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STAT5-Mediated Signals Sustain a TCR-Initiated Gene Expression Program toward Differentiation of CD8 T Cell Effectors

Grégory Verdeil,* Denis Puthier,† Catherine Nguyen, † Anne-Marie Schmitt-Verhulst,* and Nathalie Auphan-Anezin*

Poorly functional effector CD8 T cells are generated in some pathological situations, including responses to weakly antigenic tumors. To identify the molecular bases for such defective differentiation, we monitored gene expression in naive monoclonal CD8 T cells during responses to TCR ligands of different affinity. We further evaluated whether responses to weak Ags may be improved by addition of cytokines. Transient gene expression was observed for a cluster of genes in response to the weak TCR agonist. Strikingly, gene expression was stabilized by low dose IL-2. This IL-2-sustained gene cluster encoded notably transcripts for CD25, cytolytic effector molecules (granzyme B) and TNF-R family costimulatory molecules (glucocorticoid-induced TNF-R (GITR), OX40, and 4-1BB). IL-2-enhanced surface expression or function was also demonstrated in vivo for these genes. A constitutive active form of STAT5 mimicked the IL-2 effect by sustaining transcripts for the same gene cluster. Consistent with this, under conditions of low avidity TCR engagement and IL-2 treatment, endogenous STAT5 binding to 4-1BB and granzyme B promoters was demonstrated by chromatin immunoprecipitation. This study highlights those genes for which IL-2, via STAT5 activation, acts as a stabilizer of gene regulation initiated by TCR signals, contributing to the development of a complete CD8 T cell effector program. The Journal of Immunology, 2006, 176: 4834–4842.
Materials and Methods

Animals

CBA/Ca mice transgenic for the BM3.3 alloreactive anti-K\(^{b}\) TCR (tgTCR) (15), C57BL/6 (B6), C57BL/6.C-H-2\(^{bm8}\) (bm8), and CBA/Ca (CBA) mice were bred in the CIML animal facility. All animal experiments were in accordance with protocols approved by the French and European Directives.

Cell purification and culture

CD8 T cells were purified from lymph nodes of tgTCR mice by negative selection as previously described (15) and represented 90 to 98% of the enriched population. APCs were T cell-depleted irradiated splenocytes. When indicated, cultures were conducted in the presence of 10 IU/ml rIL-2 (Roche) or neutralizing anti-IL-2 (JES6) and anti-IL-2R (PC61) mAbs (both obtained from BD Biosciences). Before RNA extraction or ChIP experiments, APCs were removed by positive selection using an anti-K\(^{b}\) mAb reacting with both K\(^{b}\) and K\(^{bm8}\) (16) and anti-mouse IgGs Dynabeads (Dynal).

Adoptive transfer

The 6 × 10\(^6\) CD8tgTCR cells were injected i.v. in recipients that had been exposed to a 5 Gy irradiation 2 days before. When indicated, mice were injected i.p. with 20 \(\mu\)g of rIL-2 (Proleukin; Chiron) provided by D. Olive (Marseille).

Flow cytometric analyses

Reagents used for immunofluorescence staining were: biotin-mAb Ti98, an anti-clonotypic mAb specific for the BM3.3 TCR (7); anti-CD134, CD137, CD5, CD122, CD25, and CD86 mAbs (obtained from BD Biosciences), and anti-GITR purchased from R&D Systems.

Nuclear extract preparation and Western blotting

Nuclear and cytoplasmic extracts were prepared as described (17). Proteins (10 \(\mu\)g) were analyzed by Western blot using Abs specific for STAT5a/b (Santa Cruz Biotechnology) or phospho-STAT5 (Upstate Biotechnology).

FIGURE 1. Clusters of genes differentially expressed in the primary response of CD8 T cells to strong or weak agonists are not influenced by T cell division. A, Hierarchical clustering was applied on genes that were significantly differentially induced (taking as a factor 1.5 cut off, at least) upon stimulation of naive CD8tgTCR with B6 APCs (B6) in the absence or presence of blocking mAb to IL-2 and CD25 (B6+αIL-2); or with bm8 APCs in the absence (bm8) or in the presence of exogenous IL-2 (bm8+IL-2), for 24 h, 48 h, and 72 h. Naive CD8tgTCR cells ex vivo (0) or cultured 24 h with IL-2 (naive+IL-2) are also shown. The names of known genes are reported and identification of these cDNA clones has been verified by sequencing. The asterisks (*) indicate either expressed sequence tags (ESTs) or Riken cDNA clones or unknown clones (nonreported in Unigene). The results are shown as relative expression levels obtained after normalization and are represented with a color scale indicated at the bottom. Four major types of clusters (a-d) are shown. B, Microarray analyses were performed on RNA extracted from CFSE-labeled CD8tgTCR cells that were stimulated for 72 h with bm8 APCs as shown in Fig. 2A and either taken as a total population (bm8 total) or sorted on the basis of CFSE profiles as divided (CFSE low) and nondivided (CFSE high) cells.

