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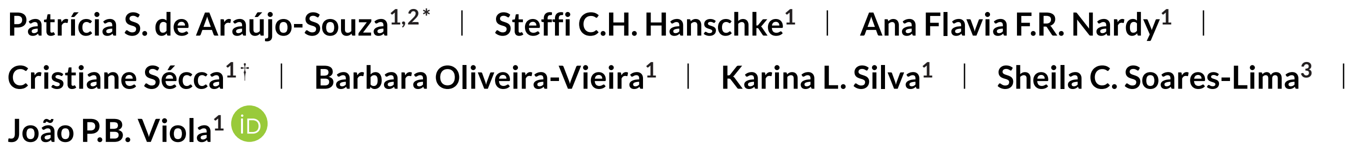
BRIEF CONCLUSIVE REPORT

Differential interferon-γ production by naive and memory-like CD8 T cells



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Abstract

CD8 T cells play a crucial role in immune responses to virus infections and tumors. Naïve CD8 T lymphocytes after TCR stimulation undergo differentiation into CTLs and memory cells, which are essential sources of IFN-γ. We investigated IFN-γ production by CD8 T cell subsets found in non-immune mice. A minor fraction of in vitro TCR-stimulated CD8 T cells produce IFN-γ, and it is regulated at the transcriptional level. Antigen inexperienced C57BL/6 mice present the coexistence of 2 populations. The main population exhibits a CD44^{low}CD122^{low} profile, which is compatible with naïve lymphocytes. The minor expresses a phenotype of immunologic memory, CD44^{hi}CD122^{hi}. Both subsets are able to produce IL-2 in response to TCR activation, but only the memory-like population is responsible for IFN-γ production. Similar to memory CD8 T cells, CD44^{hi}CD8^{+} T cells also present a higher level of the transcriptional factor Eomes and a lower level of T-bet (*Tbx21*) mRNA than CD44^{low}CD8^{+} T cells. The presence of the CD44^{hi}CD8^{+} T cell population in nonimmune OT-I transgenic mice reveals that the population is generated independently of antigenic stimulation. CpG methylation is an efficient epigenetic mechanism for gene silencing. DNA methylation at posttranscriptional CpG sites in the *Ifng* promoter is higher in CD44^{low}CD8^{+} T cells than in CD44^{hi}CD8^{+} T cells. Thus, memory-like CD8 T cells have a distinct epigenetic pattern in the *Ifng* promoter and can rapidly produce IFN-γ in response to TCR stimulation.

KEYWORDS

CD8 T cells, interferon-γ, virtual-memory cells, CpG methylation, antigen-inexperienced memory CD8 T cells

1 | INTRODUCTION

IFN-γ is an essential cytokine for immunity against intracellular pathogens and cancer. Mice lacking IFN-γ responsiveness, due to *Ifng* or *Ifngr1* disruption, show deficiencies in natural resistance to bacterial, parasitic, and viral infections.[1,2] When these mice were challenged with chemical carcinogens, they developed tumors more rapidly and at a higher frequency than wild-type (WT) animals.[3,4]

IFN-γ is produced by cells that mediate both innate and adaptative immune responses. Professional APCs, NK cells, NKT cells, and type 1 and type 3 innate lymphoid cells (ILC1s and ILC3s, respectively) are innate cell sources of this cytokine and rapidly produce IFN-γ upon activation.[5] On the other hand, lymphocytes that mediate adaptative immune responses depend on antigen-induced differentiation to produce large amounts of IFN-γ. Th1 lymphocytes present robust IFN-γ expression, in contrast to naïve CD4 and other Th effector lineage cells. Similar to CD4 lymphocytes, when naïve CD8 T cells are stimulated via their TCRs, they undergo cycles of proliferation and differentiation

Abbreviations: 3′UTR, 3′untranslated region; ARE, AU-rich elements; CD62L, cluster of differentiation 62 ligand; Eomes, Eomesodermin; HPRT, hypoxanthine phosphoribosyltransferase; ILC, innate lymphoid cells; OT-I, MHC class I-restricted OVA-specific CD8 T cells; T-bet, T-box transcription factor 21, T_{cm}; central memory cells, T_{em}, effector memory cells; T_{vm}, virtual memory CD8 T cells; Vα2, T cell receptor variable alpha 2 chain; Vβ5, T cell receptor variable beta 5 chain; WT, wild-type.

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into effector and memory cells. These differentiated cells are capable of producing high levels of IFN-γ in response to TCR activation or IL-12 and IL-18.[6]

