient mice, as described in Materials 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 (left histograms) or the partial agonist NP3R (right histograms and bar graph) in presence or absence of the NKG2D-activating Ab A10 (@NKG2D) for 4 h. CD107a expression by stimulated T IM memory cells was then assessed using flow cytometry. (A, B, and D) Data representative of three independent experiments with n = 4 mice (mean ± SEM). (C) Mean ± SEM of two independent experiments with at least n = 3 mice (*p < 0.05, **p < 0.01, unpaired Student t test).
protein in these cells, which 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_{CD8/TEM} after a 4-d treatment with IL-4C (Fig. 6). Using T_{IM} memory cells, we show that in vivo IL-4 contact inhibits memory CD8 T cell 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 cell responses given that a contribution of NKG2D expression in CD8 T cell-mediated protection 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 cell-derived soluble NKG2D ligands (9) or cytokotins: indeed, a number of yc cytokines have been shown to regulate NKG2D expression in several contexts, but these cytokotins come in two flavors, 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, which 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 favor activation of autoreactive T cells. Indeed, NKG2D expression has been associated with the development of 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 d 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 fetal spleen and thymus at 18 wk 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 Cd68, Tob1, or Klf6, are regulated by IL-4. Expression of several genes, Cdh1, Insgh7, Reck, and Gelsolin, coding for proteins controlling cell migration, is 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 IL-18bp, an inhibitor of IL-18 signaling, 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, Klrk2, Cd55, Cd84, Trat1, Ccl5, or Trem2, suggesting that IL-4 could modify the activation threshold of memory CD8 T cells, and we demonstrate that IL-4-induced downregulation 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 cell 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, thymic stromal lymphopoietin produced by epithelial cancer cells can induce the development of Th2 cells that promote tumor 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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Disclosures
The authors have no financial conflicts of interest.

References


**Supplementary Table 1: IL-4 gene expression signature of memory CD8 T cells**

The list of 245 gene symbols defining the IL-4 gene expression signature of memory CD8 T cells is presented here with additional information as the gene description, the Entrez Id and the fold change observed in the two comparisons performed (a minus symbol is added to indicate a down regulation).

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<th>FC IL-4 / Ex-vivo</th>
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**Supplementary Table 2: T\text{IM} memory cells associated gene-expression signature**

The list of 310 gene symbols defining T\text{IM} memory cells associated gene-expression signature is presented here with additional information as the gene description, the Entrez ID and the fold change observed in the two comparisons performed (a minus symbol is added to indicate a down regulation).

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<td>Sh3rf1</td>
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**Note:** Log2FC values are approximations and may not match exactly due to rounding or data representation.
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<th>Gene ID</th>
<th>Description</th>
<th>Symbol</th>
<th>Score</th>
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<tr>
<td>Sbno1</td>
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<td>243272</td>
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<td>Cdc26</td>
<td>cell division cycle 26</td>
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<td>Gcc1</td>
<td>golgi coiled coil 1</td>
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<td>5530601H04Rik</td>
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<td>Wwp1</td>
<td>WW domain containing E3 ubiquitin protein ligase 1</td>
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<td>Dgke</td>
<td>diacylglycerol kinase, epsilon</td>
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<td>Brdt</td>
<td>bromodomain, testis-specific</td>
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<td>ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
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<td>Strn</td>
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<td>Golph3</td>
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**Supplementary Table 3: Functional analysis of the IL4 signature**

Functional annotation and functional analysis of genes forming the IL4 signature were performed with the Babelomics Platform (http://babelomics.bioinfo.cipf.es/) and FatiGO, the Babelomics tool for the enrichment test. The Gene Ontology terms are regrouped in families. In a family of terms, genes appearing in a higher order functional category were not included in the more specific categories. In each list of genes the down-regulated ones are colored in red.

