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Transcriptional Control of Rapid Recall by Memory CD4 T Cells

Wendy Lai, Minjun Yu, Min-Nung Huang, Francesca Okoye, Achesah D. Keegan, and Donna L. Farber

Memory T cells are distinguished from naïve T cells by their rapid production of effector cytokines, although mechanisms for this recall response remain undefined. In this study, we investigated transcriptional mechanisms for rapid IFN-γ production by Ag-specific memory CD4 T cells. In naïve CD4 T cells, IFN-γ production only occurred after sustained Ag activation and was associated with high expression of the T-bet transcription factor required for Th1 differentiation and with T-bet binding to the IFN-γ promoter as assessed by chromatin immunoprecipitation analysis. By contrast, immediate IFN-γ production by Ag-stimulated memory CD4 T cells occurred in the absence of significant nuclear T-bet expression or T-bet engagement on the IFN-γ promoter. We identified rapid induction of NF-κB transcriptional activity and increased engagement of NF-κB on the IFN-γ promoter at rapid times after TCR stimulation of memory compared with naïve CD4 T cells. Moreover, pharmacologic inhibition of NF-κB activity or peptide-mediated inhibition of NF-κB p50 translocation abrogated early memory T cell signaling and TCR-mediated effector function. Our results reveal a molecular mechanism for memory T cell recall through enhanced NF-κB p50 activation and promoter engagement, with important implications for memory T cell modulation in vaccines, autoimmunity, and transplantation. The Journal of Immunology, 2011, 187: 000–000.

Memory T cells exhibit enhanced functional properties compared with naïve T cells in their rapid production of effector cytokines upon Ag stimulation leading to efficacious secondary immune responses (1, 2). In contrast to naïve CD4 T cells, which can only produce effector cytokines such as IFN-γ after sustained antigenic stimulation over days, memory CD4 T cells can produce IFN-γ within hours of TCR stimulation. The signaling pathways and mechanisms controlling these different effector responses and expedient memory T cell recall are not known. Although it has been established that the acquisition of primary effector capacity such as IFN-γ production is controlled by upregulation of specific transcription factors in activated naïve CD4 T cells, it is not known whether rapid IFN-γ secretion by memory T cells is controlled at the transcriptional level and/or whether differences in transcription factor mobilization or regulation play a role.

IFN-γ transcription in CD4 T cells is predominately regulated by the T-box transcription factor, T-bet. T-bet expression is both necessary and sufficient for IFN-γ production and differentiation of Th1 effector cells during a primary response from naïve CD4 T cell precursors (3). In addition, CD4 T cells from T-bet-deficient mice are substantially impaired in IFN-γ production in both primary and secondary responses (4, 5). Whether T-bet is required in wild-type memory CD4 T cells for rapid IFN-γ production and its role in secondary responses is not known.

IFN-γ production in CD4 T cells is also regulated by the ubiquitous transcription factor, NF-κB. NF-κB comprises a family of transcription factor subunits that control expression of multiple immune-related genes in both innate cells and lymphocytes (6). In resting cells, dimers or heterodimers of NF-κB subunits p50 and/or p65 (RelA) are associated with precursor and inhibitor molecules p105 and IκB, respectively (7). Ag engagement of the TCR triggers a signaling cascade resulting in inhibitor degradation and nuclear translocation of active NF-κB subunits for transcriptional regulation of multiple genes including IL-2, the high-affinity IL-2 receptor (CD25), and IFN-γ (8, 9). The role of NF-κB transcriptional activity in memory CD4 T cell function has not been defined, and the subunits important for memory T cell recall are not known.

In this study, we hypothesized that rapid recall production of the effector cytokine IFN-γ by memory CD4 T cells was regulated by the enhanced activity of transcription factors. We focused on the role of T-bet and NF-κB in memory recall because of their known role in regulating IFN-γ production during primary responses. We demonstrate that rapid IFN-γ production by Ag-specific memory CD4 T cells is regulated on the transcriptional level and occurred from a population that did not upregulate T-bet expression but was associated with NF-κB p50 nuclear translocation and increased nuclear p50 expression. Chromatin immunoprecipitation (ChIP) analyses further revealed that NF-κB but not T-bet was engaged on the IFN-γ promoter in memory CD4 T cells at early times after Ag stimulation. Moreover, inhibition of NF-κB activity by ammonium pyrrolidine dithiocarbamate (PDTC; a pharmacological inhibitor) or by a cell-permeable peptide inhibitor of NF-κB p50 translocation abrogated early recall responses by memory CD4 T cells. Our results indicate differential control of IFN-γ transcription in naïve and memory CD4 T cells and a molecular mechanism for rapid memory recall responses through NF-κB p50...
activity and promoter engagement. The identification of a transcriptional regulator for memory immune responses is important for memory modulation in vaccines, autoimmunity, and transplantation.