Cytotoxic assays

Cytotoxic activity of CD8tgTCR cells was tested on \(^{51}\)Cr-labeled (sodium chromate; New England Nuclear) specific (RMA (H-2\(^b\))) or irrelevant (L1210 (H-2\(^{d}\))) targets.

cDNA microarray analysis

The microarrays were prepared as reported previously (18) by spotting PCR products amplified from 5200 cDNA clones obtained from Soares-thymus-2NhMT, Soares-mouse-lymph-node-NBMLN and Soares-mouse-3NEMS libraries. Total RNA was isolated using Trizol reagent (Life Technologies). Complex probes were prepared from 3 \(\mu\)g of total RNA as previously described (18) with \([\alpha^{32P}]dCTP\) labeling. After hybridizations and washes, arrays were exposed to phosphor imaging plates, which were then scanned with a Fuji BAS 5000 machine (25 \(\mu\)m resolution). The hybridization signals were quantified using the Arraygauge software, and statistical analysis was applied. Mean background was calculated on blanks and subtracted. Then the median value for each microchip was calculated and used to normalize the experimental value. The same operation was done with the median value for each gene on different arrays. The ratio B6 value/bm8 value was calculated at 48 and 72 h. All genes displaying a ratio above 1.5 or below 0.6 at one (or more) time point of the kinetics were selected for further analysis. Using the Cluster program (19) (filtering 90% and log transformation), hierarchical clustering was applied on the selected genes (\(n = 737\)). Data were displayed using the Treeview program and representative groups of coexpressed genes are shown in Fig. 1. For Table I, a Student \(t\) test was applied on the 737 selected genes. Only genes with a known function that displayed at one time of the kinetics a value of \(p < 0.05\) are reported. Some genes present in Table I were not found in the clusters shown in Fig. 1. All the data have been submitted to ArrayExpress.

Retroviral infection

The retroviral vector encoding for a constitutively active form of STAT5 (STAT5CA) (20) was a kind gift from L. Van Parijs (Massachusetts Institute of Technology). Purified CD8 T cells activated with bm8 APCs were infected after 48 h with retrovirus-containing supernatants as described by Van Parijs et al. (20) after which medium was changed with the initial
TABLE I. Differential gene expression induced by TCR engagement with ligands of strong or low avidity and effect of IL-2

| Surface | Molecules | B6/bm8 | bm8 + IL-2/bm8 | B6/bm8 | bm8 + IL-2/bm8 |
|---------|-----------|--------|----------------|--------|----------------|
| II2ra   | Mn.915 (d) | 2.8 3.7 | 6.9 (e)        |        |                |
| II2r    | Mn.915    | 2.0 2.5 | 3.6            | 1.4    | 2.5            |
| Nkg7    | Mn.34613  | 3.0 2.4 | 2.8            | 1.8 2.7 |                |
| Tgfrsf9 | Mn.244187 | 1.7 2.5 | 2.7            | 1.5 1.8 | 1.3            |
| Tgfrsf18| Mn.3180   | 2.8 2.9 | 2.1            | 1.1 1.8 | 1.1            |
| Ly6c    | Mn.1583   | 3.8 5.2 | 2.0            | 2.9 2.0 | 1.0            |
| Tgfrsf4 | Mn.13885  | 1.5 1.4 | 1.6            | 1.5 1.7 | 1.0            |
| Igal    | Mn.1618   | 1.4 1.8 | 1.6            | 1.5 1.5 | 1.0            |
| C4d7    | Mn.317527 | 1.1 1.5 | 1.6            |        |                |
| Bsg     | Mn.726    | 2.0 2.4 | 1.7            |        |                |
| Selp1   | Mn.332590 | 2.2 1.4 | 1.3            |        |                |
| Igb7    | Mn.58     | 1.2 1.8 | 1.3            |        |                |
| Cta4    | Mn.390    | 2.9 2.0 | 1.1            | 2.3 2.0 | 1.7            |
| Ly6c    | Mn.788    | 1.4 1.7 | 1.1            | 1.6 1.7 | 1.1            |
| C6d     | Mn.290897 | 1.9 2.2 | 0.9            | 0.8 1.5 | 1.0            |
| B2m     | Mn.163    | 1.3 1.6 | 0.9            | 1.2 1.5 | 1.0            |
| Rxb     | Mn.1243   | 1.4 1.8 | 0.9            | 1.2 1.5 | 1.0            |
| C6d     | Mn.290897 | 2.2 1.6 | 0.8            | 1.5 1.5 | 1.0            |
| C5d     | Mn.779    | 3.5 2.0 | 0.7            | 0.6 0.9 | 0.5            |
| C5d3g   | Mn.335106 | 1.4 1.4 | 0.6            | 1.3 1.3 | 0.5            |
| C5d3g   | Mn.335106 | 1.2 1.3 | 0.6            | 1.2 1.3 | 0.5            |
| Sell    | Mn.1461   | 1.9 1.4 | 0.5            |        |                |
| Socs2   | Mn.4132   | 2.0 2.6 | 4.8            |        |                |
| Pim1    | Mn.328931 | 1.8 1.7 | 2.3            |        |                |
| Akt1    | Mn.6645   | 1.0 1.5 | 1.9            |        |                |
| Fadd    | Mn.5126   | 1.5 1.4 | 1.9            |        |                |
| Cnnx    | Mn.248827 | 1.0 1.6 | 1.6            | 1.8 2.4 | 6.0            |
| Itk     | Mn.339927 | 1.8 1.7 | 1.6            | 2.0 2.5 | 1.4            |
| Ppp3cc  | Mn.1567   | 1.4 2.0 | 1.3            | 1.5 2.3 | 1.4            |
| Hcph    | Mn.271799 | 1.8 1.6 | 1.3            | 1.1 1.4 | 0.5            |
| Hcph    | Mn.271799 | 1.9 1.7 | 1.2            |        |                |
| Bstl    | Mn.246352 | 1.3 1.7 | 1.2            |        |                |
| Ostf1   | Mn.172222 | 1.6 1.2 | 0.8            |        |                |

