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Encephalitogenic T cells that stably express both T-bet and ROR γ t consistently produce IFN γ but have a spectrum of IL-17 profiles

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Abstract

Th1/Th17 cells, secreting both IFN γ and IL-17, are often associated with inflammatory pathology. We cloned and studied the cytokine phenotypes of MBP-specific, TCR-identical encephalitogenic CD4 $^{+}$ cells in relationship to Th1- and Th17-associated transcription factors T-bet and ROR γ t. IFN γ -producing cells could be sub-divided into those that are T-bet $^{+}$ /ROR γ t $^{-}$ and those that are T-bet $^{+}$ /ROR γ t $^{+}$. The latter comprises a spectrum of phenotypes, as defined by IL-17 production, and can be induced to up-regulate IL-23R with IL-12 or IL-23. The former, *bona fide* Th1 cells, lack IL-23R expression under all conditions. *In vivo*, T-bet $^{+}$ /ROR γ t $^{-}$ and T-bet $^{+}$ /ROR γ t $^{+}$ clones induce EAE equally well.

Keywords

Th1/Th17 cells; Inflammation; Cytokines; Transcription factors; EAE/MS

1. Introduction

The observation that different sets of cytokines are secreted by distinct groups of activated CD4 $^{+}$ T lymphocytes led, over twenty years ago, to the classification of T cells as belonging to either the Th1 or Th2 subset (Mosmann et al., 1986). With the more recent addition of Th17 and Treg subsets, our perspective on the diversity of Th cells has expanded, but the lineage relationships, interactions and specific roles played by each of the different Th subsets is still an evolving and ongoing subject of investigation (Harrington et al., 2006; McGeachy and Cua, 2008; Stockinger and Veldhoen, 2007). Studies show that naïve cells cultured *in vitro* under ‘polarizing’ conditions generally develop into discrete groups, including those that make IFN γ (“Th1”), and those that make IL-17 (“Th17”), upon activation (Bettelli et al., 2006; Langrish et al., 2005; Mangan et al., 2006; Nurieva et al., 2007; Park et al., 2005; Veldhoen et al., 2006). However, among cells isolated from inflammatory conditions *in vivo*, as well as from human peripheral blood, a population of cells that can secrete both IFN γ and IL-17 is present (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Ivanov et al., 2006; Lowes et al., 2008; Nistala et al., 2008; Suryani and Sutton, 2007; Wilson et al., 2007). The lineage relationship of this set of memory cells to Th1 and Th17 subsets is still unclear, and its

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properties are not well studied, most likely because it is not generated in the polarizing conditions commonly used to obtain Th1 and Th17 subsets from naïve cells *in vitro*.

Several reports have now dispelled the notion that Th17 are solely responsible for inflammatory pathology, and confirm that both Th1 and Th17 cells can induce inflammatory disease and pathology (Cox et al., 2008; Korn et al., 2007; Luger et al., 2008; Steinman, 2008). However, recent reports again raise the issue of lineage relationships. After transferring *in vitro* polarized IL-17-producing Th17 populations into adoptive recipients, Shi et al. (Shi et al., 2008), Martin-Orozco et al. (Martin-Orozco et al., 2009) and Bending et al. (Bending et al., 2009) observed a 'phenotype switch' to IFN γ -producing Th1 cells. Because these studies utilized populations of cells, albeit polarized, highly purified and well characterized, the origin of the newly emergent IFN γ -producing cell population is not entirely clear.

In this report, we present relevant findings from our studies of a panel of cloned T cell lines. T cells cloned from immunized mice, obtained both from lymph nodes and from the central nervous system (CNS) of mice that have developed EAE, are genotypically either T-bet⁺/ROR γ t⁻ or T-bet⁺ROR γ t⁺. When characterized by cytokine production, the former (T-bet⁺/ROR γ t⁻) constitute the prototypical Th1 subset, producing exclusively IFN γ , while the latter (T-bet⁺ROR γ t⁺) present phenotypically as any or all of the subsets in question -- Th17, Th1, or Th1/Th17. While the expression of transcription factors is stable for all subsets, the antigen-induced cytokine phenotypes are, in fact, variable for cells within some cloned populations, most notably in the spectrum of IL-17 production, which may vary over time. Relative levels of T-bet and ROR γ t within each clone may be responsible for determining the available phenotype possibilities, but exogenous signaling by IL-12 and IL-23 can modulate cytokine expression in the short term. These results reflect the plasticity of a unique subset of T-bet and ROR γ t double-expressing Th cells, and may contribute to an understanding of the 'phenotype switching' often observed.