Materials and Methods

Mice
BALB/c mice (8–16 wk of age) were obtained from the National Cancer Institute Biological Testing Branch. DO11.10, DO11.10×RAG2<sup>−/−</sup>, and RAG2<sup>−/−</sup> mice (Taconic Farms, Germantown, NY) were maintained in the Animal Facility at the University of Maryland (Baltimore, MD) and at Columbia University under specific pathogen-free conditions. All animal procedures were approved by the institutional animal care and use committee for both institutions.

Abs and reagents
Fluorescently conjugated anti–IFN-γ, anti–IL-2, anti–CD25, anti–CD44, anti–CD62L, and anti–CD4 Abs were purchased from BD Pharmingen (San Diego, CA); anti–T-bet was from eBioscience (San Diego, CA), and K1-26 specific for DO11.10 TCR was from Caltag Laboratories (Burlingame, CA). CHIP grade Abs specific for NF-κB (p50) and T-bet were from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-histone H3 and anti-p50/105 Abs were from Abcam (Cambridge, MA). For Western blotting, rabbit anti–p105/50 was purchased from Abcam, anti–actin (sc-47778) from Santa Cruz Biotechnology, and mouse anti–T-bet (4B10) was purchased from BD Pharmingen. HPLC-purified OVA peptide (323–339; IASQAVHAHAEINEAGA) was synthesized by the Biopolymer Laboratory at the University of Maryland. PDTC was obtained from Sigma (St. Louis, MO), and cell-permeable p50 inhibitor and inactive control peptides were purchased from CalBiochem/EMD Biosciences (San Diego, CA).

Generation and isolation of naive and memory CD4 T cells
OVA-specific naive CD4 T cells were isolated from the spleens of DO11.10 or DO11.10×RAG2<sup>−/−</sup> mice as described (10). OVA-specific memory CD4 T cells were generated as described (10, 11) by in vitro priming of DO11.10 CD4 T cells with 1.0 μg/ml OVA peptide and APC<sup>+</sup> and adoptive transfer of the resultant primed/effector cells into RAG2<sup>−/−</sup> adoptive hosts, with persisting memory CD4 T cells recovered 2–5 mo posttransfer. Polyclonal naive and memory CD4 T cells were isolated from whole CD4 T cells based on CD4 expression using anti–CD44–conjugated magnetic MACS microbeads and separated on a MACS magnet into CD44<sup>hi</sup> (naive) and CD44<sup>lo</sup> (memory) CD4 T cell subsets, as previously described (10, 11). RAG2<sup>−/−</sup> T cells were stimulated with anti-CD3 (4 μg/ml) Abs in complete media at 37°C for 6–48 h with anti-CD28 Abs to prime the T cells, followed by anti–IFN-γ production by memory T cells is transcriptionally controlled. We compared the kinetics of effector cytokine production in Ag-specific naive and memory CD4 T cells after Ag stimulation. Naive, OVA-specific CD4 T cells were obtained from DO11.10 TCR transgenic mice, either on BALB/c or RAG2<sup>−/−</sup> backgrounds as previously described (10). OVA-specific memory CD4 T cells were generated by adoptive transfer of primed DO11.10 CD4 T cells into adoptive hosts, and recovery of persisting memory CD4 T cells from host mice occurred 2–4 mo posttransfer as described (10, 11, 19). Consistent with our earlier findings (10), antigenic stimulation of OVA-specific naive and memory CD4 T cells resulted in a Th1 cytokine profile with IFN-γ production by memory CD4 T cells (25–40%) produced IFN-γ within 6 h of T-cell activation.

Intracellular cytokine staining analysis
CD T cells were cultured with APC and 1 μg/ml OVA peptide or with anti–CD3 (5 μg/ml)/anti–CD28 (5 μg/ml) Abs in the presence of monensin (Golgistop; BD Pharmingen) added 6 h prior to cell harvest. Cytokine production was assessed by intracellular cytokine staining as described (14) and analyzed using LSR II and FACSDiva software (BD Biosciences).

Real-time PCR analysis
OVA-specific naive and memory CD4 T cells were isolated from DO11.10×RAG2<sup>−/−</sup> mice and from RAG2<sup>−/−</sup> adoptive hosts of primed DO11.10 CD4 T cells, activated in vitro with anti–CD3/anti–CD28 Abs as above, and isolated at 0–72 h. RNA was isolated from 3 × 10<sup>6</sup> to 5 × 10<sup>6</sup> cells using the RNasey Mini Kit (Qiagen, Valencia, CA), and 5 μg total RNA was used to generate cDNA using Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA). IFN-γ sequences were amplified from cDNA using primers 5′-TCTGAGCAATGAACGCTACAC-3′ (sense) and 5′-TCTTCACATCATCACATCCACTT-3′ (anti-sense) along with control HGPRT and GAPDH sequences in SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA), using the 7900 HT fast real-time PCR systems (Applied Biosystems).

