Active STAT5 Regulates T-bet and Eomesodermin Expression in CD8 T Cells and Imprints a T-bet–Dependent Tc1 Program with Repressed IL-6/TGF-β1 Signaling

Magali Grange, Grégory Verdeil, Fanny Arnoux, Aurélien Griffon, Salvatore Spicuglia, Julien Maurizio, Michel Buferne, Anne-Marie Schmitt-Verhulst and Nathalie Auphan-Anézin

*J Immunol* 2013; 191:3712-3724; Prepublished online 4 September 2013;
doi: 10.4049/jimmunol.1300319
http://www.jimmunol.org/content/191/7/3712
Active STAT5 Regulates T-bet and Eomesodermin Expression in CD8 T Cells and Imprints a T-bet–Dependent Tc1 Program with Repressed IL-6/TGF-β1 Signaling

Magali Grange,*†‡ Grégory Verdeil,*†‡ Fanny Arnoux,*†‡ Aurélien Griffon,* Julien Maurizio,§ Michel Buterne,*†‡ Anne-Marie Schmitt-Verhulst,*†‡ and Nathalie Auphan-Anezin*†‡

In adoptive therapy, CD8 T cells expressing active STAT5 (STAT5CA) transcription factors were found to be superior to unmanipulated counterparts in long-term persistence, capacity to infiltrate autochthonous mouse melanomas, thrive in their microenvironment, and induce their regression. However, the molecular mechanisms sustaining these properties were undefined. In this study, we report that STAT5CA induced sustained expression of genes controlling tissue homing, cytolytic granule composition, type 1 CD8 cytotoxic T cell–associated effector molecules granzyme B+, IFN-γ+, TNF-α+, and CCL3+, but not IL-2, and transcription factors T-bet and eomesodermin (Eomes). Chromatin immunoprecipitation sequencing analyses identified the genes possessing regulatory regions to which STAT5 bound in long-term in vivo maintained STAT5CA-expressing CD8 T cells. This analysis identified 34% of the genes differentially expressed between STAT5CA-expressing and nonexpressing effector T cells as direct STAT5CA target genes, including those encoding T-bet, Eomes, and granzyme B. Additionally, genes encoding the IL-6R and TGFβRII subunits were stably repressed, resulting in dampened IL-17–producing CD8 T cell polarization in response to IL-6 and TGF-β1. The absence of T-bet did not affect STAT5CA-driven accumulation of the T cells in tissue or their granzyme B expression but restored IL-2 secretion and IL-6R and TGFβRII expression and signaling, as illustrated by IL-17 induction. Therefore, concerted STAT5/T-bet/Eomes regulation controls homing, long-term maintenance, recall responses, and resistance to polarization towards IL-17–producing CD8 T cells while maintaining expression of an efficient type 1 CD8 cytotoxic T cell program (granzyme B+, IFN-γ+).


The differentiation of naive CD8 T cells into effector T cells (eTCs) and memory T cells is accompanied by changes in expression of genes associated with their migration, survival, self-renewal, and effector functions (1–3). Two subsets of memory T cells, effector and central memory T cells, have been characterized (4, 5). These have distinct proliferative potential, tissue homing, and functions. Gene expression profiles associated with these distinct functional subsets revealed the regulation of memory cell fate by several transcription factors (TFs) (3). Expression of two T-box family members T-bet and eomesodermin (Eomes) has been correlated with differentiation of short-lived eTCs and long-lived central memory T cells, respectively (6, 7). The antagonistic transcriptional repressors Blimp-1 and Bcl6 have also been identified as key transcriptional regulators, promoting effector and central memory T cells, respectively (8).

The cytokine receptors sharing the common γ-chain mediate activation of the JAK kinases and participate in activation of the STAT5 TF. IL-7 is involved in the maintenance of naive T cells, whereas IL-21, IL-15, and IL-2 have been shown to be important for the development and maintenance of memory stem cells, central memory T cells, and effector memory T cells, respectively (9, 10). The three memory T cell subsets demonstrate decreasing multipotency and replicative potential while acquiring increasing effector functions and the ability to home to nonlymphoid tissues. Molecular mechanisms by which STAT5 translates the effects of IL-21, IL-15, and IL-2 in defining the replicative potential and acquisition of effector functions of memory CD8 T cells remain to

*Centre d’Immunologie de Marseille–Luminy, Aix-Marseille Université, Marseille 13288, France; †INSERM Unité Mixte de Recherche 1104, Marseille 13288, France; ‡INSERM Unité de Recherche Scientifique, Unité Mixte de Recherche 7280, Marseille 13288, France; and †Technological Advances for Genomics and Clinics, INSERM Unité Mixte de Recherche 1090, Marseille 13288, France.

1M.G. and G.V. contributed equally to this work.

Received for publication February 4, 2013. Accepted for publication July 23, 2013.

This work was supported by funding from INSERM and the Centre National de la Recherche Scientifique, as well as by grants from the Association pour la Recherche sur le Cancer (to N.A.-A. and A.-M.S.-V.), the Agence Nationale de la Recherche (to A.-M.S.-V.), and the Institut National du Cancer (to A.-M.S.-V.). M.G. was the recipient of a doctoral fellowship from Association pour la Recherche sur le Cancer (to N.A.-A. and A.-M.S.-V.), the Agence Nationale de la Recherche (to A.-M.S.-V.), and G.V. held a doctoral fellowship from the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE41819) under accession number GSE41819.

Address correspondence and reprint requests to Dr. Nathalie Auphan-Anezin, Centre d’Immunologie de Marseille–Luminy, Parc Scientifique de Luminy, Case 906, 13288 Marseille Cedex 09, France. E-mail address: auphan@cimid.univ-mrs.fr

The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; ChIP-Seq, chromatin immunoprecipitation sequencing; Eomes, eomesodermin; eTC, effector T cell; GSEA, gene set enrichment analysis; GzmB, granzyme B; HPRT, hypoxanthine phosphoribosyltransferase; MFI, mean fluorescence intensity; Tc1, type 1 CD8 cytotoxic T cell; Tc17, IL-17–producing CD8 T cell; TF, transcription factor; TSS, transcription start site; wt, wild-type.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/S16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1300319
be determined. Recent studies reporting antagonistic function of STAT5 and Bcl6 (11) and STAT5-mediated regulation of T-bet expression (12) suggest that STAT5 may also be a key TF involved in effector and memory T cell fates.