CD8 T cells are crucial players in the immune response to tumor and viral infections.[7] Effector cells are short-lived and exhibit cytolytic activities involving perforin- or Fas-related mechanisms to kill target cells. Due to this distinct capacity, these cells are called CTLs. On the other hand, memory cells are long lived. Even after antigen clearance, they maintain a differentiated phenotype to allow a rapid recall to the effector state.[6] Both CTLs and memory cells readily produce IFN-γ faster than naïve cells. The importance of IFN-γ production by CD8 T cells to enhance Th1 differentiation and to control allergic inflammation has been previously shown.[8] Memory T cell compartments include central memory (T_{cm}) cells, which display homing receptors to secondary lymphoid organs ($CD62L^{hi}CCR7^{hi}$) and undergo proliferation as a consequence of antigen encounters. T_{cm} cells are a source of antigen-specific CD8 T cells. In contrast, effector memory (T_{em}) cells ($CD62L^{low}CCR7^{low}$) constitutively display effector functions, such as cytotoxicity, and are found mainly in inflamed sites.[6] Heterogeneity in the CD8 T cell pool from nonimmune individuals has been studied more recently, and the pool is not as homogeneous as previously thought.[9–11] Most of the cells exhibit a remarkably naïve phenotype ($CD44^{low}CD62L^{hi}$) and are not able to rapidly express genes involved in effector functions. In contrast, a minor CD8 T cell population displays phenotypical markers and functional characteristics of memory lymphocytes.[9,10,12] Similar to T_{cm} cells, memory-like CD8 T cells display IL-7 responsiveness and ability to proliferate in response to TCR or cytokine stimuli.[6,9] In addition to the accumulation of memory-like cells with ageing, the cells lose the capacity for TCR-dependent proliferation.[13]

Transcriptional regulation and epigenetic regulation are important in the CD8 T cell differentiation programme.[14] However, the regulation of *Ifng* expression in CD8 T cells has not been well explored. Most of the studies have focused on the molecular regulation of IFN-γ production in the CD4 T cell compartment (reviewed in[15]). There are relatively concordant data concerning the CpG methylation of the *Ifng* promoter in CD8 T cells, but fewer studies have investigated the status of other relevant sources of IFN-γ expression and epigenetic marks, such as histone modifications.[16,17] The interplay between these factors may determine CD8 T cell status. A recent single-cell transcriptional analysis revealed that memory-like CD8 T cells might also be distinguished from the naïve population by their developmental origin: fetal-derived CD8 T cells preferentially become memory-like CD8 T cells in adulthood.[11]

In this report, we identify $CD44^{hi}CD122^{hi}CD8^{+}$ T cells as the primary source of IFN-γ in the CD8 T cell compartment of nonimmune mice upon TCR stimulation. This cytokine production is regulated at the transcriptional level and is independent of previous antigen priming. This memory-like population also presents lower DNA methylation at the +12, +91, and +114 CpG sites in the *Ifng* gene, suggesting that an epigenetic mechanism may contribute to this capacity.

2 | METHODS

2.1 | Mice

C57BL/6 mice were bred and maintained at the Brazilian National Cancer Institute (INCA) animal facility (Rio de Janeiro, RJ, Brazil) in specific pathogen-free conditions. OT-I mice were bred at Isogenic Mouse Facility at the Department of Immunology, University of São Paulo (São Paulo, Brazil) and maintained at INCA's animal facility for experimentation. Male mice at 8–12 week of age were used for all experiments. All animal experiments were performed following the Brazilian Government's ethical and animal experimental regulations. The experiments were approved and conducted according to the animal welfare guidelines of the Ethics Committee of Animal Experimentation from INCA (CEUA process no. 004/13).

2.2 | Cell culture

Primary CD8 T cells were purified by negative selection according to the manufacturer's instructions (Dynal Mouse CD8 Negative Isolation Kit; Invitrogen, Carlsbad, CA, USA) from peripheral lymph node macerates (inguinal, axillary, brachial, and cervical). For all experiments, cell purity was verified by FACS analysis after isolation and always remained superior to 93%. Cells were maintained in supplemented DMEM as previously described.[18] Cell activation was performed by culturing the cells in flat-bottom well plates coated with 0.3 mg/ml anti-IgG (MP Biomedicals, Santa Ana, CA, USA) plus 1 μg/ml anti-CD3 antibodies and 1 μg/ml anti-CD28 antibodies (both BD Pharmingen, San Jose, CA, USA).

2.3 | Flow cytometry and cytokine analysis

Flow cytometry data were acquired on a BD FACSCalibur and analyzed using FlowJo software version 10.5.2 (FlowJo LLC, Ashland, OR, USA). For flow cytometry, all experiments were performed with isolated CD8 T cells, which results in a homogeneous population gated on FSC × SSC. All antibodies (anti-CD44-FITC, anti-CD69-APC, anti-CD25-APC, anti-CD122-PE, anti-CD127-PE, anti-CD62L-PerCP-Cy5, anti-IL-2-PE, anti-CD49d-FITC, and anti-Va2-APC) were purchased from eBioscience (San Diego, CA, USA), except anti-CD8-FITC, anti-IFN-γ-PE, and anti-Vb5-PE, which were purchased from BD Pharmingen. Surface staining was performed according to the manufacturer's instructions. IFN-γ and IL-2 cytokine staining was performed as previously described.[18] The antibodies were added at the final concentration suggested by the manufacturers.