\[ a,b \] Ratio of mean values of four (48h) or five (72h) independent experiments.
\[ c \] Ratio of mean values of four (bm8) or four (bm8 + IL2) independent experiments. This ratio has been used to rank the table.
\[ d \] Unigene cluster ID.
\[ e \] Ratio in bold indicates that a Student t test was applied and gave a value of p < 0.05 at 48 or/and 72 h.

Results

Transient gene expression, a characteristic of partial agonist stimulation of CD8 T cells, is stabilized by low dose IL-2, for a subset of genes.

We have previously demonstrated (8, 15) defective IL-2 production in CD8 T cells from BM3.3 tgTCR mice in response to the full agonist (15)).

ChIP

A ChIP assay kit (Upstate Biotechnology) was used according to the manufacturer’s protocols with 2.5 μg of rabbit anti-STAT5b (Zymed Laboratories) or irrelevant rabbit anti-1x8 (Santa Cruz Biotechnology) Abs. Real-time PCR was conducted on ChIP samples with the primers reported in Table II. Normalization toward the HPRT gene was applied as described (22).

Cytokines

Here, we focused on differentially induced genes defined according to the following criteria: their expression level in T cells compared with unstimulated CD8 T cells (18).

Metabolism

Transient gene expression, a characteristic of partial agonist stimulation of CD8 T cells, is stabilized by low dose IL-2, for a subset of genes.

We have previously demonstrated (8, 15) defective IL-2 production in CD8 T cells from BM3.3 tgTCR mice in response to the mutant Kbm8 (bm8), acting as a partial agonist, as compared with the isoform (B6), behaving as a full agonist. Using microarrays, we analyzed the gene expression patterns for CD8tgTCR cells that were either unstimulated or activated 24, 48, or 72 h by B6 or bm8 APCs with or without a low dose IL-2 (i.e., 10 IU/ml corresponding to the physiological amount produced in response to the full agonist (15)).

Of the 5200 cDNAs present on the microarrays, 18% were regulated (taking as a “cut off” a factor of at least 2) inactivated, as compared with unstimulated CD8 T cells (18).

Here, we focused on differentially induced genes defined according to the following criteria: their expression level in T cells stimulated by full and partial agonist differed by at least a factor of 1.5 (Fig. 1A; Table I) in a minimum of 2 (24 h) or 3 (48 and 72 h)
Table II. Potential STAT5 binding sites in promoter and first intron sequences tested by ChIP