Adaptive therapies for cancer patients based on the transfer of tumor-specific T cells have shown some success in treatment of melanoma (13) and lymphoma patients (14). However, those approaches are limited by poor survival of the transferred T cells, their limited replicative potential and capacity to infiltrate solid tumors, and their sensitivity to tumor-derived immunosuppression.

Based on the properties of the three memory CD8 T cell subsets described above, several groups have concluded that in vitro-expanded CD8 eTCs are not the best subset for adoptive therapy (15), but that less differentiated CD8 T cells might be more appropriate (16). Inusions of cytokines such as IL-2 are currently in patients to increase survival and cytolytic activity of transferred anti-tumor CD8 T cells. However, IL-2 treatments have adverse effects on patients, notably through induction of vascular leak syndrome (17). IL-2 may also activate suppressive T regulatory lymphocytes. Therefore, strategies aimed at mimicking the costimulatory effects of IL-2 while bypassing cytokine-mediated untoward effects are of interest.

Among immunosuppressive factors in the tumor microenvironment, IL-10, IL-6, and vascular endothelial growth factor, which activate STAT3, are crucial for both tumor growth and immunosuppression or T cell polarization (18), creating a “feed-forward” increased STAT3 activity both in tumor cells and in tumor-associated immune cells. In particular, aberrant IL-6/JAK/STAT3 signaling promotes inhibition of Th1 immune responses (19). For these reasons, developing CD8 effector T cells able to counteract the tumor stromal barrier and tumor-derived immunosuppression or aberrant polarization is an important challenge.

We recently reported that expression of an active form of STAT5 (STAT5CA) in CD8 T cells favors their differentiation into long-lived eTCs, manifesting phenotypic and tissue-homing properties characteristic of effector memory T cells while maintaining the high potential for Ag recall responses associated with central memory T cells. Similar characteristics were acquired whether STAT5CA-expressing cells were transferred in immunodeficient (Rag2−/−) or immunocompetent hosts (20). Of note, these properties greatly enhanced the survival and secondary responses of STAT5CA-expressing eTCs when they were transferred into tumor-bearing hosts. As compared with unmanipulated CD8 eTCs, STAT5CA-expressing eTCs very efficiently infiltrated autochthonous melanomas, maintained a type 1 CD8 cytotoxic T cell (Tc1) effector program within the tumor, and induced tumor regression (20).

To determine the molecular bases sustaining those functional properties, in this study we performed the transcriptional analysis of STAT5CA-expressing eTCs. This study reveals a long-lasting increase in the expression of both T-bet and Eomes expression. Chromatin immunoprecipitation sequencing (ChIP-Seq) analyses further indicate that T-bet and Eomes are direct STAT5 target genes. STAT5CA also dampens expression of subunits involved in IL-6 and TGF-β1–induced signaling, conferring resistance to those polarizing/immunosuppressive cytokines. Additionally, we report new insights on the interplay between STAT5 and T-bet, delineating the plasticity of effector CD8 T cell programs and their sensitivity to immunosuppression.

**Materials and Methods**

**Mouse**

Mice heterozygous for the H-2Ld/P1A35–43–specific TCR transgene (TCRP1A) (21) were kept on the Rag1−/−B10.D2 background. T-bet−/− mice on a C57BL/6 background (http://jaxmice.jax.org/strain/004648. html) were initially purchased from The Jackson Laboratory. Rag1−/−B10.D2 and Rag2−/− B6 mice were also used. All of these mice were bred in the Centre d’Immunologie de Marseille–Luminy animal facility. Animal experiments respected French and European directives.

**Cell preparation**

CD8 T cells were prepared from lymph nodes or spleen of TCRP1A Rag1−/− mice according to standard procedures. When prepared from immunocompetent B10.D2 mice, CD8 T cells were enriched using a mouse CD8 negative selection kit (Dynal/Invitrogen) according to the manufacturer’s instructions. For analysis of liver-infiltrating T cells, donor mice were anesthetized and perfused with PBS. Livers were dispersed into a single-cell suspension and passed over Ficoll-Paque solution (Amersham Biosciences).

**CD8 T cell activation and retroviral injections**

TCRP1A T cells were activated for 72 h with 10−7 M P1A35–43 (LPYLGWLVF) peptide. Polyclonal CD8 T cells were activated with coated anti-CD3 mAb (145.2C.11, 3 µg/ml) and soluble anti-CD28 mAb (37.51, 1 µg/ml). Twenty (for TCR transgenic T cells) or 40 h (for polyclonal T cells) after initial stimulation, CD8 T cells were retrovirally transduced as previously described (20). Cultures were then continued for 48 h. The retroviral transduction efficiency was ~58% for TCRP1A eTCs-STAT5CA, 31% for TCRP1A eTCs-GFP, and 6% for polyclonal CD8 eTCs-STAT5CA or eTCs-GFP. Those eTC populations were either analyzed directly or adoptively transferred in Rag1−/− syngeneic mice. In some experiments, untransduced eTCs are included as controls instead of eTCs-GFP, as we previously showed that those two T cell subsets had the same behavior upon adoptive transfer into syngeneic mice (20).

**Tumor cell lines**

P1A+ mastomastcytoma P815 and its P1A− variant P1.204 are used as stimulating cells (20). Fas-mediated cytotoxicity was measured against the leukemia L1210 expressing or not Fas.