2.4 | Cell sorting

To isolate $CD44^{low}$ and $CD44^{hi}$ populations from naïve $CD8^{+}$ purified T lymphocytes, cells were stained with an anti-CD44-APC-conjugated monoclonal antibody (eBioscience-San Diego, CA, USA) and sorted with a BD FACSMelody system according to their staining intensities. Cells were analyzed for CD44, CD62L, and CD122 expression by flow

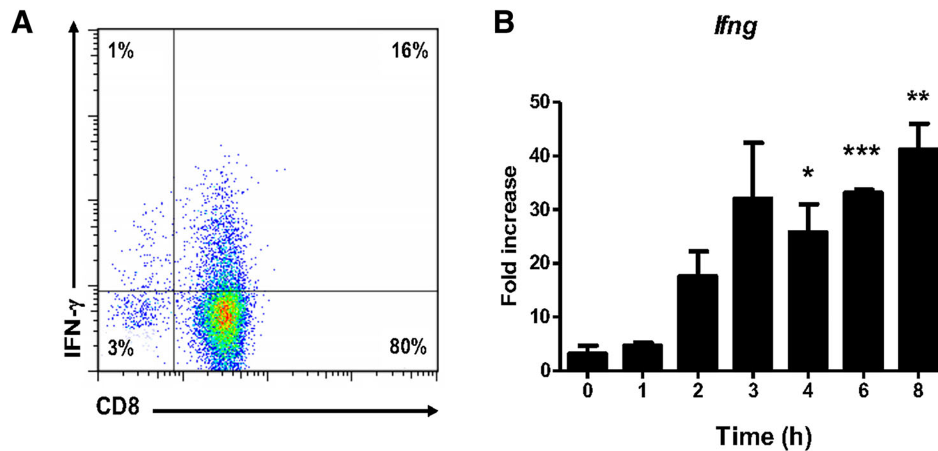

FIGURE 1 IFN-γ production by CD8 T cells. CD8 T cells were purified from lymph nodes from nonimmune C57/BL6 mice by negative selection. (**A**) Purified CD8 T cells were in vitro stimulated with PMA plus ionomycin for 6 h and intracellular production of IFN-γ was evaluated by flow cytometry. These data are representative of 3 independent experiments from a pool of 3 mice. (**B**) The indicated time points refer to purified CD8 T cell activation time in vitro with plate-bound anti-CD3 (1 μg/ml) plus anti-CD28 (1 μg/ml) compared with the lower mean obtained for unstimulated expression (0 h). *Ifng* mRNA fold induction were quantified by qRT-PCR from a pool of 8 mice. Bar graphs represent the mean of 2 independent experiments ± SE ($n = 4$). For relative quantification, the mass of cDNA used for the reaction was normalized using the *Hprt* housekeeping gene. The (*) indicates $P < 0.05$, (**) indicates $P < 0.01$, and (***) indicates $P < 0.0001$ compared with unstimulated

cytometry before (presorting) and after (postsorting) sorting to evaluate the purity obtained.

2.5 | Gene expression analysis

Total cellular RNA isolation was performed according to the manufacturer's instructions. *TRIzol®* (Invitrogen) was used for total CD8 T cells, and a RNeasy mini kit (Qiagen, Hilden, Germany) was used for CD44^{low} and CD44^{hi} CD8 T cells. After first-strand cDNA synthesis with oligo(dT)-primer (Superscript^{TM} III Reverse Transcriptase; Invitrogen), relative quantification of *Ifng*, *Eomes*, and *Tbx21* (T-bet) transcripts was performed by real-time PCR using TaqMan^{TM} Gene Expression Assay kits (*Ifng:* Mm00801778_m1, *Eomes:* Mm01351985_m1, *Tbet:* Mm00450960_m1, *Hprt*: Mm00446968_m1; Applied Biosystems, Carlsbad, CA, USA). Amplification was performed in the 7500 Real-Time PCR System (Applied Biosciences). Relative expression levels were normalized to endogenous reference *Hprt* using the $2^{-\Delta\Delta CT}$ method. The expression values were normalized to the values of unstimulated cells. All procedures were performed according to the manufacturers' suggested protocols.

2.6 | Bisulfite modification, PCR, and pyrosequencing

Genomic DNA was obtained by precipitation after Proteinase K digestion of cells (1.5×10^6). DNA samples (0.5–1 μg) were treated with the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol and modified DNA samples were stored at −20°C. DNA amplification with cIFNG1 (TAT ATT ATA AGG GTA AAA AGG GGG AGA) and cIFNG3-biotin (CCA TAA AAA AAA ACT ACA AAA CCA AAA TAC AAT A) primers was performed, and the PCR product was partially analyzed on an agarose gel. The remaining volume was used in a pyrosequencing assay using sequencing primers (Seq1: GTATAGGTGGGTATAG; Seq2: AAAAATTTGTGAAAAT; Seq3: TAGGTTATAGTTGTTA; and Seq4: TGGGTTTTTTTTTTTG). Pyrosequencing reactions were set up using the PyroGold Reagent kit (Biotage, Uppsala, Sweden) and a pyrosequencing apparatus (Qiagen; PyroMark Q96ID), according to the manufacturers' instructions. All cytosines outside CpG sites were mutated to thymidines, indicating that the bisulfite treatment was efficient. The methylation levels at the target CpGs were evaluated by converting the resulting pyrograms into numerical values according to peak height and expressed as the percentage of methylation at individual CpG sites.