2. Materials and Methods

2.1 Mice

BALB/c By mice were purchased from Jackson Laboratories and used between 4–8 wk of age. TCR-transgenic BALB mice were generated in our laboratory; the re-arranged TCR α and β chains derive from the encephalitogenic clone 3a.56, specific for the 26-mer encoded by myelin basic protein (MBP) exon 2 (Abromson-Leeman et al., 2004). Mice were maintained, and experiments were conducted, in accord with guidelines of the Committee on Care and Use of Animals of Harvard Medical School and those prepared by the Animal Committee on Care and Use of Laboratory Animals of the National Research Council (Department of Health and Human Services Publication NIH 85-23).

2.2 Reagents

MBP exon 2 peptide 26-mer was synthesized by Dr. Chuck Dahl, Biopolymers Facility, Harvard Medical School. Paired antibodies and recombinant standard cytokines for IFN γ and IL-17A ELISA assays were purchased from B-D Biosciences. Reagents for intracellular flow cytometric staining for IFN γ and IL-17A were purchased from B-D BioSciences. Antibody to mouse/human ROR γ t was purchased from eBiosciences and used according to the manufacturer's protocol. Recombinant IL-12, IL-23, and IL-21 were purchased from eBioscience and R & D. IL-6 was purchased from BD Biosciences; human TGF β 1 was purchased from eBioscience. rIL-2 is from Fitzgerald Industries.

2.3 Generation and maintenance of T cell clones

Line 173M10 derives from draining lymph nodes of an immunized T cell receptor (TCR) transgenic BALB mouse. Cells were cloned from line 173M10 by limiting dilution using as few as 0.3 cells/well or single cell sorting with a FACS Aria. T cells have been continually maintained in culture as previously described (Abromson-Leeman et al., 2004; Abromson-Leeman et al., 2007). rIL-2, 2 ng/ml, and 5% rat T-Stim without Con A (BD Biosciences) were present in all culture media. Line H was derived by purification of mononuclear cells from the CNS of a TCR transgenic BALB/c mouse immunized with exon 2 peptide for EAE induction as previously described (Abromson-Leeman et al., 2004; Abromson-Leeman et al., 2007). The line was initially cloned *in vitro* with irradiated BALB/c feeder cells and exon 2 peptide (antigen), and then maintained by restimulation every 2–4 weeks with antigen. Culture medium, including 2 ng/ml rIL-2, is replaced every 48 hours. Cloning was done by limiting dilution at 0.3 cells/well in 96 well plates; clones were maintained using the same method used for the lines.

2.4 In vitro culture of T cells for supernatants and mRNA

After initial cloning, supernatants were harvested from wells showing positive growth at 2 weeks, 40 hours after addition of 5×10^5 irradiated spleen cells and peptide, without adjusting for cell numbers in each well. Once clones were established, 2×10^5 T cells were co-cultured with 10^6 irradiated BALB/c spleen cells and either no antigen or with 10 µg/ml exon 2 peptide, in 0.2 ml complete DMEM. At 40 hours, supernatants for cytokine quantitation by ELISA were collected and pooled from duplicate wells, and diluted as necessary to obtain OD readings within the sensitivity limits of the ELISA assay, usually 1/100 for IFN γ , and 1/5 or 1/10 for IL-17. All ELISA assays were performed in duplicate. For mRNA, cells from parallel wells were harvested at 24 hours. Tri-reagent (Molecular Research Center) was used to purify RNA; the manufacturer's protocol was followed. cDNA was synthesized using Quantitect reverse transcription kit (Qiagen).

2.5 EAE induction

For adoptive disease induction, T cells were activated by co-culture with MBP exon 2 peptide and irradiated spleen cells for three days; 10×10^6 activated cells were injected i.v. into BALB/c recipients irradiated with 350 R. No pertussis toxin was used. Mice were monitored twice daily once disease onset was noted. Disease was scored as previously described (Abromson-Leeman et al., 2004). When hind limbs were completely paralyzed (score 3), mice were sacrificed.

2.6 Real-time RT-PCR

Primer pairs for real-time PCR were synthesized by IDT. Primer sequences were as follows:

Gene	Primer sequence
18S rRNA	F: 5'-CGGCTACCACATCCAAGGAA-3' R: 5'-GCTGGAATTACCGCGGCT-3'
T-bet	F: 5'-ACCAGCACCAGACA-GAGATG-3' R: 5'-ACTTGTG GAGAGACTGCAGG-3'
IFN γ	F: 5'-TCAAGTGGCATAGATGTGGAAGAA-3' R: 5'-TGGCTCTGC-AGG AT TTTTCATG-3'
IL-6	F: 5'-GAGGATACCACTCCCAACAGACC-3' R: 5'-AAGTGCATCATCGTTGTTTCATACA-3'