Promoter–reporter assays
The following promoter–luciferase constructs for transcriptional reporter assays were obtained from Agilent Technologies (Santa Clara, CA): NF-κB (NF-κB–Luc), NIFAT (NIFAT–Luc) promoter–luciferase, and the pCIS-CX negative control plasmid. Positive control pGL3 plasmid with firefly luciferase driven by the CMV promoter and the Renilla luciferase reporter vector (pRL–CMV) were obtained from Promega Corporation (Madison, WI). Whole unfraccionated CD4 T cells or CD44hi cells isolated from BALB/c mice were transfected directly or activated with anti–CD3/anti–CD28 Abs for 24 h prior to transfection with the indicated reporter constructs using nucleofection with the mouse T cell transfection kit V (Lonza, Cologne, Germany) as previously described (15). After transfection, cells were incubated overnight at 37°C 5% CO<sub>2</sub> in complete Clicks medium and subsequently lysed in Passive Lysis Buffer (Promega) provided as part of the Dual-Luciferase Reporter Assay System (Promega). An aliquot of each lysate was mixed with luciferin substrate, and within 30 s, luciferase activity was measured based on light emission at 562 nm using the Turner Devices Model TD-20/20 Luminometer (Promega), which automatically measures both firefly and Renilla luciferase activities. Readings for each sample were normalized by dividing the firefly/Renilla units, and activities within resting and TCR-stimulated T cells were expressed as the percentage of the positive control (pCMV–GL3).

ChIP and PCR
ChIP analysis of T-bet and NF-κB binding to the IFN-γ promoter was performed using the QuikChIP Assay Kit according to the manufacturer’s instructions (Imgenex Corporation, San Diego, CA). OVA-specific naive and memory CD4 T cells were activated with anti–CD3 (5 μg/ml) plus anti–CD28 (2.5 μg/ml) Abs in complete media at 37°C for 6–72 h, fixed, and lysed in SDS Lysis Buffer (Imgenex). After sonication to shear the DNA, samples were immunoprecipitated with anti-histone H3, anti-p50, anti–T-bet, or no Abs followed by protein A agarose at 4°C. ChIP sample DNA was amplified by PCR (40 cycles) using primers corresponding with consensus in the mouse IFN-γ promoter containing the NF-κB (16) or T-bet (17) binding sites. For quantitative PCR, 20 ng of total cDNA was amplified using SYBR Green as above on an Applied Biosystems 7900HT. Ct values were compared using the 8–δCt method (Applied Biosystems) using mouse IgG samples for negative controls.

Western blotting
Polyclonal naive and memory CD4 T cells were isolated to >90% purity by MACS separation as above and were either lysed directly or activated for 4–6 h with anti–CD3/anti–CD28 Abs in complete media at 37°C prior to lysis. For isolation of cytoplasmic and nuclear fractions, cells were initially lysed in buffer A (10 mM HEPES/10 mM KCl/10 mM EDTA/1 mM DTT/0.4% IGEPAL, 1 μg/ml protease inhibitor mixture) at 4°C, followed by centrifugation to yield the cytoplasmic fraction in the supernatant and nuclear fraction in the pellet. Nuclear pellets were solubilized in lysis buffer (10 mM HEPES/400 mM NaCl/1 mM EDTA/1 mM DTT, 1 μg/ml protease inhibitor mixture) at 4°C, followed by isolation of the nuclear lysate by centrifugation. Nuclear and cytoplasmic lysates were resolved on 4–20% SDS gels and immunoblotted with anti–actin, anti–T-beta, and anti-p50/105 Abs as described (10, 18).