2.7 | Statistical analysis

Statistical analysis for comparison of groups was performed using an unpaired Student's *t*-test for single comparisons using the programme GraphPad Prism (GraphPad, San Diego, CA, USA). *P* values less than or equal to 0.05 were considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | IFN-γ production by CD8 T cells upon TCR activation is accompanied by *Ifng* mRNA up-regulation

CD8 T cells are an essential source of IFN-γ in immune responses. To evaluate this production in response to TCR activation, we stimulated CD8 T cells from the lymph nodes of naïve mice and assessed intracellular IFN-γ staining. In a period of 6 h, approximately 16% of CD8 T cells produce IFN-γ (Fig. 1A). This IFN-γ production could have resulted from an increase in *Ifng* mRNA but also from post-transcriptional mechanisms.[19] The influence of AU-rich elements (AREs) within the 3′

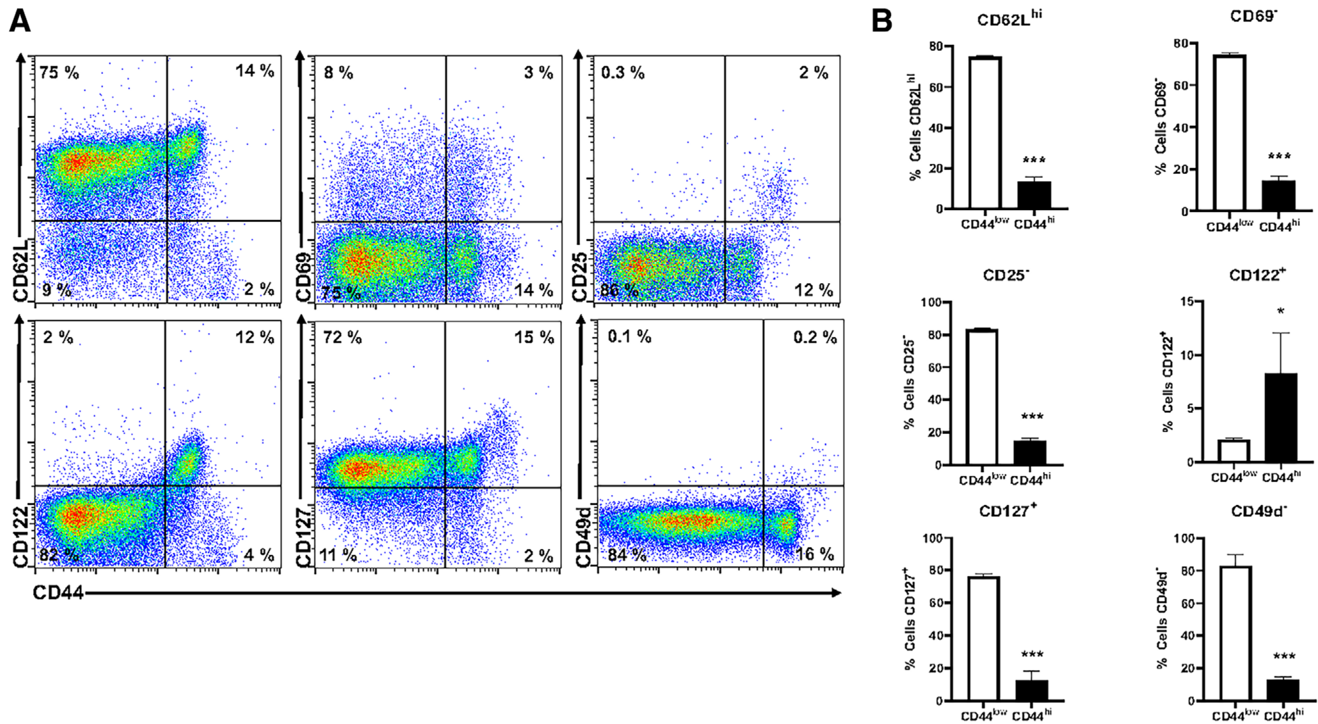

FIGURE 2 **Expression of surface markers in CD8 T cells.** CD8 T cells were purified from lymph nodes from nonimmune male C57/BL6 mice by negative selection. (**A**) Unstimulated purified CD8 T cells were analyzed for CD44, CD62L, CD69, CD25, CD122, CD127, and CD49d expression by flow cytometry. These data are representative of 3 independent experiments from a pool of 6 mice. (**B**) Percentage of CD62L^{hi}, CD69^{-}, CD25^{-}, CD122^{+}, CD127^{+}, and CD49d^{-} comparing CD44^{low} and CD44^{hi} CD8 T cell populations. Unstimulated purified CD8 T cells were gated as CD44^{low} and CD44^{hi} for analysis from a pool of 6 mice. Bar graphs represent the mean of 3 independent experiments ± SE ($n = 3$). The (*) indicates $P < 0.05$, and (***) indicates $P < 0.0001$ compared with CD44^{low} cells

untranslated region (3′UTR) on *Ifng* mRNA stability has already been shown.[20] To evaluate whether IFN-γ production results from *Ifng* gene up-regulation, we assessed *Ifng* mRNA levels from 0 to 8 h after in vitro TCR stimulation. The RT-qPCR analysis revealed that *Ifng* was rapidly induced upon TCR stimulation, with 20- and 40-fold increases observed after 2 and 6 h of stimulation, respectively (Fig. 1B). These data indicate that, in this case, IFN-γ production is, at least in part, regulated at the transcriptional level.