<table>
<thead>
<tr>
<th>GO Id</th>
<th>Go term</th>
<th>Gene names</th>
<th>Adj pval</th>
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<td>GO:0006955</td>
<td>immune response</td>
<td>Bcl2, Ccl5, Cd55, Dbn1, Dhx58, Gadd45g, Gata1, Gbp2, H2-DMa, H2-DMb2, H2-Ob, Il18bp, Il2ra, Il4ra, Irgm1, Kirk1, Lif, Lta, Lyst, Myb, Mylrf, Runx1, Tap1, Tmen173, Trat1</td>
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<td>GO:0006952</td>
<td>defense response</td>
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<td>GO:0002520</td>
<td>immune system development</td>
<td>Bak1, Bcl2, Ccl5, Cdk6, Gadd45g, H2-DMa, Il2ra, Il4ra, Lif, Lta, Myb, Runx1</td>
<td>0.0493782</td>
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<td>GO:0046649</td>
<td>lymphocyte activation</td>
<td>Gata1, Kirk1, Lyst, Treml2</td>
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<td>GO:0007160</td>
<td>cell-matrix adhesion</td>
<td>Actn2, Bcl2, Cdk6, Ddr1, Itgb7, Myb</td>
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<tr>
<td>GO:0006915</td>
<td>apoptosis</td>
<td>App, Bak1, Bcl2, Bik, Casp6, Cdh1, Eya2, F2r, Gadd45g, Gata1, Gsn, Hipk2, Il2ra, Kirk1, Lta, Lyst, Myb, Nr3c1, Prnp, Runx1, Sh3kbp1, Shf, Stk17b, Tmen173, Tnfrsf18, Xaf1</td>
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<td>GO:0007049</td>
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<td>App, Bak1, Bcl2, Bin1, Cdk6, G0s2, Gadd45g, Gata1, Hipk2, Hmg2ob, Katnb1, Lats2, Lif, Lta, Map3k8, Mcm2, Myb, Reck, Runx1, Xaf1</td>
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<td>GO:0045595</td>
<td>regulation of cell differentiation</td>
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<td>GO:0016126</td>
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<td>GO:0051084</td>
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<td>GO:0010035</td>
<td>response to inorganic substance</td>
<td>Bcl2, Casp6, Gata1, Gsn, Haao, Hvcn1, Lef1, Nr3c1, Prnp</td>
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<td>GO:0009966</td>
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<td>App, Cyth1, F2r, Hipk2, Lef1, Lif, Lta, Mbd2, Myb, Ncoa3, Nr3c1, Rasgr1, Sic20a1, Socs1, Socs2, Spata13, Tbc1d4, Tob1, Trat1</td>
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<td>GO:0016044</td>
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<td>Ap1s1, App, Bak1, Bcl2, Bin1, Clcn5, Dbn1, Gata1, Ldralp1, Lrp8, Myb, Myo6, Sh3kbp1</td>
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<td>GO:006897</td>
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<td>GO:0002684</td>
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**Supplementary Table 4: IL-4 gene-expression signature is enriched in genes regulated by STAT6**

*We used data from CD4 study [Lai Wei et al.] to confirm the link between the Stat6 transcription factor and our IL4 signature genes. Chip+ are the genes identified by chip-seq as presenting a binding site for Stat6 transcription factor in their promoters. Chip+ are thus genes directly regulated by Stat6. Reg+ are the genes identified using microarray data as being differentially expressed between WT-Th2 and STAT6KO-Th2 conditions (genes presenting a two-fold increase). Reg+ are thus genes regulated by Stat6 (directly or non-directly). Our IL4 signature is significantly enriched in stat6 bound genes in comparison with a random gene list (Chi2-test, p-value < 2.2e-16).*

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<th>Chip+/Reg+</th>
<th>Chip+/Reg-</th>
<th>Chip-/Reg+</th>
<th>Reg-/Reg-</th>
<th>Chi2-test, p-value</th>
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<tr>
<td><strong>Random 300</strong></td>
<td>5%</td>
<td>11%</td>
<td>16%</td>
<td>68%</td>
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<tr>
<td><strong>IL-4 signature</strong></td>
<td>22%</td>
<td>25%</td>
<td>18%</td>
<td>34%</td>
<td>&lt; 2.2e-16</td>
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