**In vitro stimulation with immunosuppressive/polarizing cytokines**

eTCs-STAT5CA or eTCs adoptively transferred in syngeneic Rag-deficient mice were recovered from recipients’ pooled lymph nodes and spleens. CD8 T cells from B10.D2 mice were included as control. Cells (106) were stimulated by coated anti-CD3 mAb (and soluble anti-CD28 mAb for naive CD8 T cells). Cultures were conducted in the absence or presence of TGF-β1 (R&D Systems; 5 ng/ml) for 48 h. Supernatants were collected for measurement of IFN-γ by ELISA (see below) in the absence or presence of TGF-β1 (2 ng/ml), IL-6 (5 ng/ml), anti–IL-4 (10 µg/ml), and anti–IFN-γ (10 µg/ml) for 48 h. Supernatants were collected for measurement of IFN-γ and IL-17A by ELISA (IFN-γ by AN18 and biotinylated R46A2 Abs and streptavidin-HPR (R&D Systems); IL-17A by DuoSet (R&D Systems)).

**Flow cytometry**

Abs were from BD Biosciences, except anti–granzyme B (GzmB) mAb (Invivospec). Cells (106) were analyzed on an LSRS UV or an LSRS 561 cytometer (BD Biosciences). Data were analyzed using FlowJo (Tree Star) or FACSDiva (BD Biosciences) software. For intracellular cytokine staining, CD8 T cells were stimulated ex vivo for 4 h in the presence of monensin (µM) and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences).

**Transcriptome analyses**

Naïve TCRP1A T cells, untransduced TCRP1A eTCs, and TCRP1A eTCs-STAT5CA recovered from spleen and lymph nodes after transfer in congenic Rag1−/− B10.D2 hosts were positively selected using anti-CD8 magnetic beads (Miltenyi Biotec). The same was done for T-bet−/− eTCs-STAT5CA recovered after transfer in congenic Rag2−/− B6 hosts. Cell purity ranged from 95 to 99%. In vitro–activated (72 h) TCRP1A eTCs-STAT5CA and eTCs-GFP were FACs-sorted on the basis of GFP expression. Two to four independent biological replicates have been included in the transcriptomic analysis (see Supplemental Fig. 1). Statistically significant differences in mRNA abundance between samples were determined using the moderated t test of Bioconductor package linear models for microarray data. We selected probes showing at least a log2FoldChange value >0.5 or <−0.5 with a p value <0.05. A Venn diagram (22) showed the distribution of genes significantly differentially expressed between two cell populations. The gene set enrichment analysis (GSEA) method from the Massachusetts Institute of Technology (http://www.broad.mit.edu/gsea) (23) was used to statistically test whether a set of genes of interest was distributed randomly in the large list of genes.
sorted on the basis of their expression level for TCRP1A eTCs-STAT5CA over TCRP1A eTCs with a p value <0.05.Both C5 genes annotated by the same Gene Ontology terms; Supplemental Table I) and C7 (immunologic signature; see Supplemental Table II) sets of genes of interest were used for these analyses. Microarray data have been submitted to National Center for Biotechnology Information Gene Expression Omnibus database: (http://www.ncbi.nlm.nih.gov/projects/geos/query/acc.cgi?acc=GSE41819).

Quantitative RT-PCR

Total RNA was isolated using an RNasey kit (Qiagen). RNA samples (1 μg) were reverse-transcribed using random primers and SuperScript II reverse transcriptase (Life Technologies, Grand Island, NY). Quantitative real-time PCR was done by mononuclear product detection (SYBR Green; Stratagene) of cDNA. mRNA expression levels were normalized to the housekeeping hypoxanthine phosphoribosyltransferase (HPRT) gene using the 2−ΔΔCt method in which ΔCt was the difference between the mean Ct value of triplicates of the sample and of the HPRT control. Values normalized to those of the naive CD8 T cell sample are shown. Primer sequences are: IFN-γ, forward, 5′-CCAGAACCGACGCAGACGAAAGG-3′, reverse, 5′-GGGACACCTTCGATGAGGCTA-3′; GzmB, forward, 5′-TCCAAACAGGCAGCTGTCCT-3′, reverse, 5′-GTTGGTTGTTGACAGCAGTTGCT-3′; T-bet, forward, 5′-CAACAACCCCCGGGAGACG-3′, reverse, 5′-CCCCCACAAGCTTGTACGAG-3′; Eomes, forward, 5′-CCCTATGGGTCAATTCC-3′, reverse, 5′-CCAGAACCACCTGCAACGAA-3′; HPRT, forward, 5′-AGCCCTTGTGCTCTCAAGG-3′, reverse, CTGTAATAATGCTACGATGAGG-3′.

ChIP and ChIP-Seq

ChIP of crosslinked chromatin was performed essentially as previously described with minor modifications (24). Cells extracts from 50 × 10⁶ cells were sonicated 12 times, using the S-4000 Sonifier (Misonix, Farmingdale, NY) with 30-s pulses, to obtain DNA fragments between 200 and 500 bp in length. Chromatin extracts were prewashed with 50 μl magnetic beads (Invitrogen) for 1 h at 4°C in a rotating wheel. Precipitation was then carried out overnight at 4°C using 10 μg anti-STAT5a Ab (R&D Systems) complexed to 50 μl magnetic beads. After reverse crosslinking, enriched DNA fragments were extracted with phenol/chloroform and recovered using the QIAquick PCR purification kit (Qiagen). For ChIP-Seq, sequencing of two STAT5a ChIP and input DNA samples was performed on an AB SOLiD v4.0 (Life Technologies) according to the manufacturer’s protocol. Sequence read mapping to version 9 build 37 (mm9) of the mouse genome was achieved with BFAST (v0.7.0). Only reads with a mapping score ≥120 were kept. Average values from the two replicates or for the input sample were summed up in nonoverlapping 50-bp bins along each chromosome and normalized per 10 million reads to generate Wiggle files, using an R custom script. Wiggle files were visualized using the Integrated Gene Browser tool (http://www.affymetrix.com). Significant STAT5-bound regions were retrieved using the HOMER peak-calling tool (v4.1; http://bioinformatics.weizmann.ac.il/homer/) with default settings (false discovery rate, 0.001; local and input fold enrichment, 4.0). Only peaks found in both replicates were elongated to 204 and 189 bp for replicates 1 and 2, respectively. The height of the peaks was corroborated by a recent whole-genome study illustrating the relationship between NK cells and T cells (31). Expression of some NK cell receptors was confirmed by cell surface staining (not shown), but the functional consequences of this expression on CD8 T cells are still unclear (32).