Results
Rapid IFN-γ production by memory T cells is transcriptionally controlled
We compared the kinetics of effector cytokine production in Ag-specific naive and memory CD4 T cells after Ag stimulation. Naive, OVA-specific CD4 T cells were obtained from DO11.10 TCR transgenic mice, either on BALB/c or RAG2<sup>−/−</sup> backgrounds as previously described (10). OVA-specific memory CD4 T cells were generated by adoptive transfer of primed DO11.10 CD4 T cells into adoptive hosts, and recovery of persisting memory CD4 T cells from host mice occurred 2–4 mo posttransfer as described (10, 11, 19). Consistent with our earlier findings (10), antigenic stimulation of OVA-specific naive and memory CD4 T cells resulted in a Th1 cytokine profile with IFN-γ as the predominant effector cytokine (data not shown) and distinct kinetic profiles. Naive CD4 T cells exhibited peak IFN-γ production after 48–72 h of stimulation, with negligible IFN-γ production at 6–24 h poststimulation (Fig. 1A). By contrast, a high frequency of memory CD4 T cells (25–40%) produced IFN-γ within 6 h of Ag activation, with peak IFN-γ production at 24 h, demon-
Materials and Methods
isolated as in
and memory CD4 T cells. OVA-specific naive and memory CD4 T cells
and IFN-γ in resting naive and memory CD4 T cells, the level of IFN-γ
over a similar kinetic window of activation (Fig. 1) resulted in peak IFN-γ
and activated OVA-specific naive and memory subsets by real-
generated stimulation for memory CD4 T cells and after 72 h for naive
CD4 T cells (Fig. 2A). TCR/CD3 stimulation of polyclonal CD4 T
T-bet was upregulated in both naive and memory CD4 T cells after 48 h,
although T-bet upregulation was greater in polyclonal memory compared
with naive CD4 T cells after 24 h of activation (Fig. 2B). These results show enhanced upregulation of T-bet expression after TCR stimulation of memory compared with naive

stratating the enhanced kinetics and magnitude of effector cytokine production that is a distinguishing feature of memory CD4 T cells.

We investigated whether the rapid production of IFN-γ from memory CD4 T cells was due to production of new transcripts or derived from preexisting IFN-γ transcripts. To distinguish these possibilities, we quantitated the level of IFN-γ mRNA in resting and activated OVA-specific naive and memory subsets by real-time PCR over a similar kinetic window of activation (Fig. 1B). In resting naive and memory CD4 T cells, the level of IFN-γ mRNA was below detection, indicating that neither subset harbors significant levels of preformed IFN-γ transcripts. However, the kinetics and magnitude of IFN-γ-specific mRNA differed vastly between the two subsets. Notably, memory CD4 T cells exhibited a dramatic increase and peak levels of IFN-γ transcripts after only 6 h of TCR stimulation, whereas stimulation of naive CD4 T cells resulted in peak IFN-γ mRNA content after 24–48 h (Fig. 1B and inset). In addition, memory CD4 T cells produced >10-fold more IFN-γ mRNA at peak time points compared with activated naive CD4 T cells (Fig. 1B and inset). These results indicate that the enhanced kinetics and magnitude of IFN-γ production by memory CD4 T cells is controlled at the level of mRNA and that preformed mRNA for IFN-γ is not notably present in memory CD4 T cells.

Lack of constitutive or rapidly upregulated T-bet expression in memory CD4 T cells

We hypothesized that rapid production of IFN-γ mRNA in memory CD4 T cells could be due to elevated expression of the T-bet transcription factor that is required for IFN-γ transcription by Th1 effector cells (3). However, we found intracellular T-bet expression to be comparably low/negative in resting Ag-specific naive and memory CD4 T cells (Fig. 2A), as well as in resting polyclonal naive (CD44lo) and memory (CD44hi) CD4 T cells (Fig. 2B). After activation with Ag, there was minimal upregulation of T-bet in OVA-specific naive and memory CD4 T cells at 6 h, with maximal T-bet upregulation observed after 48 h of antigenic stimulation for memory CD4 T cells and after 72 h for naive CD4 T cells (Fig. 2A). TCR/CD3 stimulation of polyclonal CD4 T cells with anti-CD3/anti-CD28 Abs resulted in maximal T-bet upregulation in both naive and memory CD4 T cells after 48 h, although T-bet upregulation was greater in polyclonal memory compared with naive CD4 T cells after 24 h of activation (Fig. 2B). These results show enhanced upregulation of T-bet expression after TCR stimulation of memory compared with naive

FIGURE 1. Kinetics of IFN-γ secretion and transcript expression in naive and memory CD4 T cells. A, Rapid and enhanced production of IFN-γ in Ag-stimulated memory versus naive CD4 T cells. OVA-specific naive and memory CD4 T cells (see Materials and Methods) were stimulated with OVA peptide and APC for 0–72 h, and IFN-γ production was determined by intracellular cytokine staining. Results are expressed as percentage of Ag-specific IFN-γ+ cells gated on CD4+KJ1-26+ cells at each time point, from one experiment representative of five independent experiments. B, IFN-γ transcript expression in resting and activated naive and memory CD4 T cells. OVA-specific naive and memory CD4 T cells isolated as in A were activated with anti-CD3/anti-CD28 Abs for 6–72 h, and IFN-γ-specific mRNA was amplified by quantitative real-time PCR (see Materials and Methods). Results are expressed as IFN-γ transcript-levels relative to control HPRT transcripts. Inset shows level of IFN-γ mRNA in naive CD4 T cells with reduced scale for direct comparison of peak mRNA production.