3.2 | Unprimed memory-like population produces IFN-γ upon TCR activation

We then characterized the surface markers found on CD8 T cell subsets from nonimmune mice (Fig. 2). We assessed the CD44 status of purified CD8 T cells and observed a predominant CD44^{low} population. It is expected for naïve T cells, because CD44 is an adhesion molecule up-regulated after TCR activation involved in T cell homing to peripheral tissues. But we also found that approximately 15% of CD8 T cells were CD44^{hi}, similar to conventional memory cells (Fig. 2). Individual unbiased cytometry analysis of other markers, associated with recirculation and access to secondary lymphoid organs, revealed that both CD44^{low} and CD44^{hi} populations are similar CD62L^{hi}, CD69^{-}, CD25^{-}, CD127^{+}, and CD49d^{-} (Fig. 2), but in contrast to CD44^{low} population, which is also CD122^{-}, most of the CD44^{hi} cells are CD122^{+} (Fig. 2).

To compare the IFN-γ production in CD8 T cell subpopulations, intracellular cytokine staining after in vitro TCR stimulation was performed in whole CD8 T cells (Fig. 3A). In fact, CD44^{hi}CD8^{+} T cell produces IFN-γ, while both CD44^{low} and CD44^{hi} produce IL-2 (Fig. 3A). Our hypothesis is that CD44^{hi} cells produce higher IFN-γ than CD44^{low} population. But as T cells rapidly up-regulate CD44 after TCR activation, it would be also possible that CD44^{low} CD8 T cells became CD44^{hi} and then produces IFN-γ. To exclude this possibility, in Fig. 3C, we have first sorted CD8 T cells according to CD44 expression and then stimulated them, demonstrating that CD44^{hi}CD8^{+} T cell produces IFN-γ (Fig. 3B and 3C). We can conclude that CD8 T cells that were already CD44^{hi} in mice were the responsible for IFN-γ production. On the other hand, both CD44^{low} and CD44^{hi} populations can account for IL-2 production (Fig. 3B and 3C). These data suggest that differential cytokine production is restricted to IFN-γ, which is related to the effector response.

We also analyzed *Ifng* mRNA levels in CD44^{hi} and CD44^{low} sorted cells (Fig. 4A) after in vitro TCR stimulation. *Ifng* expression was more than 150× higher in CD44^{hi} CD8^{+} T cells than in CD44^{low} CD8^{+} T cells. Considering this difference in *Ifng* mRNA between memory-like (CD44^{hi}) and naïve (CD44^{low}) populations, we investigated whether Eomes and T-bet could be differentially involved in the regulation of *Ifng* expression in CD8 T cell subsets (Fig. 4B). Eomes and T-bet are crucial transcription factors involved in *Ifng* expression and play fundamental roles during CD8 T cell differentiation.[21] T-bet is associated

FIGURE 3 **IFN-γ and IL-2 production by CD8 T cells subpopulations.** CD8 T cells were purified from lymph nodes from nonimmune C57/BL6 male mice by negative selection. (**A**) Purified CD8 T cells were in vitro stimulated with PMA plus ionomycin for 6 h. After stimulation, intracellular production of IFN-γ and IL-2 were evaluated in CD44^{low} and CD44^{hi} by flow cytometry. These data are representative of 2 independent experiments from a pool of 3 mice. (**B**) Unstimulated purified CD8 T cells were sorted according to CD44 fluorescent-labeled antibody staining. After sorting, CD44^{low} and CD44^{hi} subpopulations were stimulated as in A and analyzed for intracellular production of IFN-γ and IL-2 cytokines by flow cytometry. These data are representative of 2 independent experiments from a pool of 6 mice. (**C**) Percentage of cells producing IFN-γ and IL-2 sorted CD44^{hi} and CD44^{lo} subpopulations. Bar graphs represent the mean of 2 independent experiments ± SE