Comparing adoptively transferred eTCs-STAT5CA and eTCs, GSEA performed with the C5 gene set (Supplemental Table I) showed a positive enrichment score for transcripts involved in cell cycle and DNA replication, consistent with the high potential for secondary responses of eTCs-STAT5CA (20) (see below) or encoding components of intracellular organelles such as microtubules, cytoskeleton, and secretory vesicles in correlation with the high cytolytic activity of the eTCs-STAT5CA, a characteristic corroborated by a programmed cell death/cell-mediated cytotoxicity gene cluster. Interestingly, when GSEAs were done with the recently released C7 “immunological signature” gene set (Supplemental Table II), a positive enrichment score was found for transcripts involved in T effector versus memory CD8 T cells, naive versus T effector CD8 T cells, and unstimulated versus IL-2 (or IL-15)–stimulated NK cells.

In both microarray and cytometric analyses, eTCs-STAT5CA demonstrated a GzmB expression (Fig. 2B–D) and exhibited a higher cytolytic activity than their control counterparts (20) (Fig. 2E). FasL was not constitutively expressed on eTCs-STAT5CA but could rapidly be induced upon Ag triggering (Fig. 2F), due perhaps to the high level of FasL transcript (Fig. 1A), contributing to Fas-dependent cytotoxic activity when encountering its target (Fig. 2E). Whether the high level of transcripts encoding IFN-γ (Fig. 1A) influenced the kinetics of the secondary response by eTCs-STAT5CA was evaluated through a kinetic study of in vitro restimulation in response to peptide-loaded APCs (Fig. 2G). Although no constitutive cytokine production was detected in those cells, a larger fraction of eTCs-STAT5CA than control eTCs efficiently secreted IFN-γ after activation (Fig. 2H). Additionally, eTCs-STAT5CA were also found to secrete CCL3 and TNF-α but not IL-2 (Fig. 2H). Altogether, STAT5CA regulated a gene expression program that induced very responsive Tc1.

Briefly, after 72 h in vitro culture, control untransduced (TCRP1A eTCs) or GFP-transduced (TCRP1A eTCs-GFP) and STAT5CA-transduced (TCRP1A eTCs-STAT5CA) were adoptively transferred into congenic Rag-1−/− B10.D2 mice for prolonged periods to establish a pool of memory T cells.

Using a whole-genome transcriptomic analysis, in this study we define the distinct gene expression program between untransduced and STAT5CA eTCs: those two eTC subsets were analyzed after 3 d of in vitro culture and at later time points (20–68 d) after transfer. Compared to naive T cells, adoptively transferred eTCs-STAT5CA and untransduced eTCs showed 1526 and 1001 upregulated genes, respectively, with 608 genes being shared between these two subsets (Fig. 1A). Among this shared group, Tbx21 (encoding T-bet) and Eomes were present, although at a higher level in the eTCs-STAT5CA (Fig. 1A; genes are marked with an asterisk). This was also the case for some known T-bet target genes (GzmB, Ifng, Cxcr3, Il2rb) (29, 30) and for Fasl. Additionally, compared with naive T cells, eTCs-STAT5CA expressed higher levels of transcripts encoding effector molecules (GzmB, GzmK, Pff1, Tfnp), molecules regulating cytokine signaling (Socs1, Socs2, Cish) and costimulatory receptors (Tnffr18, Gltir) and Tnfrsf9 (4-1BB). Specific staining corroborated the high expression of proteins GTR and 4-1BB on eTCs-STAT5CA (Fig. 2A). The observed relative increased expression of a large collection of transcripts for killer cell lectin-like receptors on eTCs-STAT5CA was in agreement with a recent whole-genome study illustrating the relationship between NK cells and T cells (31). Expression of some NK cell receptors was confirmed by cell surface staining (not shown), but the functional consequences of this expression on CD8 T cells are still unclear (32).

In both microarray and cytometric analyses, eTCs-STAT5CA demonstrated a GzmB expression (Fig. 2B–D) and exhibited a higher cytolytic activity than their control counterparts (20) (Fig. 2E). FasL protein was not constitutively expressed on eTCs-STAT5CA but could rapidly be induced upon Ag triggering (Fig. 2F), due perhaps to the high level of FasL transcript (Fig. 1A), contributing to Fas-dependent cytotoxic activity when encountering its target (Fig. 2E). Whether the high level of transcripts encoding IFN-γ (Fig. 1A) affected the kinetics of the secondary response by eTCs-STAT5CA was evaluated through a kinetic study of in vitro restimulation in response to peptide-loaded APCs (Fig. 2G). Although no constitutive cytokine production was detected in those cells, a larger fraction of eTCs-STAT5CA than control eTCs efficiently secreted IFN-γ after activation (Fig. 2H). Additionally, eTCs-STAT5CA were also found to secrete CCL3 and TNF-α but not IL-2 (Fig. 2H). Altogether, STAT5CA regulated a gene expression program that induced very responsive Tc1.