FIGURE 2. Early IFN-γ production by memory CD4 T cells is not associated with upregulation of T-bet expression or promoter engagement. A, T-bet expression in resting and Ag-stimulated naive and memory CD4 T cells. OVA-specific naive and memory CD4 T cells were stimulated with OVA/APC as in A and T-bet expression assessed by intracellular staining with T-bet-specific Abs. Histograms are gated on CD4+KJ1-26+ cells. B, T-bet expression in resting and TCR-stimulated polyclonal naive (CD44lo) and memory (CD44hi) CD4 T cells. Histograms shown are gated on live CD4+ T cells. C, T-bet expression as a function of IFN-γ production in resting and Ag-activated OVA-specific naive and memory CD4 T cells as in A. T-bet and IFN-γ expression are shown gated on CD4+KJ1-26+ cells. Results are representative of five independent experiments.
subsets only after sustained stimulation (24–48 h) and beyond the time point for rapid recall.

We further evaluated whether IFN-γ production from naive or memory CD4 T cells was occurring from cells that had upregulated T-bet (Fig. 2C). In OVA-speciﬁc naive CD4 T cells, IFN-γ production was detected 24–48 h after antigenic stimulation exclusively from the T-bethi population (Fig. 2C, top row), consistent with the requirement for T-bet expression for Th1 generation. By contrast, in memory CD4 T cells, IFN-γ production at early times of antigenic stimulation (6–24 h) occurred from T-betlo cells and was only associated with T-bethi cells after sustained (48–72 h) Ag stimulation (Fig. 2C, bottom row). This coordinated analysis of T-bet and IFN-γ production revealed that early IFN-γ production by memory T cells occurred from distinct populations relative to T-bet expression, with rapid IFN-γ production occurring in the absence of sustained T-bet expression or upregulation.

NF-κB transcriptional activity is detected at early times after TCR stimulation in CD4 T cells

We investigated whether the transcription factors NF-κB or NFAT, previously shown to be involved in IFN-γ transcription in cell lines or primary T cells (8, 9), were activated at early times after TCR stimulation in CD4 T cells using luciferase reporter assays. We initially performed this analysis in whole, un fractionated CD4 T cells from BALB/c mice, as we previously showed that the population producing IFN-γ at early times after activation corresponds with memory phenotype CD44hi cells (10). We transfected resting and anti-CD3/anti-CD28–stimulated CD4 T cells with promoter–reporter constructs in which luciferase was driven by NF-κB–binding (NF-κB–Luc) or NFAT-binding promoter elements (NFAT [IL-2]–Luc) or with a constitutively active CMV promoter (pGL3) as a positive control and a promoterless luciferase plasmid (pCIS–CK) as a negative control. All luciferase constructs were cotransfected with a plasmid expressing Renilla luciferase (pRL–CMV) for normalization of luciferase activity between samples using the dual luciferase reporter assay (see Materials and Methods). In resting CD4 T cells, there was negligible NFAT activity and a low level of basal NF-κB activity. After TCR stimulation for 24 h, NF-κB activity increased substantially (3- to 4-fold), whereas NFAT activity was not significantly higher than that of the negative control (Fig. 3A), consistent with other studies that also found negligible NFAT activity in Th1-like effector cells or after 24-h activation of primary mouse T cells (20). We also fractionated the memory (CD44hi) subset from total CD4 T cells and found a similar low, yet detectable level of NF-κB activity in resting-memory-phenotype CD44hi CD4 T cells, upregulation of NF-κB transcriptional activity within 24 h of TCR stimulation of this subset (Fig. 3B), and no detectable NFAT activity at this time point (data not shown). Together, these results indicate that NF-κB but not NFAT activity is readily triggered early after TCR engagement of memory CD4 T cells.