I-A ^d	F: 5'-CAACCACCACAACACTCTGG-3' R: 5'-ATCTCCAGCATGACCAGGAC-3'
TNF α	F: 5'-CATCTTCTCAAAATTCGAGTGACAA-3' R: 5'-TGGGAGTAGACAAGGTACAACCC-3'
IL-17F	F: 5'-CAAAACCAGGGCATTCTGT-3' R: 5'-ATGGTGCTGTCTTCCTGACC-3'
IL-12R β 2	F: 5'-AGTCA-CCAACCTGTCCCTTG-3' R: 5'-GAACAGGCCACAGTTCCATT-3'
IL-23R	F: 5'-AAGGCTTTTCGGAACCTCAT-3' R: 5'-TTCC-AGGTGCATGTCATGTT-3'
ROR γ t	F: 5'-CCGCTGAGAGGGCTTAC-3' R: 5'-TGCAGGAGTAGGCCACATTACA-3'
FasL	F: 5'-AAG-AAGGACCACAACACAAATCTG-3' R: 5'-CCCTGTAAAA-TGGGCCACACT-3'
IL-17A	F: 5'-GCTCCAGAAGGCCCTCAGA-3' R: 5'-AGCTTTCCCTCCGCATTGA-3'
IL-22	F: 5'-GTCAACC-GCACCTTTATGCT-3' R: 5'-CATGTAGGGCTGGAACCTGT-3'
GAPDH	F: 5'-TGACCACCAACTGCTTA-3' R: 3'-GGATGCAGGGATGATGTT-5'

Quantitative real-time PCR was done using the Roche Light Cycler 480 in a 96-well format, with manufacturer's reagents for SYBR green detection. Standard curves were initially used to ensure linearity between Ct (cycle threshold) values and relative gene levels. The equation used to calculate fold up- or down-regulation of gene expression in comparison with a baseline condition after normalization of each sample to the housekeeping gene, 18S rRNA, is:

$$2^{(Ct \text{ target gene in experimental group} - Ct \text{ of target gene in control group}) / (Ct \text{ housekeeping gene in experimental group} - Ct \text{ housekeeping gene in control group})}$$

for Figures 4 and 5. Calculation of fold changes in the CNS (Figure 8) necessitates the use of GAPDH as housekeeping gene; control values derive from the mean expression of target genes in CNS tissue from four uninjected BALB/c mice.

3. Results

3.1 Ex vivo T cells constitute a spectrum of IFN γ /IL-17-producing cells

T cell lines were established both from lymph nodes of MBP peptide immunized TCR transgenic mice and from CNS of transgenic mice that developed EAE after immunization. All lines thus derived secrete both IFN γ and IL-17 when restimulated with irradiated spleen cell feeder and MBP peptide (Abromson-Leeman et al., 2007) and data not shown). To examine the cellular origins of the two cytokines, two such lines were cloned; cytokines secreted by each clone, when stimulated with antigen, were quantitated by ELISA. A total of 210 clones were derived from the combined clonings, and clones from either line displayed a similar spectrum of results upon first examination. Twenty seven clones made IL-17 but no IFN γ , 110 made IFN γ but no IL-17, and 73 made both IFN γ and IL-17 (Figure 1).

Upon subsequent rounds of stimulation, however, cytokine profiles changed for many of these clones. Figure 2 depicts multiple rounds of stimulation for four groups of cells, each exemplified here with two clones. Clones in groups I and II produced only IFN γ , and no

detectable IL-17. Clones in group III secrete both IFN γ and IL-17 with each round of stimulation, although the amounts vary, and the IL-17 level was highest with the initial restimulation. These can be referred to as 'Th1/Th17'. Although the two clones shown are from the lymph node-derived line, at least one clone from the CNS line (clone H24, data not shown) also continued to make both IFN γ and IL-17. Clones in group IV made IL-17 with the first and second rounds of stimulation, but little to none thereafter. Thus this group includes clones that we initially phenotyped as 'Th17' (e.g. M10.26) and 'Th1/Th17' (e.g. M10.49).

Intracellular flow cytometric staining of clones in group III, i.e. double producing cells, shows furthermore, that even within a 'clone', there is a spectrum of antigen-dependent cytokine production. Thus at the time tested, 42% of cells within M10.66, and 67% of M10.116 cells made only IFN γ , 10 and 31%, respectively, made both IFN γ and IL-17, and 11% of M10.66 (and none of M10.116) were making only IL-17 (Figure 3A).

We next examined the effects of exogenously added cytokines in modulating IFN γ and IL-17 levels (Figure 3B). In the absence of both antigen (left panels) and exogenous cytokines (medium group), cytokine detection was minimal (<13 ng/ml IFN γ and <0.16 ng/ml IL-17). The addition of IL-12, however, induces secretion of significant levels of IFN γ . IL-21, added exogenously to non-antigen stimulated cells in this group, induces a much lower, but consistent, level of IFN γ secretion. Exogenous IL-23 leads to a low, but reproducible, rise in IL-17 production. In the presence of antigen (right panels), as previously shown, levels of both IFN γ and IL-17 are high (note expanded scale). IFN γ levels are modulated downward with IL-23, as well as with TGF β /IL-6 treatment. Conversely, IL-17 levels fall dramatically in the presence of exogenous IL-12. These data reinforce the notions of variability and plasticity of IFN γ and IL-17 protein production by the population of dual-producing cells, and begin to address the external signals that influence relative levels of cytokine production.