Results

STAT5CA expression in vivo—transferred CD8 T cells induces a sustained Tc1 gene expression program

We derived long-lived eTCs by retroviral expression of a mutated STAT5α1*6 (28), referred to as STAT5CA hereafter, in in vitro–activated CD8 T cells expressing a transgenic TCR specific for the P1A encoded cancer germine Ag (TCRP1A), as described (20).
FIGURE 1. Long-term expression of STAT5CA sustains a large gene expression program in CD8 T cells. (A) Venn diagram showing the distribution of genes with significant changes (logFC value >0.5 or <-0.5; p value <0.05): TCRP1A eTC-STAT5CA/naive TCRP1A T cells (blue circle) and untransduced TCRP1A eTC/naive TCRP1A T cells (yellow circle). For each subset the most significant genes (highest ratio) are reported as well as their fold increase in parentheses. For some of the 608 common genes, numbers in parentheses are relative to the fold increase in (Figure legend continues)
STAT5CA expression induces either sustained gene regulation or time-dependent accumulation of transcripts, leading to concomitant expression of T-bet and Eomes

Among the 100 genes displaying the highest positive enrichment between TCRP1A eTCs-STAT5CA and TCRP1A eTCs after adoptive transfer, we selected those relevant to T cell functions and activation as well as those included in GSEA clusters (cell cycle, DNA replication, microtubules/cytoskeleton, and JAK/STAT signaling) shown in Supplemental Table I. Two groups of transcripts with distinct expression kinetics were identified. In the type 1 group (Fig. 1B), transcripts were already upregulated at 72 h after activation in both eTCs and eTCs-STAT5CA and their expression was either sustained at the same level or slightly increased 20 d later in in vivo–transferred eTCs-STAT5CA, while being respectively reduced or maintained at the same level in control eTCs. This cluster included transcripts involved in DNA replication, cell cycle, microtubules/cytoskeleton (dotted lines), as well as Gzmb, il2rb, Tnfrsf9, Tnfrsf18, and STAT5a. Tbx21 (T-bet) and its target genes il2rb and Cxcr3 were not included in this figure because they were not ranked within the 100 genes with highest enrichment. However, their expression profile was similar to that of the type 1 gene cluster (see Fig. 2B, 2C for T-bet) that also included the T-bet target gene gzmnb and il12rb2 (Fig. 1B). This pattern of gene expression is consistent with a role for STAT5 in the stabilization of a Tc1 gene expression program initiated by TCR stimulation (33, 34).

In the type 2 group (Fig. 1C), transcripts were strongly upregulated from 72 h to day 20 after activation in eTCs-STAT5CA, although this was not (or to a much lower extent) observed in control eTCs. This latter gene cluster included Eomes, Ifng, Tnf, and killer cell lectin-like receptors. We confirmed by qRT-PCR the enrichment of these target genes and downmodulation of IL-6Rα and the gp130 signaling subunit, as well as transcripts for TGFβRII. Staining of eTCs-STAT5CA and control eTCs or naive CD8 T cells confirmed the reduced expression of IL-6Rα and gp130 (Fig. 3A) surface proteins on STAT5CA-expressing T cells. As compared with naive CD8 T cells, TGFβRII was also expressed at a lower level at the surface of eTCs-STAT5CA (Fig. 3B).

Identification of direct STAT5CA target genes

To identify regulatory regions occupied by STAT5 TF in ex vivo–purified memory TCRP1A eTCs-STAT5CA, we performed ChIP using an anti-STAT5a Ab followed by deep sequencing (ChIP-Seq). Two independent preparations of TCRP1A STAT5CA-eTCs were directly (ex vivo) fixed for ChIP-Seq assays without any cytokine treatment. Results from these two independent experiments were merged to define common identified peaks, which included 7320 significantly bound DNA regions associated with 6969 different genes in the TCRP1A eTCs-STAT5CA (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE41819).

Most bound regions were found at >5 kb from the transcription start sites (TSS) (Fig. 4A), suggesting preferential binding to putative distal enhancer regions. The distribution of STAT5a-bound regions was similar to the one previously reported for 3 d in vitro–preactivated T cells restimulated with IL-2 (12, 40). However, as compared with recent cytokine-driven STAT5a activation, long-lasting expression of STAT5CA might induce its participation in transcriptional complexes of a different nature.

We compared the 6969 STAT5a-associated genes identified by ChIP-Seq with the 3039 transcripts that were significantly different between TCRP1A STAT5CA-eTCs and TCRP1A eTCs in the transcriptomic analyses: 1043 (34%) of those transcripts were found to be STAT5a target genes (Fig. 4B). Thus, a substantial proportion of the genes whose expression is modulated in TCRP1A eTCs-STAT5CA are likely to be directly controlled by STAT5a.

STAT5 is known to bind to TTCN/N(G/A)GAA (where “N” indicates any nucleotide) IFN-γ–activated site motifs. We found that 88.8% of STAT5a-bound regions contained at least one STAT5 motif. A focused analysis of STAT5-bound regions found by ChIP-Seq was conducted on the upregulated Tbx21 and Eomes genes and downregulated Tgfb2 and il6st genes (Fig. 4C, Supplemental Table III). These analyses showed three bound regions around the Tbx21 (T-bet) gene, at positions −12.3, +35.2, and +39.4 kb. The upstream site was recently reported to bind STAT5a in CD4 T cells incubated with IL-2, or STAT4 in CD4 T cells...
incubated with IL-12 (12), in line with the observation that IL-2/STAT5 and IL-12/STAT4 may have overlapping actions in CD4 Th1 differentiation, with both contributing to T-bet expression. We also identified two enriched STAT5a-bound regions around the Eomes gene at positions 23.3 and +14.6 kb. For the repressed genes, two peaks were found upstream (23.3 and -23.0 kb) of Tgfbr2 and two in its intronic region (+55 and +66 kb). Likewise, three regions upstream of il6st were identified in the STAT5a ChIP at positions -27, -15.5, and -3.3 kb. No significant STAT5a binding was found on the GzmB gene (data not shown). Finally, we identified two major peaks located at 253 and -274 kb upstream of the Gzmb TSS. A minor peak (GzmB no. 3) at position -240 bp was found, in agreement with our previous study using classical ChIP on in vitro–activated CD8 eTCs (34). We also performed