| Sequence Name | Forward Primer 5′→ | Reverse Primer 5′→ | STAT5 Consensus Site Localization | STAT5 Consensus Site |
|---------------|-------------------|--------------------|---------------------------------|---------------------|
| CD5           | GGGCAGGGATTTCCATGCTG | GTTCCCTGTTGGCAATGAGC | +8248 | TCCACGAGA |
| Lt-α Ctrl     | CGCCACCAATGTCACACTCTT | GGAATCGAGGAGACCTCTCA | / | / |
| Lt-α          | TGTGACAGCTATAAGGTCAG | AAAGCTGGCCTGCTAAGA | -846 | TCCGGGAA |
| GzmB Ctrl     | TTTGCGCATTTACACCACTGCG | TGATTTGTTGCTACTT | / | / |
| GzmB          | ACCTCACCCTTCATGCTTG | TACATTGATTACCTTTCTTG | -144 | TCCAGAGA |
| G1TR          | CGACCTAGCTGGTGGCTCTT | GCTTCTTGGCCTCAACTGACAA | -354 | TTAGAGGA |
| 4–1BB-2       | TCCCTGCCACACCTCCCAAT | CTTGCTTGGCCAGGCTCTCTAA | -5808 | TCCGGGAGA |
| 4–1BB-3       | AGCAATGGACCACCAAGGCACTG | TCACCAAGGCTACCTTC | -5431 | TCGAGGAGA |
| CD25 Ctrl     | GCTGCGAGAGACCTTATAAGT | CTGTGGAGCCTGGAGGTT | -1989 | TCGAGGAGA |
| CD25 (PRR-4)  | TGCCACACACAGAAGCTAA | ACCGAGCTTGAGACCAAGA | +2533 | TCCAGAGA |
| HPRT (ref)    | TGCTGACACTCCTGCGATTACA | CTTGGCCTGGTGAAGCTATT | / | / |

*Conserved noncoding sequences from human and mouse were obtained as described (55). Briefly, 15 kb upstream region and first intron sequences and cDNA sequences of studied genes were obtained from the University of California at Santa Cruz (UCSC) Genome browser. To identify exons on DNA sequences, Spidey (NCBI) was used. Underlined nucleotide indicates a mismatch compared with the consensus STAT5 sequence.

**Position from the coding ATG (+1)**.

independent experiments (see Materials and Methods). A small cluster of genes were induced exclusively in response to the full agonist (Fig. 1Aa). These genes included CD5 and CD6 and their level of expression was not affected by addition of exogenous IL-2. A second set of genes coding for chemokines (CCL3, CCL4; Fig. 1Ab) showed an early expression that peaked at 24 h, and decreased thereafter whether IL-2 was present or not. For a third set of genes, the poor up-regulation of their expression in response to bm8 was enhanced by IL-2 (Fig. 1Ac). Interestingly, a main cluster was characterized by low level transcript expression in naive CD8 T cells, with sustained expression (still positive at 72 h) in response to the full agonist but with transient increase (at 24 and 48 h) after activation by the partial agonist (Fig. 1Ad). Importantly, for this cluster, addition of exogenous IL-2 during the activation with bm8 APCs restored a stable pattern of gene expression (Fig. 1Ad). This concerted regulation by TCR and IL-2R signals applied to the CD25 gene, as already described (23–25) but also to genes encoding molecules involved in cytolytic effector function (GzmB), cell surface molecules (Tnfrsf18 (GITR), Tnfrsf9 (4-1BB), Tnfrsf4 (OX40)), cytokines (Lt-2α, -4837), components of signaling pathways (the proviral integration site 1 serine/threonine kinase (Pim-1), calmodulin-1, adenylate kinase-2; Socs2 (as reported by Kovanen et al. (26)); Fadd; Itk) and cell survival components (Bcl2, Caspase 3). In contrast, IL-2 failed to complement expression of genes encoding other signaling partners (such as Hcph and Ppp3cc) or cell cycle molecules (Cdkn2d). Additionally, IL-2 also contributed to down-regulation of a set of genes (Sell, Klf3, Bcl2a1a, Ltb) (Table I).

**IL-2 corrects late CD25 expression but does not affect the delayed Ag-driven proliferation in response to the partial agonist**

CD8tgTCR cell division was heterogeneous in response to the partial agonist, part of the population having divided and appearing blastic at day 3 after stimulation, whereas around 20% of the cells remained undivided (Fig. 2A), despite their acquisition of the CD44 activation marker (8). Therefore, the gene expression profiles of divided and nondivided populations, sorted on the basis of CFSE labeling 3 days after activation with bm8 APCs were compared (Fig. 1B). For the differential gene clusters identified here, patterns were similar in all bm8-stimulated cell populations. The distinct gene regulation in response to full versus partial agonists