3.2 Transcription factor expression in cells making IFN γ $\pm$ IL-17

ROR γ t was identified as a critical and requisite transcription factor for Th17 cell development and IL-17 production by naïve cells (Ivanov et al., 2006), while T-bet is known as the 'master' regulator of Th1 cell development and IFN γ production (Szabo et al., 2000). We measured relative expression levels of these two transcription factors by real-time quantitative PCR. Results shown in Figure 4A, for each of the clones presented in Figure 2, depict expression levels of both T-bet and ROR γ t. T-bet is well expressed by all eight clones, although its level is slightly less for Group III clones M10.66 and M10.116 (Ct values of 24.0 $\pm$ 0.1 and 23.5 $\pm$ 0.1) than for all others (Ct's ranging from 21.2 $\pm$ 0.1 to 22.5 $\pm$ 0.1). Dramatic distinctions in ROR γ t are observed. While clones M10.1, H35, M10.77, and M10.87 all make IFN γ and no IL-17, they differ in ROR γ t expression. M10.1 and H35 (Group I) do not express significant ROR γ t (Ct levels $\geq$ 32). In contrast, despite making only IFN γ , clones M10.77 and M10.87 (Group II) do express ROR γ t, with Ct values of 23.6 and 22.9. The IFN γ - and IL-17-producing clones M10.66 and M10.116 (Group III) have the highest levels of ROR γ t, with Ct's of 22.2 and 22.5. They are also the only group in which the ROR γ t transcript level is higher than the level of T-bet transcript. Finally, Group IV clones M10.49 and M10.26, which produce IFN γ and have variable IL-17 production, also clearly express ROR γ t transcripts.

3.3 Signalling by IL-12 or IL-23 differentially modulates Th1- and Th17-related gene programs in ROR γ t⁺/T-bet⁺ clonal populations

We next examined whether expression of transcripts for transcription factors and cytokines can be modulated by exposure to IL-12 or IL-23, in the presence and absence of antigen, in the IFN γ /IL-17-producing clones M10.66 and M10.116. In the absence of antigen (left panels, Figure 4B), IL-12 slightly upregulates T-bet gene expression, in addition to up-regulating

IFN γ gene expression. Inversely, IL-12 down-regulates both ROR γ t and IL-17 expression. IL-23 leads to an increase in IL-22 gene expression.

With antigen stimulation, T-bet increases, and with it, the IFN γ expression goes up. ROR γ t expression tends to decrease with antigen stimulation. The effects of exogenously added IL-12 and IL-23 on the two clones are not consistent when antigen is present; results may possibly be complicated by the endogenous production of these cytokines by APC when T cells are activated. ROR γ t expression levels are, however, consistently down-regulated in the presence of IL-12, as they are without antigen, and IL-22 expression is increased with the addition of IL-23.

Since the effects of IL-12 and IL-23 modulation can be directly observed on T cells without addition of APC, studies of these genes and others characteristic of either 'Th1' or 'Th17' programs were extended to include rested clones M10.1 (group I) and M10.77 and 87 (Group II), in addition to M10.66 and M10.116. Figure 5 depicts fold changes in gene expression for clones from groups I, II, and III after incubation with either cytokine. In resting M10.1 (T-bet⁺/ROR γ t⁻), IL-12 induced a 7-fold increase in IFN γ up-regulation, but no other significant changes in gene expression. IL-23 had no effects, a result that is not unexpected, since these Th1 clones lack IL-23R gene expression (as judged by RT-PCR). In resting Group II clones M10.77 and M10.87 (T-bet⁺/ROR γ t⁺ and IFN γ ⁺/IL-17⁻), addition of IL-12 dramatically reduced ROR γ t expression (445- and 390-fold, respectively) and increased IL-23R expression 485- and 125-fold, respectively; these changes are magnified by the negligible baseline levels of IL-23R expression. Exogenous IL-23 also increased IL-23R expression, although not as dramatically (61- and 9-fold, respectively). In response to IL-12, Group III clones M10.66 and M10.116 (T-bet⁺/ROR γ t⁺ and IFN γ ⁺/IL-17⁺) both up-regulated Th1-related genes (T-bet, IFN γ , FasL, IL-12R β 2) 5- to 12-fold, and down-regulated Th17-related genes ROR γ t, IL-17A, IL-17F (> 12-fold). In response to IL-23, both clones down-regulated FasL (3–10-fold), and up-regulated IL-17F and IL-22 (> 7-fold). Group III clones M10.66 and M10.116 have a baseline expression of IL-23R mRNA that ranges from 400- to 1600-fold higher than that of clones in Group II (Ct of 24 as compared with Ct >32 for Group II clones).