FIGURE 2. (A) Ex vivo TCRP1A eTCs-STAT5CA show increased expression of activation markers. Rag-1−/− B10.D2 mice were injected with 2 × 10⁵ TCRP1A eTCs-STAT5CA 23 d earlier. Splenocytes were analyzed for expression of the reported activation markers on CD8 GFP+ TCRP1A eTCs-STAT5CA. Naive TCRP1A T cells are included as controls. (B–D) Long-term expression of STAT5CA induces upregulation of T-bet and Eomes TFs. (B) Quantitative RT-PCR on RNA samples isolated from the following T cells: purified naive CD8 T cell from B10.D2 mice; (B) 72 h in vitro–activated untransduced (control) or STAT5CA-transduced TCRP1A (sorted GFP+ cells); (C) purified TCRP1A eTCs-STAT5CA from Rag-1−/− B10.D2 mice injected 20 d earlier. Values (2−ΔΔCt) normalized to those of the naive CD8 T cell sample are shown. Results are representative of two or three experiments done in triplicate. Statistical analyses were performed with a Mann–Whitney test using GraphPad and a two-tailed p. *p < 0.05, **p < 0.01, ***p < 0.001. (D) TCRP1A eTCs and TCRP1A eTCs-STAT5CA from Rag-1−/− B10.D2 mice injected 20 d earlier were stained for GzmB, Eomes, and T-bet. On gated CD8+ T cells, their respective expression (with the corresponding MFI) is shown overlaid on an isotype control (gray). Results are representative of three experiments. (E and F) TCRP1A eTCs-STAT5CA can use both secretory and death receptor pathways for target killing. (E) Splenocytes from Rag-1−/− B10.D2 mice injected with 10⁴ untransduced TCRP1A eTCs (day 53) or 2 × 10⁵ TCRP1A eTCs-STAT5CA (day 46) were used in a cytolytic assay. CD8+ T cells were cocultured during 5 h with targets negative (L1210; not shown; lysis never >8%) or positive (L1210-Fas) for Fas surface expression. For the latter, three different conditions were tested: medium, to detect constitutive Fas-mediated killing; peptide P1A, to reinduce FasL expression, which also triggers GzmB/perforin-mediated lysis because the targets express H-2Ld; peptide P1A plus EGTA plus MgCl₂, to reinduce FasL expression while inhibiting GzmB/perforin lysis. (F) Naive TCRP1A T cells and TCRP1A eTCs in vitro activated for 72 h and splenocytes of Rag-1−/− B10.D2 injected 46 d earlier with TCRP1A eTCs-STAT5CA were stimulated for 4 h with either ionomycin/PMA or tumor cells expressing (P1A+, P511) or not (P1A−, P1.204) the P1A Ag and then stained for FasL. The fold increase between FasL surface expression on activated cells relative to cells cultured in medium is reported. (G and H) TCRP1A eTCs-STAT5CA manifest rapid and potent IFN-γ secretion upon secondary stimulation; those cells produce IFN-γ, TNF-α, and CCL3 but not IL-2. TCRP1A eTCs-STAT5CA and TCRP1A eTCs were adoptively transferred into Rag-1−/− B10.D2 mice. Thirty days later CD8+ T cells recovered from pooled lymph nodes and spleens were stimulated with P1A peptide–pulsed APCs and then stained for IFN-γ (percentage IFN-γ+ among gated CD8+ T cells is represented as a function of time; means ± SEM are shown from three independent experiments; statistical analyses were done with an unpaired t test [GraphPad]; **p < 0.01 by a two-tailed p test); or (H) for 4 h with anti-CD3 bound on a FcRg+ (P1.204) tumor. Percentage cytokine+ cells among gated CD8+ T cells are shown. Data are representative of at least three independent experiments.
classical ChIP assays for all of the identified peaks using three independent TCRP1A STAT5CA-eTCs preparations (Fig. 4D) and thus validated the ChIP-Sequencing data.

Altogether, a direct regulation of both T-bet and Eomes might be at the basis of the particular phenotype and functional properties of the eTCs-STAT5CA maintained in vivo, whereas direct regulation of il6st and Tgfr2 genes may contribute to the dampening of their sensitivity to IL-6 and TGF-β.

T-bet deficiency did not compromise STAT5CA-induced differentiation of long-lived eTCs and their accumulation in tissues

We next evaluated whether the promotion of long-lived CD8 eTCs in tissues was dependent on T-bet. For this purpose, polyclonal CD8 T-bet-deficient T cells were recovered from B6 T-bet−/− mice, activated by anti-CD3/CD28, and transduced or not with STAT5CA. After adoptive transfer in Rag-2−/− B6 hosts, the number of recovered T cells from livers was higher for eTCs-STAT5CA compared with eTCs for both wild-type (wt) and T-bet-deficient cells, whereas differences in recovery were not significant in the spleens (Fig. 5A). This enhanced tissue accumulation of STAT5CA-transduced T cells may result from a combination of increased tissue-homing, proliferation, and survival in a T-bet-independent fashion. Additionally, GzmB was equivalently expressed in T-bet-deficient and in wt eTCs-STAT5CA recovered from the spleen (Fig. 5B, 5C). In both cases, the level of GzmB was higher than in the corresponding eTCs.

Transcriptomic analysis (Fig. 6A) of T-bet−/− eTCs-STAT5CA determined that most of the transcripts induced by STAT5CA were still upregulated in absence of T-bet at a similar level (Il2ra, Ccl3, Tnf, Socs1, Gmnc, Cish, Eomes) or at a lower level (Gemb, Ifng, Fasl, Socs2, Gzma, Gzmk, Rab27b, Cxcr3). Some of these results have been validated by cytometry (Fig. 5B, 5C, and data not shown). However, we noticed a relatively higher Eomes protein (Fig. 5B, 5C) than transcript (Fig. 6A) expression level, suggesting that posttranscriptional mechanisms might control expression of the corresponding protein. Altogether, this analysis showed that STAT5CA expression in CD8 eTCs contributes in a manner independent of T-bet to their increased accumulation in peripheral tissues and to sustained expression for a cluster of genes. The question may be raised as to whether for some of these increased transcripts Eomes expression compensates for lack of T-bet. Indeed, T-bet−/− CD8 T cells exposed to lymphocytic choriomeningitis virus in vivo had reduced expression of Cxcr3 and GzmB as compared with wt CD8 T cells, but CD8 T cells lacking both T-bet and Eomes expression failed to develop into Cxcr3+GzmB+ effector cells (29).