**FIGURE 2. IL-2 increases late, but not early, Ag-driven proliferation and CD25 expression in CD8 T cells responding to the partial agonist.** A, CFSE-labeled CD8tgTCR cells were cultured with B6 or CBA or bm8 APCs in the absence or presence of IL-2 (10 IU/ml). After the indicated periods of time, CFSE labeling was analyzed on gated CD8 T cells. The percentage of nondivided cells is reported. At day 3, cell culture yields (%) were: B6, 6.18 ± 0.83; bm8, 3.26 ± 0.28; bm8+IL-2, 3.75 ± 1.30; CBA, 0.99 ± 0.50; CBA+IL-2, 1.03 ± 0.52. B, CD8tgTCR cells were cultured as in A over a period of 4 days. After the indicated periods of time, percentage of CD25+CD122+ gated CD8 T cells was measured in two independent experiments. Mean of fluorescence intensity of CD25 is also reported (in brackets).
therefore relied on the strength of TCR engagement and was not perturbed by the cells in the bm8-stimulated T cell population that had not divided.

Fig. 2A also shows that addition of exogenous IL-2 did not modify CD8tgTCR proliferation at day 3 while it reduced slightly the percentage of nondivided cells by day 4. However, the presence of IL-2 compensated for the defective CD25 expression at day 3 and 4, but not up to day 2, an early time at which CD25 up-regulation is strictly dependent on the strength of TCR engagement (Fig. 2B).

To further evaluate whether strong TCR signaling alone was capable of inducing mRNAs found in clusters c and d (Fig. 1A), blocking Abs to IL-2 and IL-2R were added to B6-stimulated CD8tgTCR cells (Fig. 1A, B6xIL-2). Inhibition of the IL-2R pathway resulted in decreased expression of genes shown in Fig. 1A, c and d. It should be noted that all the observed effects of IL-2 depended on TCR engagement as no alteration in gene expression was observed when IL-2 alone was added to naive T cells (Fig. 1A, naive/IL-2).

Expression of TNF-R superfamily members GITR, 4-1BB, and OX40 is regulated by both TCR and IL-2R signals in vitro and in vivo

Genes encoding TNF-R superfamily members GITR, 4-1BB, and OX40 were among those up-regulated in response to B6 Ag, but only transiently in response to bm8 (Table I). For GITR, we observed a 10-fold higher cell surface expression of the molecule in response to full compared with partial agonist (Fig. 3A), 4-1BB and OX40 surface expression was readily induced by B6 but very poorly by bm8 (Fig. 3A). However, for all three genes, the switch from a transient to a sustained pattern of expression upon addition of IL-2 to bm8 APCs (Table I) was correlated with an increased expression of the protein at the surface of the CD8tgTCR cells (Fig. 3A). Also in agreement with the gene expression data (Fig. 1Aa), surface expression of CD5 was not influenced by the presence of IL-2 in vitro (Fig. 3A).

To evaluate whether strong TCR signaling alone was capable of inducing these costimulatory molecules, blocking Abs to IL-2 and IL-2R were added to B6-stimulated CD8tgTCR cell cultures (Fig. 3B). This resulted in decreased expression of CD25, GITR, 4-1BB, and OX40 molecules, indicating that expression of all these molecules is controlled by both TCR and IL-2R signals.

In a previously established in vivo protocol of activation, naive CD8tgTCR cells acquired the CD25<sup>+</sup> phenotype in response to full or partial agonist, and CD25 up-regulation was restored upon injection of rIL-2 (7), mirroring the effects observed in vitro (Fig. 2B). We further tested whether the regulation of cell surface expression of costimulatory molecules would be similar in this setting. Naive CD8tgTCR cells were adoptively transferred into irradiated B6 and bm8 recipient mice (7) and splenocytes analyzed after 3 days. Fig. 4A reports defective induction of CD25, GITR, OX40, and 4-1BB when recipient mice expressed the bm8 partial agonist, compared with a
higher level of these surface molecules upon transfer in B6 recipient mice. However, in both situations, CD8tgTCR cells were activated as demonstrated by up-regulation of CD44, which remained marginal upon transfer into syngeneic (CBA) mice. Importantly, we observed that exogenous IL-2-enhanced expression of these surface molecules during the in vivo activation of the CD8 T cells in the bm8-expressing host. IL-2 treatment together with TCR engagement was required, because no such effect of IL-2 was observed upon the control transfer of CD8 T cells in CBA mice. The fact that exogenous IL-2 appeared slightly less efficient in vivo (Fig. 4A) than in vitro (Fig. 3A) may be due to its short half-life in vivo.