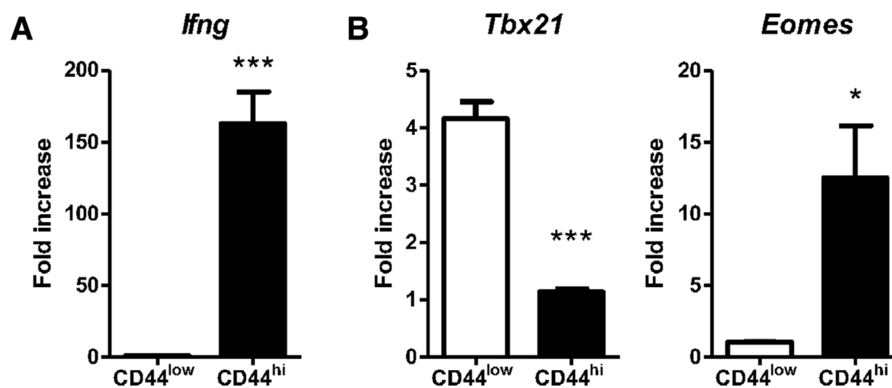

FIGURE 4 **Different mRNA levels in CD8 T cells CD44^{low} and CD44^{hi} cells.** CD8 T cells were purified from lymph nodes from nonimmune C57/BL6 male mice by negative selection sorted according to CD44 fluorescent-labeled antibody staining as described in Fig. 3. Then, cells were in vitro stimulated with PMA plus ionomycin for 4 h and total mRNA was extracted. *Ifng* (**A**), T-bet (*Tbx21*) and *Eomes* (**B**) mRNA fold induction were quantified by qRT-PCR from a pull of 3 mice. The data refer to purified CD8 T cell compared with the lower mean obtained for CD44^{low} (*Ifng* and *Eomes*) or CD44^{hi} (*Tbx21*) expression. Bar graphs represent the mean of 2 independent experiments ± SE ($n = 4$). For relative quantification, the mass of cDNA used for the reaction was normalized using the *Hprt* housekeeping gene. The (*) indicates $P < 0.05$, and (***) indicates $P < 0.0001$ compared with CD44^{low} cells

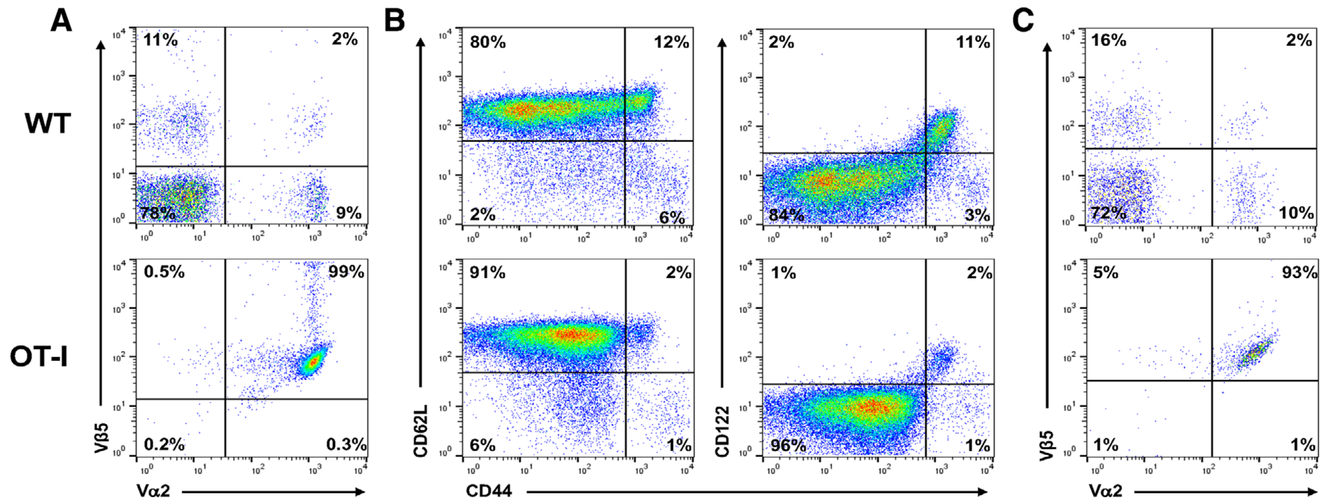

FIGURE 5 **T CD8 CD44^{hi} cells in OT-I TCR transgenic mice.** CD8 T cells were purified from lymph nodes from nonimmune C57/BL6 (WT) and OT-I TCR transgenic male mice by negative selection. Unstimulated purified CD8 T cells were analyzed for (**A**) Vα2 and Vβ5, (**B**) CD44, CD62L, and CD122 expression by flow cytometry. (**C**) Unstimulated purified CD8 T cells were analyzed for Vα2 and Vβ5 gated in CD44^{hi}CD62L^{hi} T cells. These data are representative of 3 independent experiments ($n = 3$)

with an effector phenotype, whereas Eomes expression is increased in central memory[21] and memory-like CD8 T cells.[22,23] CD44^{hi} CD8^{+} T cells presented higher level of *Eomes* mRNA than CD44^{low} CD8^{+} T cells. Previous results support the importance of *Eomes* expression in memory-like CD8 T cells. Eomes is required for virtual memory cell (T_{vm}) development since this population is not found in IL-15- or Eomes-deficient mice, but it does occur in T-bet-deficient mice.[22] CD122 expression in T_{vm} cells was also shown to be Eomes dependent.[22] Martinet et al.[24] also showed that the type I IFN signaling pathway led to the activation of Eomes and a reduction in the memory-like cell pool in IFNRA-deficient mice. It is possible that innate stimuli, such as type I IFNs, favor the expansion of T_{vm} cells, which are superior to naïve cells in playing an effector role when conventional memory cells are not available. In contrast, we observed that upon TCR stimulation, CD44^{hi}CD8^{+} T cells presented lower levels of T-bet (*Tbx21*) mRNA than CD44^{low}CD8^{+} T cells (Fig. 4). Previous studies which performed RT-PCR ex-vivo analysis observed higher T-bet detection in splenic T_{vm} cells than in naïve counterparts.[22,23] It has been suggested that the presence of T-bet in this cell type might explain the antigen-independent IFN-γ production.[12] Previous studies reported that, like conventional memory cells, T_{vm} cells could secrete IFN-γ in response to IL-12 and IL-18 stimuli.[9,24] The plasticity of this subset to produce IFN-γ may be an advantage in the initial response against infection. Although we just assessed Tbet (and Eomes) expression after in vitro TCR stimulation, it is possible that *Tbx21* expression may be modulated by TCR stimulus.

