

**T-helper 1 cells facilitate the entry of T-helper 17 cells to the
central nervous system during experimental autoimmune
encephalomyelitis[#]**

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Running title: Th17 cells do not initiate EAE

Abstract

It has recently been proposed that experimental autoimmune encephalomyelitis, once considered the classical Th1 disease, is predominantly Th17-driven. Here we show that myelin-reactive Th1 preparations, devoid of contaminating IL-17+ cells, are highly pathogenic. In contrast, Th17 preparations lacking IFN- γ + cells do not cause disease. Our key observation is that only Th1 cells can access the non-inflamed CNS. Once Th1 cells establish the EAE lesion, Th17 cells appear in the CNS. These data shed important new light on the ability of Th1 versus Th17 cells to access inflamed versus normal tissue. As the IL-17-triggered release of chemokines by stromal cells could attract many other immune cells, allowing Th17 cells to access the tissues only under conditions of inflammation may be a key process limiting (auto)immune pathology. This has major implications for the design of therapeutic interventions, many of which are now aiming at Th17 rather than Th1 cells.

Introduction

Experimental autoimmune encephalomyelitis (EAE) had long been considered the prototypic Th1-mediated autoimmune disease (1, 2), until recent findings suggested a primary role for Th17 cells in this model (3, 4). This paradigm-shift has sparked a rapid and remarkable change in emphasis in the search for disease-modifying drugs, away from the Th1 pathway, towards the Th17 pathway (5-8). It is therefore important to have a clear understanding of the relevance of each of these pathways in autoimmune pathology. This requires an assessment of the ability of Th1 or Th17 cells to initiate pathology upon adoptive transfer in the absence of each other.

With purity of Th phenotype in mind, we have evaluated the EAE-initiating capacity of Th1 versus Th17 populations. Our results indicate that Th1 cells have a preferential ability to access the non-inflamed CNS and that the pathology they cause promotes the subsequent infiltration of Th17 cells.

Results

Th1 cells initiate EAE upon passive transfer and promote the recruitment of Th17 cells to the CNS

Using draining lymph nodes from mice immunized with myelin oligodendrocyte glycoprotein peptide 35-55 (pMOG), our emphasis was to generate the cleanest possible Th1 and Th17 preparations. Stimulation with pMOG alone induced a mixed response dominated by IFN- γ production and a small proportion of cells producing IL-17 or co-producing IFN- γ and IL-17 (Fig 1a). Addition of IL-12 and IL-18 maximised IFN- γ production and repressed IL-17, giving the highest ratio of IFN- γ to IL-17 producers (Fig 1a). Although IL-23 induced the highest frequency of IL-17-producers (\sim 40% of cells), it did not reduce the overall proportion of IFN- γ producers (\sim 20% of cells made IFN- γ , either alone or in combination with IL-17). TGF- β was required to suppress IFN- γ production. The IL-6+IL-23+TGF- β combination therefore produced the most highly polarized Th17 population (Fig. 1a). As exemplified in Fig 1b, administration of Th1 cells alone, or Th1 together with Th17 cells, led to clinical disease, whereas Th17 cells alone were not pathogenic. Similarly, populations generated in the presence of IL-6 and TGF- β (no IL-23) did not cause EAE alone, and did not significantly modulate the course of disease when co-transferred with Th1 cells (Fig 1c).

To assess their comparative capacity to home to the target organ, Th1-polarized and Th17-polarized cells were sourced from CD45.1 CD90.2 donors and CD45.2 CD90.1 donors, respectively, for injection into CD45.2 CD90.2 hosts. Both Th1 and Th17 populations were clearly identifiable in the spleen (Fig 2a) with Th17 preparations at significantly higher frequencies than Th1 preparations when transferred either singly or in tandem (Fig. 2a). The picture from the CNS was

the reverse of that from the spleen (Fig d). A strong Th1 infiltrate was clearly identifiable (around 100-fold enriched, as a percentage of total CD4+ cells, compared to the spleen). Th17 cells were not identifiable in the CNS if transferred alone, but were present when transferred together with Th1 effectors (Fig. 2d). We conclude that Th1 lymphocytes have a superior capacity to home to the CNS and orchestrate inflammation and cellular recruitment thereby facilitating the entry of Th17 cells.