Regulation by IL-2 of CTL effector function in vitro and in vivo

To evaluate whether the duration of expression of genes encoding effector molecules has functional consequences, we measured CTL activity upon in vitro stimulation of naive CD8tgTCR cells. Fig. 3D shows that effector cells obtained following B6 stimulation displayed higher cytolytic activity than those responding to bm8, unless exogenous IL-2 was provided in the culture. In the latter case, real-time PCR demonstrated that addition of IL-2 led to a strong up-regulation of GzmB mRNA, equivalent to the level induced by B6, whereas the level of perforin transcript was only partially complemented by IL-2 (Fig. 3C). In the in vivo setting, we also observed an IL-2-dependent complementation of the weak cytolytic activity induced in CD8tgTCR cells transferred in bm8, compared with B6 recipient mice (Fig. 4B). These results demonstrated that IL-2- and TCR-mediated signals have a synergistic effect on expression of components that are limiting for cytolytic activity both in vitro and in vivo.

Molecular bases for IL-2-induced complementation of weak TCR-mediated signaling: STAT5 complements TCR signals for the induction of TNF-R family members GITR, 4-1BB, and OX40, as well as GzmB, Pim-1, Lta-α, and CD25

Ligand-induced heterodimerization of the IL-2Rβ and γc cytoplasmic domains activates Jak1 and Jak3, which phosphorylate tyrosine residues on the IL-2R β-chain, which in turn serve as docking sites for STAT5 (23) and Shc. STAT5, a member of the signal transducers and activators of transcription (STAT) is important in modulating T cell functions through the IL-2R (27). Moreover, TCR stimulation has been shown to directly activate STAT5 in some experimental settings (28), but not in others (29). Hence, it

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**FIGURE 5.** In synergy with weak TCR engagement, STAT5CA mimics the effects of low dose IL-2 on the expression of a panel of transcripts and surface molecules. A, CD8tgTCR cells were cultured with medium (1, 2) or B6 (3, 6) or bm8 (4, 5) APCs without addition (1, 3, 4) or in the presence of rIL-2 (2, 5) or mAb to IL-2 and CD25 (6). After 72 h, nuclear (top) and cytoplasmic (bottom) extracts were analyzed by Western blot using anti-phospho-STAT5 or STAT5 Abs, respectively. B, C, and D, After 48 h of culture with bm8 APCs in the absence of IL-2, CD8tgTCR cells were infected with retrovirus-containing supernatants and cultured for an additional 48 h. B and C, GFP+ cells transduced with either STAT5CA or mock vectors were sorted, and their RNA content was used for real-time PCR (B) and microarray analysis (C). D, Immunofluorescence is shown for GFP+ gated cells transduced with either STAT5CA (bold line) or mock (dotted line) vectors, as well as control nontransduced GFP- cells (in gray). E, CD8tgTCR cells were activated for 72 h with bm8 APCs in the presence of mAb to IL-2 (bm8) or in the presence of rIL-2 (bm8 + IL-2). ChIP with anti-Stat5b or irrelevant (IgG) Abs were analyzed by real-time PCR. The 2-ΔΔCt values (2-ΔΔCt = (ΔΔCt TARGET − ΔΔCt HPRT) [bm8 + IL-2] − (ΔΔCt TARGET − ΔΔCt HPRT) [bm8]) were calculated for at least three independent experiments. For a given target gene, a Student t test was applied on the ChIp of the STAT5 consensus sequence compared with a control sequence (*, p < 0.05; **, p < 0.01). ChIPs with irrelevant Ab showed no significant difference between the two conditions (not shown).
was of particular interest to investigate how this transcription factor underwent phosphorylation and nuclear translocation upon strong or weak TCR engagement. Phospho-STAT5 was absent in the nucleus of unstimulated CD8tgTCR cells but was translocated in low quantities at 24 and 48 h (data not shown) and in high amounts at 72 h following activation by B6 APCs (Fig. 5A). In contrast, phospho-STAT5 was barely detectable in nuclei of bm8-stimulated T cells at all time points (Fig. 5A; only visible with long exposure at 72 h; not shown). Importantly, when exogenous IL-2 was added together with bm8 APCs, a high level of phospho-STAT5 was translocated at 72 h, a time when expression of mixed CD25/CD122 IL-2R (Fig. 2B) was also observed. IL-2 failed to induce phospho-STAT5 in unstimulated CD8tgTCR cells, consistent with the absence of CD25 (Fig. 2B). To evaluate whether strong TCR signaling alone was capable of modulating STAT5 activation, blocking Abs to IL-2 and IL-2R were added to B6-stimulated CD8tgTCR cell cultures. The amount of phospho-STAT5 detected in the nucleus in these conditions was strongly reduced (Fig. 5A, lane 6).