3.3 | Memory-like CD8 T cells are found in nonimmune OT-I transgenic mice

The existence of memory-like CD8 T cell populations was initially assumed to result from T cell responses to gut microbiota or exposure to unrelated pathogens.[12] It was probably due to the similarity of some surface markers between memory-like and conventional memory (antigen-experienced) CD8 T cell populations, such as the CD44^{hi} and CD122^{+}. To verify whether this memory-like population can be found in the absence of antigenic stimulation, we compared purified CD8 T cells from OT-I transgenic mice with those from C57BL/6 WT mice. OT-I transgenic mice present MHC class I-restricted, OVA-specific CD8^{+} T cells due to Tcra-V2 and Tcrb-V5 gene insertions.[25] Figure 5A shows that all the CD8 T cells from OT-I mice were Vβ5 and Vα2, whereas only a minor fraction (2%) of the CD8 T cells from the WT mice expressed Vβ5 and Vα2 TCRs. Unbiased flow cytometry analysis (Fig. 5B) revealed the existence of CD44^{hi}CD122^{+}CD8^{+} T cell population in unstimulated OT-I transgenic mice, which presents the same TCR Vα and Vβ profile as naïve CD8 T cells (Fig. 5C). These data suggest that memory-like CD8 T cell development is not dependent on antigen recognition. It agrees with data showing the presence of a memory-like population in models from homeostatic proliferation in pathogen-free mice and germ-free mice, and even mice fed with an elemental diet free of potential food antigens.[9,12] Our results also support the previous data that memory-like populations arise from precursors with an unrestricted TCR repertoire.[9,24] Recently, it was described that T_{vm} cells might possess a TCR repertoire with increased self-reactivity.[26] It is possible that in a diverse TCR repertoire pool, the antigen affinity favors memory-like development, but our data support that it is not necessary for memory-like T cell development. Taken together, these data suggest that the memory-like CD8 T cell population occurs independently of antigenic stimulation and may depend on cytokine stimuli.

3.4 | Memory-like CD8 T cell population exhibits less CpG methylation at the *Ifng* promoter than naïve cells

During CD8 T cell differentiation, large-scale changes occur at the transcriptional level that coordinate the expression of genes associated with effector function, survival, and self-renewal.[21] *Ifng*