These data both point out the spectrum of responses to exogenous signaling that can only be dissected with monoclonal populations as shown here, and serve to illustrate the plasticity of T-bet⁺/ROR γ t⁺ T cells, specifically illustrating their responsiveness to external signals in implementing more of a T-bet/IFN γ -oriented phenotype (Th1), or a ROR γ t/IL-17/IL-22 (Th17) pattern of gene expression.

Intracellular ROR γ t protein expression by clones from groups I, II, and III is shown in Figure 6. The genotypically T-bet⁺/ROR γ t⁻ clone H35 (Group I) has no detectable ROR γ t protein expression beyond background staining, while T-bet⁺/ROR γ t⁺ clones M10.87 and M10.77 (Group II) and M10.66, M10.116 (Group III), express ROR γ t protein above background levels. Protein staining also shows that clones M10.66 and M10.116, Group III clones that always produce both IFN γ and IL-17 have a higher level of ROR γ t (MFI's of 22 and 24) than Group II clones M10.87 and M10.77 (MFI's of 13 and 8, respectively), that make IFN γ but no detectable IL-17 (Figure 2). This 2–3 fold difference in protein level is mirrored in differences in mRNA expression (Fig. 4A). Thus while clearly positive both genotypically and phenotypically for ROR γ t, quantitative differences in ROR γ t expression might account for the phenotypic differences observed in IL-17 production and dominance of a 'Th1' versus a 'Th1/Th17' profile.

3.4 Encephalitogenicity and in situ gene expression by ROR γ t⁺/T-bet⁺ T cells

T cell clones of all groups described in this report are capable of inducing EAE in syngeneic naïve recipient mice. The kinetics of onset and severity of disease are similar for all groups.

In this BALB/c model, early signs of disease (cachexia, hind limb weakness and ataxia) are apparent by day 7–8 post-transfer, and progress to scores of 3 (cachexia and hind limb paralysis) by days 9–10 for both Th1 and Th17/Th1 recipients. CNS tissue sections (Figure 7) show typical mononuclear infiltrates of lymphocytes and macrophages in white matter of lumbar spinal cord, cerebellum, and lateral medullary areas of brain. Infiltrates, composed mostly of lymphocytes and macrophages, are both meningeal and perivascular, extending into parenchymal areas. There are no obvious histological differences between disease induced by Th1 (ROR γ t-negative) versus Th1/Th17 (ROR γ t+) clones.

mRNA was purified from CNS tissue (brainstem and spinal cord) of mice injected with either H35 (T-bet⁺/ROR γ t⁻, IFN γ only) or M10.66 (T-bet⁺/ROR γ t⁺, IFN γ /IL17⁺) before onset of clinical signs (day 5), and on subsequent days through day 10, at which time all mice were sacrificed with scores of ≥ 3 . Quantitative PCR for TCR α and β transgenes confirmed the presence of T cells in the CNS on day 5 in both groups; levels of transgene-specific TCR transcripts continued to rise, reaching plateau levels on days 7–10 for H35, and on days 9–10 for M10.66 (data not shown). Transcripts for T-bet and ROR γ t, IFN γ and IL-17, as well as inflammatory cytokines IL-6 and TNF α , and MHC Class II were quantitated by real-time PCR, and fold increase in CNS of clone recipients as compared with the averaged mean expression in a group of 4 normal controls (Figure 8). Data are also shown for CNS tissue from 5 individual recipients of Group IV clones, collected on day 10, at the beginning of peak disease.

On day 5, before onset of any visible clinical disease, the presence of T cells is confirmed not only by TCR transcripts (V α is up over 20-fold in the H35 recipient, and over 8-fold in the M10.66 recipient, data not shown), but by their characteristic transcription factors. T-bet, but not ROR γ t, is increased in the H35 recipient, while both T-bet and ROR γ t are increased in the M10.66 recipient. At this time point, IFN γ expression is increased in both groups. IL-17, however, is not detected at this early point in the M10.66 recipient.

By day 6, however, IL-17 expression is beginning to increase in the M10.66 group, and reaches plateau levels on days 7–10. IFN γ , as well as other markers of inflammation – IL-6, TNF α , and MHC Class II, reach plateau levels on days 9–10 in M10.66 recipients. Tissue from H35 recipients, shows maximal expression of IFN γ , IL-6, TNF α , and MHC Class II by day 7, and levels remain about the same through day 10.

CNS tissue from Group IV clones, those that have both transcription factors and variable IL-17 production, shows the presence of both T-bet and ROR γ t transcripts, and high levels of IFN γ , IL-6, and TNF α expression, however there are no detectable IL-17 transcripts. These cells were injected into recipients at a time when they were still producing IL-17 *in vitro* (i.e. just after the first round of restimulation shown in Figure 2 for Group IV clones), yet the *in vivo* cytokine profile appears more like a Th1 profile, with the exception that ROR γ t can be detected, confirming the ROR γ t⁺/T-bet⁺ nature of these cells that now appear to have ‘changed’ to a Th1 phenotype. These data show that the subset of ROR γ t⁺/T-bet⁺ cells that have variable production of IL-17 but always produce IFN γ , may appear phenotypically as either Th17, Th17/Th1 or Th1.