Nuclear translocation of T-bet and NF-κB after TCR stimulation in naive and memory CD4 T cells

We investigated whether T-bet or NF-κB transcription factors were differentially expressed and/or mobilized to the nucleus in naive or memory CD4 T cells. We analyzed T-bet and NF-κB p50/p105 expression in the cytoplasm and nuclear fractions of polyclonal naive and memory CD4 T cells in the resting state and after different time points of TCR stimulation (Fig. 4). Consistent with the flow cytometry results, we did not detect T-bet expression in resting naive or memory CD4 T cells in the cytoplasm or nucleus. T-bet expression appeared as a faint band in the cytoplasmic fraction in 6-h–stimulated naive and memory CD4 T cells with maximal cytoplasmic T-bet expression after 48 h for both subsets (Fig. 4, top gel). In the nucleus, T-bet was not present in resting or 6-h–activated naive or memory CD4 T cells, with nuclear T-bet detected only after 24–48 h of activation for both subsets (Fig. 4, bottom gel). We analyzed expression of NF-κB p50, which is expressed as a complex with its precursor p105 inhibitor molecule in the cytoplasm (p50/p105) and as an active p50 form in the nucleus (6). Both resting naive and memory CD4 T cells had a low level of cytoplasmic p50/p105 that increased after 6–48 h of activation (Fig. 4A). In the nucleus, a faint NF-κB p50 band was detected in nuclear lysates of resting naive and memory CD4 T cells (no p105 was present, data not shown); however, after 6 h of stimulation, memory CD4 T cells exhibited a 1.8-fold increase in nuclear p50 by densitometry, whereas naive CD4 T cells maintained the same level of nuclear p50 as in the resting state (Fig. 4B). We also found a greater increase in p50 after 0–6 h stimulation of OVA-specific memory compared with naive CD4 T cells (Supplemental Fig. 1). These results indicate that at the time point for rapid recall (6 h after stimulation), NF-κB, but not T-bet, is present in the nucleus of naive and memory CD4 T cells and exhibits a greater-fold upregulation in memory CD4 T cells.

Rapid engagement of NF-κB but not T-bet on the IFN-γ promoter in memory CD4 T cells

To evaluate further the transcriptional mechanisms controlling early IFN-γ production by memory CD4 T cells, we used ChIP analysis to compare T-bet versus NF-κB p50 engagement on the
IFN-γ promoter in resting and activated naive and memory CD4 T cells. We evaluated whether a 360-bp fragment corresponding with the T-bet binding site on the IFN-γ promoter (17) was present in anti–T-bet immunoprecipitates (IPs) from OVA-specific naive and memory CD4 T cells before and after stimulation with anti-CD3/anti-CD28 Abs (used to avoid contaminating APC) (Fig. 5A). For the ChIP assay; no Ab IPs served as negative controls (−), anti-histone H3 IPs were positive controls (+), and the input DNA ("N") prior to immunoprecipitation was the positive PCR control. In naive T cells, both NF-κB and p50 were detected in anti–NF-κB IPs, whereas only NF-κB was detected in anti-p50 IPs (Fig. 5A). These results indicate similar delayed kinetics of T-bet binding to the IFN-γ promoter after TCR stimulation of naive T cells as detected by Western blot (Fig. 4). Second, the ChIP results show that mobilization of NF-κB is bound to the IFN-γ promoter in naive and memory CD4 T cells. OVA-specific memory CD4 T cells were isolated and prepared for ChIP analysis. Gel shows a 388-bp product corresponding with the NF-κB binding site on the mouse IFN-γ promoter that was PCR-amplified from IPs using protein A agarose alone (lanes marked "−"), anti-histone H3 (lanes marked "+"), or anti-p50 (NF-κB) Ab (lanes marked "κB"). C. Quantitative comparison of NF-κB versus T-bet binding to the IFN-γ promoter in naive and memory CD4 T cells. ChIP analysis was performed on resting and activated naive (N) and memory (M) CD4 T cells as in A and B, except real-time quantitative PCR was used for amplification. Graph shows the average fold induction over negative control IPs from four independent experiments.

For NF-κB ChIP, we used anti-p50 Abs to immunoprecipitate NF-κB and amplify out a 388-bp fragment corresponding to the NF-κB binding site of the IFN-γ promoter (16). By ChIP analysis, we detected a 388-bp band in anti-p50 IPs in memory CD4 T cells that were stimulated for 6–48 h but not in resting memory CD4 T cells (Fig. 5B). We further analyzed quantitative differences in the temporal binding of NF-κB p50 versus T-bet on the IFN-γ promoter in naive and memory CD4 T cells, using real-time quantitative PCR analysis of NF-κB p50 or T-bet ChIP (Fig. 5C). In naive CD4 T cells, both NF-κB p50 and T-bet showed maximal association to the IFN-γ promoter after 48 h of TCR stimulation (Fig. 5C). In memory CD4 T cells, there was increased engagement of NF-κB p50, but not T-bet, on the IFN-γ promoter 6 h after TCR stimulation, but after 48 h of TCR stimulation, both NF-κB and T-bet bound to the IFN-γ promoter (Fig. 5C). These quantitative ChIP results reveal two important insights into transcriptional control of rapid recall in memory T cells: First, they demonstrate that NF-κB is bound to the IFN-γ promoter at early times (6 h) poststimulation only in memory and not in naive CD4 T cells, despite the presence of nuclear p50 in 6-h–activated naive cells as detected by Western blot (Fig. 4). Second, the ChIP results show that mobilization of NF-κB on the IFN-γ promoter occurs earlier than T-bet in memory CD4 T cells. NF-κB activity and p50 translocation is required for rapid recall of memory CD4 T cells