T-bet deficiency in STAT5CA-expressing eTCs leads to a diversification of effector functions

This transcriptomic analysis also pointed toward a subset of genes that is regulated by STAT5CA in a strictly T-bet–dependent manner. This subset included some of the known T-bet target genes: Klf4, Ppif, and other upregulated (Gzmm) and downregulated transcripts (Ii7r, Iil6ra) (Fig. 6A). Importantly, iil2 transcripts appeared to be upregulated in T-bet−/− eTCs-STAT5CA (Fig. 6A). This observation was validated by measuring cytokine production. Indeed, T-bet−/− eTCs-STAT5CA recovered the ability to secrete IL-2 (Fig. 6B–D), as compared with wt eTCs-STAT5CA, which failed to do so. We also observed that STAT5CA expression greatly enhanced the amount of accumulated IFN-γ whether the responding T cells were T-bet sufficient or deficient (Fig. 6E). Untransduced wt CD8 eTCs were also able to secrete IFN-γ and IL-2, although the mean fluorescence intensity (MFI) value for IFN-γ was lower than in cells expressing STAT5CA (Fig. 6B). Finally, untransduced T-bet−/− CD8 eTCs had nearly lost the ability to produce IFN-γ, whereas a small subset of cells had the capacity to secrete IL-2 (Fig. 6B–D).

Altogether, these results suggest that T-bet−/− eTCs-STAT5CA might be less differentiated than wt eTCs-STAT5CA with preserved IL-2 secretion akin to that seen in central memory CD8 T cells.

T-bet deficiency in STAT5CA-expressing eTCs leads to sensitivity to IL-6/TGF-β1 and promotes IL-17 secretion

Transcriptomic analysis had indicated a milder downmodulation of Iil6ra, Il6st, and Tgfr2 in T-bet-deficient as compared with wt eTCs-STAT5CA as compared with naive CD8 T cells (Fig. 6A).
FACS analysis confirmed a lower expression of the IL-6Rα-chain on wt as compared with T-bet–deficient STAT5CA-expressing cells (Fig. 7A). Downregulation of IL-6Ra expression thus appears to depend on T-bet.

The genetic ablation of T-bet has been shown to strongly favor IL-17 expression both in CD4 and CD8 T cells (29, 41), with Th17/IL-17–producing CD8 T cells (Tc17) differentiation being induced by IL-6 and TGF-β1. We therefore evaluated levels of IL-17 induced by IL-6/TGF-β1 treatment of the wt and T-bet–deficient eTC-STAT5CA populations (Fig. 7B). Whereas T-bet-deficient STAT5CA-expressing cells produced high amounts of IL-17, wt eTCs-STAT5CA failed to produce this cytokine. We also observed that wt eTCs were unable to produce IL-17 upon IL-6/TGF-β1 treatment. This result suggests that T-bet represses differentiation of Tc17. Indeed, T-bet−/− eTCs were converted to IL-17 producers under polarizing conditions. It thus appears that T-bet–deficient eTCs, whether they express STAT5CA or not, conserve some functional plasticity and are reprogrammed as Tc17 producers upon IL-6/TGF-β1 treatment. In contrast, wt eTCs-STAT5CA appear to be fully differentiated and resistant to polarizing IL-6/TGF-β1 cytokines.
**Discussion**

STAT5 is a TF capable of inducing both positive and negative gene regulation owing to its capacity to recruit either transcriptional coactivators or corepressors (42). STAT5CA might additionally modify the chromatin structure for efficient TF recruitment on the targeted loci (43). Indeed, STAT5 function is modulated by its interaction with cofactors (44) or with other TFs bound on neighboring sites (36). In CD8 (33, 34) and CD4 (45) T cells, sustained activation of STAT5 was shown to be crucial for the stabilization of gene expression programs. Sustained activation of STAT5 was also shown to be important in nonhematopoietic cells (46) for chromatin remodeling and transcription of the β-casein gene. Transcriptomic analyses of CD8 eTCs expressing STAT5CA in this study identify a cluster of genes demonstrating an early and sustained expression (cluster 1; Fig. 1B) and also a cluster of genes exhibiting a time-dependent accumulation (cluster 2; Fig. 1C).

Whether a relationship of causality exists between early expression of some of the genes from type 1 cluster and subsequent expression of some genes of the type 2 cluster remains to be determined. The pattern of expression of the type 1 gene cluster, including Tbx21/T-bet, is consistent with a role for STAT5CA in the stabilization of a Tc1 gene expression program initiated by TCR stimulation (33, 34). For Eomes expression, STAT5CA appeared to induce a time-dependent accumulation of the transcripts (type 2 cluster). These patterns of T-bet and Eomes gene expression are consistent with the recent description of an IL-2–dependent expression of Eomes in CD8 T cells several days after removal of the TCR stimulus, whereas T-bet was induced in response to TCR engagement (47).

High GzmB expression by CD8 eTCs-STAT5CA may result from direct STAT5-mediated regulation (34) (Fig. 4); indirect regulation through increased T-bet/Eomes expression, as well as decreased Bcl6 expression, as the latter TF is a known repressor of GzmB transcription (39); and/or complementation between the T-box members T-bet and Eomes (Fig. 5). Indeed, although not constitutively activated, CD8 eTCs-STAT5CA were capable of enhanced secondary responses. This may be due to their increased expression of transcripts encoding effector molecules allowing rapid recall responses.

**FIGURE 5.** STAT5CA expression induces differentiation of long-lived eTCs independently of T-bet. (A–C) Rag-2−/− B6 mice were injected with (2 × 10^6) T-bet−/− or wt CD8 eTCs or (10^6) T-bet−/− or wt CD8 eTCs-STAT5CA. At day 47, absolute numbers of CD8 T cells recovered in liver and spleen are shown in (A) (mean of four experiments). In (B), expression of GzmB and Eomes on the gated splenic CD8 eTCs are reported together with their MFI, compared with an isotype control (gray). In (C), the fold increase for GzmB and Eomes MFI between T-bet−/− eTCs-STAT5CA and wt eTCs-STAT5CA is shown for independent donor mice.
correlation was also observed in CD8 effector memory T cells developing in the absence of CD4 help (6), suggesting that the level of T-bet/Eomes not only controls CD8 effector memory T cell functions (cytolysis, IFN-γ) but also, directly or indirectly, controls the fate of these cells, possibly through the induction of senescence. Altogether, the concomitant T-bet<sup>hi</sup>/Eomes<sup>hi</sup> expression by CD8 eTCs-STAT5CA may explain their responsive memory rather than terminal effector fate, as suggested by others (48). Indeed, sustained STAT5, T-bet, and Eomes expression are correlated with a superior efficacy of STAT5CA-expressing eTCs.
for antitumor adoptive therapies as compared with control eTCs, even when the latter is associated with IL-2 complex infusions (20), a protocol that might only induce transient STAT5 activation.