4. Discussion

While there is little remaining doubt as to the importance of T cells of the ‘Th17’ and ‘Th1/Th17’ subsets in inflammatory and pathological conditions both in experimental mouse models and in human disease, the precise role played by these cells is complicated by questions as to their lineage and degree of relatedness to each other and to Th1 cells (Gocke et al., 2007; Mathur et al., 2006). Studies of cells isolated from inflammatory conditions have often described Th1/Th17 cells, i.e. cells making both IFN γ and IL-17 (Acosta-Rodriguez et al.,

2007; Annunziato et al., 2007; Chen et al., 2007; Cosmi et al., 2008; Ivanov et al., 2007; Suryani and Sutton, 2007; Wilson et al., 2007), and three recent reports describe ‘conversion’ of ‘Th17’, or IL-17-producing cells, to ‘Th1’, or IFN γ -secreting cells, during the disease induction process *in vivo* (Bending et al., 2009; Martin-Orozco et al., 2009; Shi et al., 2008). We have studied cells with Th1/Th17 properties by making antigen-specific monoclonal populations; our results, reported here, may contribute to understanding these recent reports by allowing a detailed focus on the nature of these cells.

Ex vivo T cell lines, isolated from either lymph node or CNS of MBP peptide-immunized TCR-transgenic BALB/c mice (the CNS line from a mouse with EAE) were cloned; the resulting lines of ‘homogeneous’ clones revealed a remarkable level of heterogeneity with regard to IFN γ and IL-17 production, but absolute stability with regard to expression of lineage-specifying transcription factors T-bet and ROR γ t. A spectrum of (antigen-dependent) cytokine-secreting phenotypes was initially observed -- cells secreting only IFN γ , only IL-17, or both IFN γ and IL-17. While all clones grew equally well, the IL-17-only phenotype (exemplified in Figure 2 by clone M10.26) was short-lived; all these cells, within one or two additional rounds of antigen-stimulation *in vitro*, began secreting IFN γ . In some cases, IL-17 production was intermittently observed in addition to the IFN γ , while in others, IFN γ became the only cytokine produced. Similarly, IL-17 production by some clones that made both IFN γ and IL-17 on first analysis became intermittent, but cells continued making vigorous IFN γ responses (e.g. M10.49). A number of clones that initially made both IFN γ and IL-17 maintained the IFN γ and IL-17 dual-producing phenotype (e.g. M10.66 and M10.116, shown in Figure 2). Cells that initially made only IFN γ retained this phenotype (Groups I and II). Analysis of transcription factor expression, however, further divided this group into T-bet⁺ROR γ t⁻ (*bona fide* Th1 cells, as in Figure 2, Group I) and T-bet⁺ROR γ t⁺ cells, exemplified by M10.77 and M10.87 in Figure 2.

Thus clonal analysis has allowed us to ‘zoom in’ on this population of ROR γ t⁺/T-bet⁺ cells in a way that is not possible with an uncloned population, even when highly purified. The composite picture of the monoclonal populations reflects the multitude of phenotypes that contribute to observed plasticity (Lee et al., 2009; Spolski and Leonard, 2009; Wei et al., 2009). Our data show that these cells may present, phenotypically, as ‘Th17’, ‘Th1’, or ‘Th1/Th17’ cells. The heterogeneity of cytokine phenotype is even further underscored by intracellular staining and flow cytometric analysis at the single clone level, as shown by two examples of Th1/Th17 (i.e. M10.66 and M10.116) in Figure 3. 24 hours after stimulation with antigen, a majority fraction of each of these IFN γ +/IL-17+ clones is producing only IFN γ . A smaller fraction, 10% of M10.66 and 31% of M10.116 is, in fact, simultaneously producing both cytokines, and only M10.66 includes 11% of cells making only IL-17. Exogenous signaling by IL-12, IL-23, or IL-21 affects relative production of IFN γ and IL-17, both in the absence and presence of antigen, as shown in Figure 3B. Not surprisingly, IL-12 increases IFN γ and diminishes IL-17, while IL-23 increases IL-17 and diminishes IFN γ . IL-21 is qualitatively similar to IL-12, although its effects are less dramatic. The inverse effects of IL-12 and IL-23 on gene expression again confirm the notion that dual-positive cells such as M10.66 and M10.116 are able to respond to either one or the other cytokine by implementing the corresponding T-bet or ROR γ t program, while decreasing expression of the other. Thus IL-12 increases T-bet, IFN γ , FasL and IL-12R β 2 expression, while decreasing expression of ROR γ t, IL-17A, and IL-17F. IL-23, conversely, decreases FasL expression, and increases IL-23R, IL-17 and IL-22 expression. Interestingly, Group II clones such as M10.77 and M10.87 respond to IL-12 by dramatically decreasing ROR γ t mRNA expression, but their level of T-bet and related genes is already high, so an additional increase does not occur. They do, however, upregulate IL-23R in response to IL-12, even more so than in response to IL-23; the functional consequences of increased IL-23R expression remain to be determined. The

ROR γ t-negative Th1 clone, M10.1, expresses none of the ROR γ t-related genes including IL-23R; co-culture with IL-23 effects no changes in expression of any of its genes.