To determine a functional requirement for NF-κB in memory CD4 T cell recall that was suggested by the molecular analyses above, we selectively inhibited NF-κB activity and assayed its effect on memory T cell recall and signaling. Activation of OVA-specific memory CD4 T cells with Ag (OVA/APC) in the presence of the...
pharmacologic NF-κB inhibitor PDTC (21, 22) resulted in near complete inhibition of early IFN-γ production at 6 and 24 h post-antigen stimulation (Fig. 6A). In addition, PDTC also inhibited Ag-driven CD25 upregulation (Fig. 6A, right panel), indicating that NF-κB is required for early recall responses by memory CD4 T cells.

To rule out secondary effects of PDTC on TCR-mediated events, we also used a cell-permeable, peptide-specific inhibitor of NF-κB p50 translocation (see Materials and Methods) to determine whether activation of NF-κB p50 was required for memory CD4 T cell signaling and function. As shown in Fig. 6B, activation of memory CD4 T cells for 6 h resulted in IFN-γ production that was unperturbed by the presence of a control cell-permeable peptide but reduced by >50% in the presence of a similar concentration of p50 inhibitor peptide (Fig. 6B). Importantly, the p50 inhibitor peptide also inhibited other early TCR-driven events in memory T cell activation including TCR downregulation, measured by surface expression of the KJ1-26 clonotype, and CD25 upregulation (Fig. 6C). These results demonstrate regulation of early memory CD4 T cell signaling and effector function via NF-κB p50 translocation.

Discussion

We present here new molecular and functional evidence for control of memory CD4 T cell recall by the transcription factor NF-κB.

Specifically, we demonstrate that IFN-γ production by Ag-specific and polyclonal memory CD4 T cells is associated with an increase in NF-κB activity and p50 subunit expression, but not nuclear expression of the T-bet transcription factor that is required for IFN-γ production during Th1 differentiation. Moreover, there was an increase in NF-κB p50 but not T-bet binding to the IFN-γ promoter in memory CD4 T cells after short-term stimulation by ChIP analysis. We further demonstrate that rapid IFN-γ production by Ag-stimulated memory CD4 T cells requires NF-κB transcriptional activity and that early memory T cell signaling and activation specifically requires nuclear translocation of the p50 subunit. Together, our findings provide a molecular basis for the anamnestic response by memory T cells.

We demonstrate that rapid IFN-γ production by memory CD4 T cells is controlled at the transcriptional level, as resting memory CD4 T cells displayed negligible expression of IFN-γ transcripts by real-time PCR, yet were substantially increased at 6 h after stimulation. Similarly, the lack of early IFN-γ production by naive CD4 T cells was mirrored by a lack of significant IFN-γ transcription until later times after stimulation. Our results are consistent with earlier results using IFN-γ promoter–luciferase reporter mice that showed IFN-γ promoter activity (i.e., luciferase expression) only in memory CD4 T cells early after stimulation and not in naive T cells (23). Differential transcription factor usage by naive and memory CD4 T cells has also been explored in additional types of promoter–luciferase transgenic mice. These studies established that AP-1 transcriptional activity was equivalent in naive and memory CD4 T cells (24), but accumulation of NFAT activity was required at later times (after 36 h) for IL-2 production (25). A role for NFAT or other transcription factors in early IFN-γ production was not identified in these studies, and we similarly did not find early induction of NFAT activity in memory CD4 T cells that correlated with early recall responses.

T-bet is both necessary and sufficient for Th1 differentiation and IFN-γ production resulting from stimulation of naive CD4 T cells (3, 5) and is the main controlling factor for IFN-γ production in CD4 T cells (26). We initially hypothesized that increased expression or upregulation of T-bet might control rapid IFN-γ production by memory CD4 T cells; however, results obtained through three different approaches together indicate that rapid recall by memory CD4 T cells is likely independent of T-bet transcriptional activity. First, by intracellular flow cytometry, we did not detect T-bet expression in resting or short-term activated Ag-specific or polyclonal memory CD4 T cells or an association with T-bet expression and rapid memory CD4 T cell IFN-γ producers. Second, by Western blot analysis, we were able to detect nuclear T-bet only after sustained (24–48 h) activation of both naive and memory T cells, indicating similar delayed kinetics of nuclear T-bet accumulation for both subsets. Finally, ChIP analysis revealed a similar late kinetics of T-bet binding to the IFN-γ promoter in activated naive and memory CD4 T cells. Our results are certainly consistent with findings that terminally differentiated Th1 effector cells did not exhibit a similar requirement for T-bet for IFN-γ production compared with primary Th1 effector (27, 28), due to stable T-bet–induced chromatin modifications at the IFN-γ locus (28).