Whether STAT5 plays a role in the sustained gene regulation observed upon addition of IL-2 in conditions of suboptimal TCR engagement was further addressed by transducing bm8-activated CD8tgTCR cells with retroviruses driving the expression of a constitutively active form of STAT5 (STATCA) (20). In bm8-activated and retroviral transduced GFP+ CD8tgTCR cells cultured in the absence of added IL-2, expression of STATCA induced the expression of CD25, GITR, 4-1BB, OX40, GzmB, Lt-α, and Pim-1 both at the level of mRNA (Fig. 5, B and Cb) and protein (Fig. 5D). In contrast, we also identified a subset of genes, including Prf1, regulated by IL-2 independently of STAT5 (cf Fig. 1Aa and Fig. 5C). Finally, the expression of CCL3, CCL4, CD5, CD6, and CD44, which were not regulated by IL-2 signaling (Fig. 1A), were not affected by STATCA (Fig. 5C). Control transduction with the mock GFP vector did not alter surface expression of all these markers. Altogether, activation of STAT5 appeared to be a main contributor to the IL-2R-driven signal acting in synergy with a TCR engagement.

Bioinformatics analysis allowed us to identify conserved STAT5 binding sites (GAS-like sequences) in the 5′ regions and in the first intron of the genes reported in Table II. To further analyze the mechanism by which STAT5 could implement transcription initiation by weak TCR engagement, we performed ChIPs using anti-STAT5b mAb on CD8tgTCR cells stimulated by bm8 APCs in the presence or absence of IL-2 (Fig. 5E). Among the three potential STAT5 binding sites identified in the promoter region of 4-1BB, one (4-1BB 2) was significantly enriched (p < 0.01) with the anti-STAT5 mAb compared with a control sequence (4-1BB Ctrl) in condition of culture with IL-2. For GITR, a partially degenerated STAT5 motif overlapping an Ets-1 binding site was identified, but anti-STAT5 failed to enrich this particular sequence. No STAT5 consensus sites were found in the promoter and the first intron of the OX40 gene. Altogether, these ChIP experiments allowed us to establish that 4-1BB is a direct target gene of STAT5, whereas further analysis will be required to establish how GITR and OX40 gene expressions are modulated by STAT5CA (Fig. 5, B–D). Additionally, we characterized occupied STAT5 consensus sequences in the promoter of the GzmB gene that had not previously been described. In these ChIP experiments, the PRR-4 element in the first intron of CD25 (24) and the GAS-like element in the promoter of the Lt-α gene (30) were taken as positive controls, whereas the CDS sequence appeared not to be regulated by STAT5 in accordance with the STAT5CA transduction data (see Fig. 5, B–D).

**Discussion**

**Synergy between engagement of low avidity TCR and IL-2R affects early differentiation in addition to late proliferation of naive CD8 T cells**

We addressed the question of the influence of strength of TCR signal on the development of a functional program in naive alloreactive CD8 T cells in a 2- to 4-day window after antigenic stimulation. The expression and composition of the IL-2R complex was found to be differentially regulated by strength of TCR signal during that period (Fig. 2B). This allowed us to characterize a first step involving autocrine or paracrine IL-2 that controls divergence in the fate of naive CD8 T cells. In this study, cDNA microarray analysis revealed an important feature of IL-2 on primary activated CD8 T cells, namely a costimulatory effect on sustained regulation of Ag-induced gene expression for a subset of genes that included CTL effector molecules and TNF-R family members (GITR, 4-1BB, and OX40) thought to be important for T cell costimulation. IL-15 was unable to substitute for IL-2 at that stage (not shown).

Importantly, in vivo activation of the naive CD8tgTCR cells following adoptive transfer into irradiated recipients expressing either B6 or bm8 alloantigens resulted in distinct function (Fig. 4B) and phenotype (Fig. 4A) associated with profoundly divergent outcomes, with induction of a lethal GVHR in the former situation and a nonaggressive long-term maintenance in the latter situation (7). Interestingly, in a GVHR model, engagement of GITR by an agonist ligand on CD8 T cell effectors was recently shown to induce increased mortality (31). Both sets of data are coherent with a contribution of GITR expression on CD8 T cells to the severity of GVHR. Depending on whether therapy protocols aim at dampening CD8 T cell responses as in GVHR, or at increasing their efficiency as in anti-tumor reactions, the effect of IL-2 on initial Ag-driven CD8 T cell differentiation including increased effector function and up-regulation of the TNF-R molecules such as GITR, 4-1BB, and OX40 should be avoided or promoted (31–34). Indeed, IL-2 treatment of tumor-bearing mice also leads to enhanced expression of these TNF-R molecules on tumor-specific CD8 T cells (G. Verdel, unpublished results).