Intracellular staining with antibody to ROR γ t confirms the presence of ROR γ t protein in clones expressing ROR γ t mRNA, and its absence from a Th1 clone (H35). The fluorescence intensity, higher in Group III clones than in Group II clones, mirrors the quantitative differences in ROR γ t expression observed in RT-PCR. It is tempting to speculate, based upon these results, that levels of ROR γ t and/or relative levels of ROR γ t and T-bet, provide an indicator of the likelihood of phenotypic manifestation of the ROR γ t-related program.

Finally, the *in vivo* ability of ROR γ t-expressing clones to induce EAE was compared with Th1 clones, which we have previously reported to be strongly encephalitogenic (Abromson-Leeman et al., 2007). Both T-bet⁺ROR γ t⁻ Th1 clones and T-bet⁺ROR γ t⁺ clones induce strong inflammatory disease in brain and spinal cord of naïve recipients. Kinetics of disease development, and histological and clinical presentation, are virtually indistinguishable. Quantitative PCR for TCR transgenes and for characteristic transcription factors shows that both Th1 clone H35 and Th1/Th17 clone M10.66 have reached the CNS by day 5, prior to onset of clinical signs of disease. As the disease process progresses, mRNA for their characteristic cytokines becomes increasingly abundant, reaching plateau levels by days 7–10. Clones from Group IV (those that made IL-17 when initially cloned, have largely stopped producing IL-17 but intermittently will still make some IL-17 *in vitro*) also induce disease in all recipients but failure to detect IL-17 transcripts indicates that IL-17 production does not appear to play a role. Likely candidates for pro-inflammatory cytokines, such as IFN γ , IL-6, and TNF α are all present at high levels. Thus clones in this group, at the time of injection, were phenotypically Th17 or Th1/Th17, but their *in situ* profile of cytokine production is indistinguishable from a Th1 profile. That these are not Th1, however, is evident from their continued expression of ROR γ t in addition to T-bet.

In summary, we present in this report our results studying MBP peptide-specific monoclonal populations of CD4⁺ memory/effector cells, comparing ROR γ t⁺/T-bet⁺ cells with ROR γ t⁻/T-bet⁺ populations. Transcription factor expression is a stable characteristic of these clones, while antigen-stimulated cytokine production (i.e. phenotype) is not fixed. A spectrum of phenotypes is discernable within the group of ROR γ t⁺/T-bet⁺ cells, including those that phenotypically resemble Th1 cells, to Th1/Th17 cells, to Th1/Th17 whose IL-17 production is variable. (Cells making only IL-17 initially all began producing IFN γ by the next round of activation). Intraclonal heterogeneity in phenotype is also evident – at any given time point, cells of a single clone may produce IFN γ alone, IL-17 only, or both, in response to antigen stimulation. The spectrum of possibilities for each cell may be determined by the relative levels of T-bet and ROR γ t present in a given clone. Within each population, however, exogenous cytokines such as IL-12 and IL-23 can strongly influence the observed cytokine phenotype by transiently altering the balance of the T-bet- versus the ROR γ t-directed program. Levels of IL-23R expression, which play a role in responsiveness to IL-23, are in turn influenced both by the constitutive level of ROR γ t as well as by the modifying effects of IL-23 and IL-12. Results presented here may shed some light on previously unclear lineage relationships and on the fundamental nature of plasticity and multiplicity of phenotypes of ROR γ t⁺/T-bet⁺ cells.

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Figure 1.

Profile of IFN γ and IL-17 secreted by 181 LN-derived clones (Line M10) and 29 CNS-derived clones (Line H). Levels of IFN γ and IL-17 in 40 hour supernatants of individual cloned T cells were quantitated by ELISA after first cloning lines derived from either BALB/c TCR-transgenic lymph nodes, 10 days after immunization, or CNS, at onset of clinical signs of EAE. Among LN-derived clones, 97 made IFN γ only, 19 made IL-17 only, and 65 made both cytokines. Among CNS-derived clones, 13 made IFN γ only, 8 made IL-17 only, and 8 made both cytokines.

Figure 2.