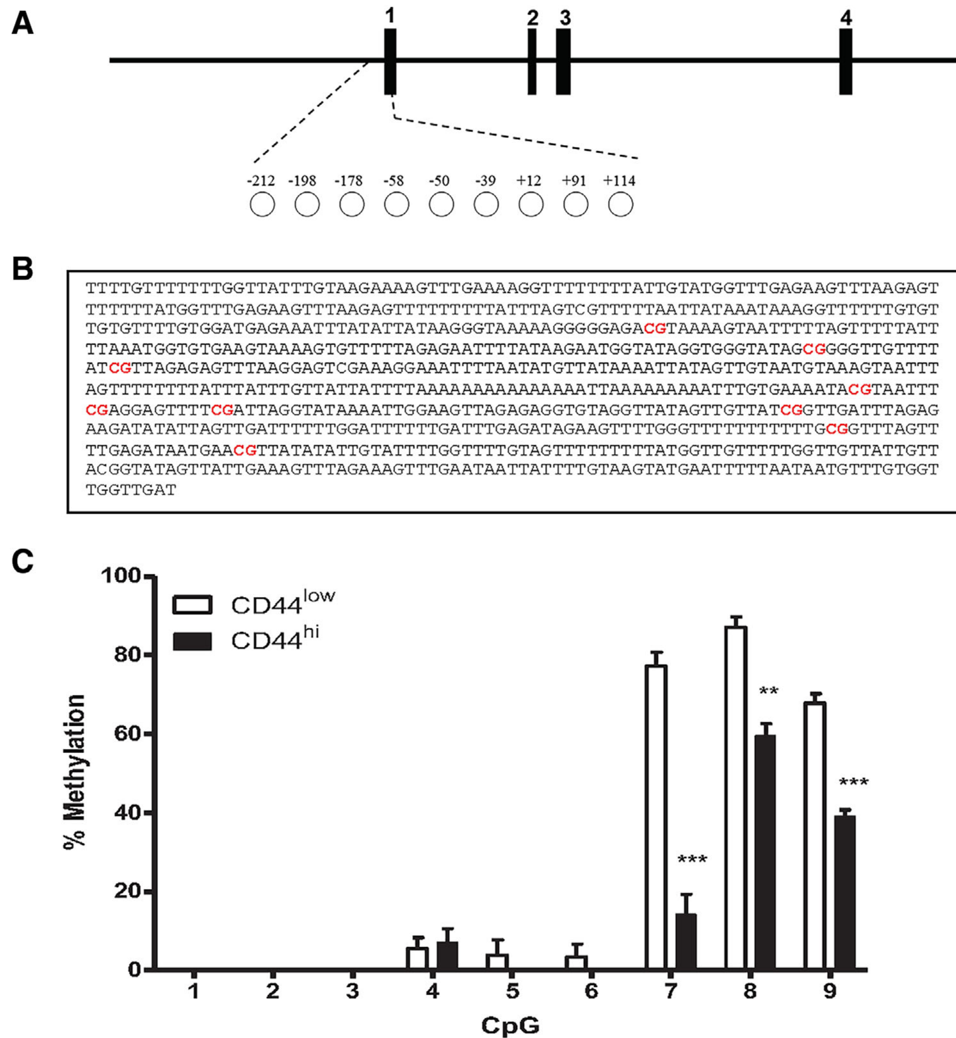

FIGURE 6 **CpG methylation of the *Ifng* gene promoter.** (**A**) Scheme of the mouse *Ifng* locus. Exons are shown as black boxes. CpG sites located at the *Ifng* promoter are indicated. The numbers correspond to their distance relative to the transcription start site (+1) of the murine *Ifng* (**B**) DNA sequence of murine *Ifng* promoter with CpG sites highlighted (red). (**C**) CD8 T cells were purified from lymph nodes from nonimmune C57/BL6 mice by negative selection and sorted according to CD44 fluorescent-labeled antibody staining as described in Fig. 3. Genomic DNA was extracted and modified with sodium bisulfite, followed by PCR using specific primers for murine *Ifng* promoter and submitted to pyrosequencing to evaluate CpG Methylation. These data are representative of 3 independent experiments from a pool of 6 mice. Bar graphs represent the mean of 3 independent experiments ± SE ($n = 3$). The (**) indicates $P < 0.01$, and (***) indicates $P < 0.0001$ compared with CD44^{low} cells

expression can be regulated by the selective actions of transcriptional factors and by epigenetic mechanisms.[27] DNA methylation of CpG sites within the *Ifng* promoter was previously shown to be associated with the ability to produce IFN-γ in CD8 T lymphocytes.[28] Effector cells had a completely unmethylated promoter,[28,29] whereas posttranscriptional CpG sites were significantly methylated in naïve cells.[28] In contrast, conventional memory cells were partially methylated at posttranscriptional CpG sites. CpG methylation sites in this population is more evenly distributed throughout *Ifng* promoter.[28] We then evaluated the methylation state of 9 CpG sites surrounding the *Ifng* transcriptional start site (Figs. 6A and 6B) in memory-like and naïve CD8 T cells. CpG sites located at the untranscribed region of the *Ifng* promoter (positioned at −212, −198, −178, −58, −50, and −38) were unmethylated in both CD8 T cells analyzed, similar to that observed by Winders et al.[30] Nevertheless, we found that the +12 CpG site was methylated in approximately 80% of CD44^{low} CD8^{+} T cells, whereas less than 20% of the methylation at this site was found in CD44^{hi}CD8^{+} T cells (Fig. 6C). Sites + 91 and +114 also differed between CD8 T cell subsets analyzed. We identified higher IFN-γ production in memory-like CD44^{hi} CD8^{+} T cells than in CD44^{low} CD8^{+} T cells, which presented a lower CpG methylation level at the +12, +91, and +114 posttranscriptional sites. This difference was not previously found when CpG methylation at the *Ifng* promoter was compared between CD44^{hi} and CD44^{low} CD8 T cell clones,[31] because the fragment analyzed did not include +91 and +114 CpGs. In fact, consistent unmethylation throughout the *Ifng* promoter was observed in CD44^{hi} CD8 T cell clones, but CD44^{low} clones exhibited more variability in IFN-γ gene methylation, several clones presented nearly all CpG methylated.[29] One possibility is that these differences may be attributed to the establishment of clones.

In summary, we characterized a population of CD8 T cells from unmanipulated C57BL/6 mice able to produce IFN-γ in response to TCR activation faster than naïve cells. This memory-like population that shares functional properties with antigen-primed memory CD8 T cells in the naïve pool of antigen-inexperienced mice may confer advantages in the initial response against infection. We also found a lower methylation in posttranscriptional CpG sites of *Ifng* promoter in memory-like than naïve cells. These data support the hypothesis that differential CpG methylation of posttranscriptional sites in the *Ifng* promoter may play a role in mRNA expression in memory-like cells.

AUTHORSHIP

P.S.A.S. performed experiments, analyzed the data, contributed to experimental design, and drafted the manuscript. S.C.H.H. performed most of the experiments, analyzed the data, and participated in the discussions. C.S. helped with experiments and participated in the discussions. A.F.F.R.N., B.M.V., and S.L. performed experiments of pyrosequencing. K.L.S. performed the cell sorting. J.P.B.V. conceived the study design and supervised the study.

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DISCLOSURES

The authors declare no conflicts of interest.

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