Interestingly, genes involved in T cell proliferation such as myc, cyclins D2, D3, and E, previously described to be transcriptionally regulated by a PI3K pathway activated by IL-2R signals in a CTLL line (35) were not among the genes regulated in synergy with TCR signaling. This may be related to the fact that although IL-2 efficiently induces proliferation of T cell lines or preactivated T cells (36, 37), initial Ag-induced cell division appeared independent of IL-2 for naive T cells (Fig. 2A, day 3) (38). However, IL-2 had a slight effect on late cell division by bm8-stimulated CD8tgTCR cells (Fig. 2A, day 4).

Also, our study is distinct from previous work describing IL-2 responsive genes on optimally preactivated T cells or T cell lines (26, 36, 39, 40). Indeed, no study has systematically examined the modification of gene expression profiles to be attributed, respectively, to TCR and IL-2R in naive CD8 T cells during the first 72 h of their primary response. Consequently, our study uniquely examines the role of the initial strength of TCR engagement for commitment to effector cells and defines a distinct gene profile that highlights those genes that are regulated synergistically by low dose IL-2 with a limiting TCR signal. Those circumstances may be particularly relevant for vaccination protocols to weakly antigenic tumors (41).
Transient gene expression is a signature for partial CD8 T cell activation

Strikingly, we found that a main feature of gene expression profiles in the response of naive CD8 T cells to a partial agonist was their transience. The question whether this characteristic is 1) directly due to the low avidity TCR stimulation, and/or 2) indirectly linked to the defect in IL-2 production that abrogates synergistic pathways between TCR and IL-2R signaling was further addressed in this study.

Consistent with the first possibility, the expression of some early induced genes such as CD5 and the chemokines CCL3, CCL4, and XCL1, failed to increase or to become sustained in the presence of IL-2. This may be due to defective early signaling involving the ERK pathway in response to partial agonist, as previously suggested (15, 42). Indeed, it has been proposed that the means by which triggering of the same tgTCR on a monoclonal CD8 population by either a strong or a weak agonist could lead to different outcomes might rely on the intensity and duration of intracellular signaling. In particular, some sensors including early immediate gene products appear to act as general integrators for sustained ERK activation (43).

STAT5 acts as a stabilizer of TCR-induced gene expression for a subset of genes

The expression of a large number of genes, however, appeared regulated sequentially by TCR- and IL-2R-mediated signals. These included genes already known to be regulated by IL-2 such as CD25 (44), Pim-1 (45), and GzmB (46) and also genes for which such dual control has not been described. Particularly intriguing is the case of the TNF-R family members GITR, 4-1BB, and OX40. Their genes are located in contiguity on the same chromosomes in mice (Chr4) and man (Chr1), whereas the corresponding proteins, characterized by homologous intracellular segments devoid of death domains, are thought to be involved in CD4 and/or CD8 T-cell costimulation (47–50). Herein, we report that three TNF-R gene members have their expression 1) regulated by the strength of TCR engagement and 2) modulated by IL-2R signaling through the activation of STAT5. This transcription factor was further shown to bind conserved motifs in the promoter region of the 4-1BB, GzmB, and Lt-α genes (Fig. 5E and Table II).

We have stressed the regulation of genes belonging to the TNF-R superfamily and CTL effector function, but a thorough analysis of the genes that become accessible to the transcriptional machinery upon weak TCR engagement will require further analysis. Among these other candidate genes, the regulation of gene expression and function of oncostatin M, a Th1-produced cytokine that regulates hematopoietic progenitor cell homeostasis (51) might be of particular interest.

The sequential gene accessibility model

We have identified STAT5 binding sites in the 5’ regions of some TNF-R family members that may be candidate sites in a sequential gene accessibility model. In the face of transient TCR-induced signaling that characterizes the response to weak agonists, it will be interesting to establish which genes become accessible to the transcriptional machinery upon weak TCR engagement, and which factors (including STAT5) may contribute to stabilizing TCR-initiated gene expression. In Th2-polarizing Cd4 T cell cultures, signaling through STAT5 2 days after Ag stimulation is required to prepare T cells to produce Th2 cytokines upon subsequent challenge (52). Mechanistically, collaboration between the TCR-induced GATA-3 and the IL-2-dependent activation of STAT5 has been proposed to stabilize the accessibility of the IL-4 gene (53).

There may thus be a general scheme for STAT5 in stabilizing gene expression in T cells. In B lymphocytes, STAT5b activation is a control point where a decision between plasma or memory cell fate is made (54). STAT5 thus appears to control secondary decisions in adaptive immunity involving CTL, Th2, or B cells.

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Disclosures

The authors have no financial conflict of interest.

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