Secreted levels of IFN γ and IL-17 by eight T cell clones, shown for first 3–5 rounds of restimulation after cloning. With the exception of the first round of stimulation, supernatants were collected and pooled from duplicate wells, 40 hours after addition of irradiated BALB spleen cells and MBP exon 2 peptide (10 μ g/ml). Cytokines were quantitated in duplicate by ELISA, as detailed in Materials and Methods. Variance between duplicate wells was <20% for all positive samples. Intervals between rounds of stimulation were approximately 2 weeks.

Figure 3.

Expression and modulation of IFN γ and IL-17 in double producing clones.

A, Intracellular staining of IFN γ /IL-17-secreting clones M10.66 and M10.116. T cells were harvested 24 hours after activation with peptide antigen and irradiated BALB/c spleen cells, incubated with Brefeldin A for 2 hours, surface stained with FITC-CD4, followed by permeabilization and staining with APC-anti-IFN γ and PE-anti-IL-17 antibodies. Shown are percentages of CD4 $^{+}$ activated T cells staining with indicated anti-cytokine antibodies in each quadrant. B, Cytokine pre-treatment modulates IFN γ and IL-17 protein expression. IFN γ /IL-17-secreting clones were co-cultured with either TGF β +IL-6 (5 and 30 ng/ml, respectively), IL-12 (10 ng/ml), IL-23 (50 ng/ml), or IL-21 (50 ng/ml) for seven days, harvested, washed, and added to irradiated BALB/c spleen cells without (left) or with 10 μ g/ml antigen (right), together with the same exogenous cytokine used in pre-culture conditions. IFN γ and IL-17 secreted by 40 hours were quantitated by ELISA.

Figure 5.

Modulation of gene expression induced by IL-12 and IL-23. Real-time PCR quantitation of gene expression in resting T cell clones (no antigen stimulation for ≥ 2 months), cultured with IL-12 (black bars) or IL-23 (gray bars) for 4 weeks. Fold changes were calculated as in Materials and methods, normalizing to 18S rRNA. Ct values >32 were assigned values of 32 for calculation purposes.

Figure 6.

Intracellular detection of ROR γ t protein. Resting T cell clones were harvested from *in vitro* culture, permeabilized, and stained for ROR γ t protein using PE-anti-ROR γ t antibody (bold lines); PE-isotype control is also shown (thinner lines). The percentage of positive cells shown below histograms is calculated by subtracting the percentage positive with PE-isotype control from the percent positive with PE-ROR γ t antibody; mean fluorescence intensity of staining with anti- ROR γ t antibody is shown below histograms.

Figure 7.

H & E staining of CNS sections from recipients of Th1 (Group I) and Th1/Th17 clones (Groups II and III). All mice were sacrificed at onset of neurological signs of disease; tissues from two mice in each group were harvested for histological analysis. **M10.1** (Group I), Perivascular and parenchymal lymphocytic infiltration in cerebellar white matter (10×). Higher magnification (60×) shows mostly lymphocytes, some scattered macrophages. Meninges of lumbar spinal cord are focally infiltrated with lymphocytes; parenchyma has focal dense infiltrates (10×). Higher magnification (60×) shows dense meningeal infiltrate (right); parenchyma is infiltrated with lymphocytes and a few macrophages. **M10.77** (Group II), Lateral medulla (cochlear nucleus) is densely infiltrated by macrophages (10×). Parenchyma

has been replaced by sheets of confluent macrophages; notably few lymphocytes are observed (60×). In low lumbar spinal cord, there is dense infiltration of both meninges and parenchyma by lymphocytes (10×). Lymphocytes densely infiltrate meninges, and spinal cord white matter is densely infiltrated by macrophages (60×). **M10.66** (Group III), Diffuse perivascular and parenchymal infiltration of lymphocytes and macrophages in cerebellar white matter (×10). Severe perivascular and parenchymal infiltration of cerebellar white matter with macrophages and lymphocytes (×60). Spinal cord white matter is infiltrated with macrophages and lymphocytes, extending from meninges and into parenchyma perivascularly (10×). On high magnification, very dense infiltration of lymphocytes are observed in meninges, and extending into parenchyma, where numerous macrophages are also observed (60×). **M10.116** (Group III), Perivascular infiltration of lymphocytes in cerebellar white matter (10×). Lymphocytes and macrophages are observed infiltrating into parenchymal white matter of cerebellum (60×). Spinal cord is infiltrated with lymphocytes in sub-meningeal regions, and extending a short distance into parenchyma (10×). Lymphocytes and macrophages are observed in sub-meningeal and parenchymal regions of spinal cord (60×).

Figure 8.

Real time PCR quantitation of genes expressed in CNS tissue (spinal cord and brainstem) of recipients of indicated T cell clones. CNS tissues were harvested on indicated days post-injection. Each bar corresponds to one recipient. Bars represent fold increase in indicated transcripts as compared with averaged expression in 4 normal BALB/c control mice, after normalization of each with GAPDH. Group IV includes one recipient each of clones M10.26, M10.28, M10.46, M10.49, and M10.52.