Our results establish a role for NF-κB transcriptional activity and, in particular, translocation of the p50 subunit in rapid IFN-γ by memory CD4 T cells. In T cells, active forms of NF-κB comprise heterodimers of p50/p65 subunits or homodimers of p50 that need to be liberated from the associated inhibitor molecules in the cytoplasm (IκB and p105, respectively) prior to nuclear translocation and activation (29, 30). We found that translocation of p50 to the nucleus occurred at early times (from 5 to 60 min) in

FIGURE 6. NF-κB transcriptional activity and p50 translocation is required for rapid recall of memory CD4 T cells. A, OVA-specific memory CD4 T cells were stimulated with OVA/APC for 6 and 24 h in the presence of PDTC (25 μM) or PBS control, and T-bet expression, IFN-γ production, and CD25 induction were analyzed by flow cytometry gated on CD4+KJ1-26+ T cells. Results are representative of five experiments. B, OVA-specific memory CD4 T cells were activated for 6 h with anti-CD3/anti-CD28 Abs in the presence of media alone or media plus cell-permeable control peptide or p50 inhibitor peptide (see Materials and Methods), and IFN-γ production was assessed. Results are representative of three independent experiments with the average inhibition by p50i = 55 ± 11%. C, Memory CD4 T cells activated as in B showing downregulation of Ag-specific TCR expression (clonotype KJ1-26) and upregulation of CD25 expression after 6 h of activation with OVA/APC in the presence of control or p50i inhibitor peptide.
both memory and naive CD4 T cells (data not shown) with a greater increase in nuclear p50 observed between 0 and 6 h of activation of memory compared with naive CD4 T cells (Fig. 4). In addition, nuclear p50 was only found associated with the IFN-γ promoter in memory CD4 T cells and not in naive CD4 T cells at the 6-h activation time point. Together, the Western blot and ChIP analyses suggest that alterations in the IFN-γ promoter of memory CD4 T cells may facilitate access of NF-κB for transcriptional activation of the IFN-γ gene. Epigenetic modifications have also been identified in memory CD8 and CD4 T cells in the promoter regions of IFN-γ and other genes that are rapidly expressed upon secondary challenge (31–33), which may account for the ability of NF-κB to engage readily the IFN-γ promoter in memory but not naive CD4 T cells.

Our finding that IFN-γ transcripts were not detected in resting memory T cells and that TCR-mediated NF-κB p50 activation was required for rapid IFN-γ production by memory CD4 T cells together support TCR-mediated signaling control of early memory T cell recall. We previously showed in a model of TCR-mediated signaling ablation in memory T cells that deletion of the key TCR-coupled linker/adaptor molecule in memory T cells abrogated TCR-mediated rapid recall (34). In this study, we show that TCR-mediated triggering in wild-type memory T cells exhibits a preferential downstream requirement for p50 translocation to initiate early IFN-γ production. A role for p50 activation and translocation in memory T cells is also suggested by studies in gene-targeted mutant mice. Mice expressing a degradation-resistant p105 inhibitor subunit (preventing p50 translocation) exhibited profound defects in the development of memory T cells (35), and conversely, mice lacking p105, but expressing active p50, exhibited increased proportions of memory CD4 T cells (36). Furthermore, mice deficient in both p50/p105 also exhibited defects in T cell-mediated protective responses to an intracellular pathogen (37). Given the multiple immune abnormalities associated with global manipulation of NF-κB expression (6, 37), a precise analysis of the in vivo role of NF-κB subunits in memory responses in future studies will require manipulation of NF-κB expression specifically in memory CD4 T cells using targeted ablation approaches we have previously employed (34).

Our results provide new insights into the molecular control of amnestic immune responses. We reveal at least two pathways controlling memory T cell responses: a T-bet–dependent pathway for immediate recall regulated by NF-κB and a T-bet–dependent pathway for IFN-γ production at later times (38). Given the ubiquitous role of NF-κB in promoting rapid inflammatory cytokine production in innate immune cells, we propose that memory CD4 T cells have co-opted an innate type of signaling pathway for their rapid effector responses through the TCR. The efficient mobilization of NF-κB to relevant accessible promoters in memory T cells may therefore regulate immediate recall, with implications for memory modulation in vaccines and autoimmune diseases.

Disclosures

The authors have no financial conflicts of interest.

References