The polarization of pMOG-reactive donor populations is stable

Stability of cytokine phenotype confirmed that the disease seen with Th1 populations was not because of residual IL-17 production, and that lack of disease with Th17 preparations did not reflect a loss of IL-17 production. Among cells recovered from the spleen and the CNS the phenotype of both populations appeared stable (Fig 2e-h). It is important to emphasise that donor Th1 cells extracted from the CNS of diseased mice did not show any capacity for IL-17 production (Fig 2h).

Th17-polarized myelin-reactive T cell receptor transgenic T cells can induce EAE, but only in the presence of IFN γ -producing cells.

The current understanding that IL-6+TGF- β is the key combination for driving Th17 differentiation of naïve cells has come from either anti-CD3 stimulation of polyclonal populations or Ag stimulation of naïve TCR transgenic cells. Thus far the use of naïve T cells differentiated in the presence of these cytokines has not been described in an EAE model. We made use of the Tg4 mouse that expresses a transgenic T cell receptor (TCR) recognizing the Ac1-9 peptide of myelin basic protein (MBP) (9). We generated Th1- and Th17-polarized populations by

primary in vitro stimulation of CD4⁺CD62L^{hi}CD25⁻ cells taken from Tg4xCD45.1 mice for transfer into B10.PL (CD45.2) recipients (Fig 3a). Th1-polarized cells (devoid of IL-17 producers) efficiently induced severe, often fatal EAE (Fig 3b). The number of Th1 blasts transferred correlated well with the severity of EAE. EAE could be induced with as few as 1×10^6 . Numbers beyond 4×10^6 led to high mortality (data not shown). In this model, Th17-polarized populations could occasionally induce some EAE, but this was mild disease with delayed onset and reduced severity (Fig 3b). Furthermore, despite stringent Th17-polarization pre-transfer (Fig 3a), those donor cells recovered from the CNS consistently displayed a capacity for IFN- γ production (Fig 3c). There was a striking shift in the IL-17⁺:IFN γ ⁺ ratio in these populations from $\sim 200:1$ at the time of transfer to $\sim 3:1$ upon extraction from the CNS. In contrast, Th1 donor cells recovered from the CNS did not show any capacity for IL-17 production upon restimulation ex-vivo (Fig 3c). The Th1 phenotype was very stable during preclinical stages, through to the peak of disease (days 5-14, data not shown). In contrast "Th17" cells recovered from the CNS had a propensity for IFN- γ production, suggesting a degree of instability upon re-encounter with Ag (discussed below).

The data shown in Fig 2 indicated that transferred IL-17 producing cells did not have an inherent fragility in vivo (they accumulated in large numbers in the spleen). Nevertheless, we sought to maximise their opportunity for clonal expansion and disease initiation by transfer into lymphopenic B10.PLxRAG2^{-/-} hosts (Fig 3 d-f). Th17 preparations could induce disease in RAG2^{-/-} recipients (Fig 3e). However, the disease induced with Th1-effectors was always more rapid, with higher mortality. Using the Th1 passive transfer system we have recently reported that host-derived CD4⁺foxp3⁺ cells are recruited to the inflamed CNS and play a key role in recovery from EAE (10). The absence of these cells in

lymphopenic hosts therefore is probably an important factor contributing to this high mortality. Importantly, cells taken from the diseased CNS after transfer of Th17-polarized populations clearly showed the presence of IFN- γ -producing cells. In fact, as illustrated in Fig 3f, IFN- γ producing cells were found to markedly outnumber IL-17 producing cells in the CNS of these mice.