Comparison of secondary responses of T-bet–deficient and wt eTCs-STAT5CA further highlights the complex interplay between T-bet and STAT5. IFN-γ secretion appeared to be T-bet–dependent but can be compensated directly by STAT5CA and/or indirectly by STAT5CA-induced Eomes. IL-2 secretion was found inhibited by STAT5CA expression in a T-bet–dependent manner (Fig. 6), in agreement with the reported repressive function of T-bet on IL-2 gene transcription in CD4 Th1 cells (49). Importantly, these results suggest that T-bet–deficient eTCs might be less differentiated than wt eTCs-STAT5CA with preserved IL-2 secretion akin to that seen in central memory CD8 T cells. Recent studies (10, 16) point to the superior therapeutic potential of less differentiated CD8 T cells in adoptive therapies. The polyfunctional characteristics of T-bet–/– eTCs-STAT5CA might therefore be considered useful features for this purpose. However, a main limitation for adoptive therapies is tumor-derived immunosuppression that dampens antitumor responses of both endogenous and transferred CD8 eTCs. In human melanoma cells with the BRAF(V600E) mutation, production of immunosuppressive cytokines IL-6, IL-10, and vascular endothelial growth factor A depended on activation of both the BRAF/MAPK and STAT3 pathways (50). In a mouse model (TiRP) of autochthonous melanomas (51), aggressive melanoma development was associated with increased levels of several immune-modulating cytokines in the sera (52), including IL-6, a well-known inducer of STAT3 (19). Indeed, pSTAT3 was detected in tumor cells and in some hematopoietic cells infiltrating the TiRP melanoma tumors (52). Additionally, those TiRP tumors were infiltrated by T cells with low levels of GzmB effector molecules and presenting an “exhausted” programmed death-1 phenotype (52). We recently showed (20) that adoptive transfer of tumor-specific eTCs-STAT5CA was efficient at inducing melanoma regression. Within the TiRP tumors, eTCs-STAT5CA maintained a GzmB expression, suggesting acquisition of resistance to the local and systemic immunosuppression or reprogramming (53). Transcriptomic analyses of eTCs-STAT5CA in this study revealed a STAT5-mediated repression of IL-6Ra, IL-6st, and TGFβRII subunits. Although IL-2 was previously shown to decrease IL-6R expression on CD4 Th1 T cells (12), in this study we show that the use of an active form of STAT5 provides long-term IL-6R repression on CD8 eTCs in a T-bet–dependent manner (Fig. 7). Additionally, STAT5CA also induced stable repression of TGFβRII.

We show that genetic loss of T-bet abrogates IL-6R and TGFβRII repression and increases their sensitivity to these cytokines, leading to the differentiation of Tc17 (Fig. 7). As a consequence, the concerted action of STAT5CA and T-bet contributes to repression of IL-6Ra and TGFβRII expression, without compensation by STAT5CA-induced Eomes expression. A competition between binding of STAT5 (repressive) and STAT3 (permissive) on the IL-17 promoter has recently been reported for CD4 T cells (54). In this study, we report the incapacity of STAT5CA expression to mediate IL-17 repression in absence of T-bet. However, the loss of T-bet results in increased IL-6Ra expression, a possible increased STAT3 signaling in the absence of T-bet may overcome the STAT5CA repressive effects on IL-17 transcription (54).

From the therapeutic point of view, polyfunctional T-bet–/– eTCs-STAT5CA might appear more appealing than fully differentiated Tc1 wt eTCs-STAT5CA. However, the restored sensitivity of T-bet–/– eTCs-STAT5CA to IL-6 and TGF-β1 dampens the curative potential of those cells. As a general feature, T-bet might lead to imprinting of a Tc1 functional program and blocking of subsequent cytokine-induced polarization, whereas STAT5CA might amplify the effector functions by sustained regulation of the corresponding genes (e.g., Ifng, Gzmb) and decreased sensitivity of CD8 eTCs to IL-6/TGF-β1 through sustained downmodulation of their receptors. A direct regulation by STAT5CA of the expression of those receptors is suggested by our ChIP-Seq/ChIP data identifying regions of bound STAT5 around the Ifng and Tgfb2 genes in long-term in vivo-maintained eTCs-STAT5CA (Fig. 4).

In conclusion, direct gene regulation by STAT5CA induces a combined central memory/effector memory T cell gene transcription profile with concomitant high expression of T-bet and Eomes. These eTCs-STAT5CA are endowed with long-term maintenance, efficient recall responses, and stable downmodulation of receptors mediating IL-17 polarization. Altogether, our data further indicate the importance of testing both functional responses and imprinting in T cells for adoptive therapy, as tumor-derived cytokines might polarize candidate T cells to be used for this purpose.

Acknowledgments

We acknowledge Pascal Barbry and Chihem Moreihlon from the Plateforme Transcriptome Nice–Sophia Antipolis, Institut de Pharmacologie Moléculaire et Cellulaire, Sophia Antipolis, France. We acknowledge the Centre d’Immunologie de Marseille–Luminy bioinformatics platform (headed by Sebastien Jagger) and personnel funded by Cancéropôle Pro-
Disclosures
The authors have no financial conflicts of interest.

References

Downloaded from http://www.jimmunol.org/ by guest on October 8, 2017

The Journal of Immunology

3723
factor eomesodermin enables CD8+ T cells to compete for the memory cell