Signature cytokine production is more stable in Th1 than Th17 populations

We assessed the stability of in vitro-primed Th1 and Th17 populations following re-stimulation in the absence of exogenous polarizing cytokines. Both populations continued to produce their signature cytokine, but not the opposing cytokine, in the absence of secondary Ag stimulation (Fig 4a,b). We never observed IL-17 production by Th1 preparations, even at high Ac1-9 concentrations (Fig 4a). In contrast high dose Ac1-9 could provoke IFN- γ production by Th17 preparations (Fig 4b,c). Ag-induced TNF- α production was equivalent in Th1- and Th17-polarized cells (Fig 4d), whereas the latter produced greater amounts of IL-6 (Fig 4e).

Th17-polarized cells do not show increased IL-10 production or foxp3 expression

It has recently been reported that, in addition to IL-17 production, TGF- β and IL-6 promote IL-10 production, which limits the pathogenic activity of T effectors (and may even confer suppressive activity), whereas IL-23 promotes production of IL-17 alone, conferring pathogenicity (11). We consistently included IL-23 in our Th17 polarizing conditions alongside TGF- β and IL-6 in order to maximize pathogenic potential. Furthermore, upon restimulation, Th1-polarized Tg4

populations always produced higher levels of IL-10 than their Th17-polarized counterparts (Fig 4f). Similarly, Th1-polarized populations from pMOG-primed mice produced IL-10, whereas this was negligible in Th17 preparations (Fig 4g). Therefore IL-10 production by Th17-polarized cells does not account for their lack of pathogenicity in this instance. Our Th17 preparations were uniformly foxp3-negative (Fig 4h). Therefore we have no evidence for a regulatory component that would inhibit EAE on transfer of Th17 preparations. This conclusion is supported by the fact that their inclusion did not suppress the pathogenic activity of Th1 populations (Fig 1b). Populations polarized using only IL-6 and TGF- β did not significantly attenuate disease induced by Th1 effectors (Fig. 1c). Thus our Th1-polarized populations were not sensitive to suppressive activity of such cells, as reported by McGeachy et al using IL-23-conditioned pathogenic populations (11). Expression of the lineage-specific transcription factors T-bet and ROR γ t in our pre-transfer populations (following primary polarization) showed elevated T-bet expression in Th1 and elevated ROR- γ t expression in Th17 preparations respectively (Fig 4i).

Discussion

Our data provide a picture of Th1 effectors preferentially infiltrating the non-inflamed CNS to initiate inflammation which facilitates the recruitment of Th17 cells. These data are clearly at odds with the recent paradigm-shift that has led to the role of Th1 cells in EAE being questioned. This has been based largely on active disease models using CFA immunization in gene knockout mice, in which effects can be complex. For example, exaggerated EAE in IL-12(p35)^{-/-} (3) is complicated by their lack of IL-35, which has a reported role in regulatory T cell function (12). To get a definitive picture of their comparative pathogenic

potential it is imperative to transfer disease with Th1 or Th17 cells, avoiding complications that might arise from the use of CFA in the host, and also to use populations that are as devoid of the opposite population as possible. The literature lacks clear evidence for a pure Th17 population, totally deficient in IFN- γ production, having any capacity to initiate CNS-autoimmune disease. Previous studies (4) used IL-23-expanded, IL-17-producing preparations that clearly included contaminating IFN γ + cells (4,11). Using such mixed donor populations, the contribution of IFN- γ and IL-17 producing cells could not be determined. By using traceable populations with truly distinct cytokine secretion profiles, we have been able to dissect the comparative migratory potential and functional stability of Th1 versus Th17 cells with greater exactitude than previously achieved.

Three recent reports of actively-induced EAE show that the majority of IL-17-producing CD4+ cells in the CNS also produce IFN- γ (13-15). We also see these cells after culture with IL-23 alone (Fig 1a) but not after culture with IL-23, IL-6 and TGF- β . However, whenever we saw disease after Th17 transfer, IFN- γ producing cells were always over-represented in the CNS compared to their frequency at the time of transfer (Fig 3). The ability of Th17-polarized populations to gain IFN- γ producing capacity upon subsequent antigenic stimulation (Fig 4) may lead to the organ specific enrichment of IFN- γ + and IFN- γ +/IL-17+ cells in the CNS as has been seen following transfer of Th17 polarized populations (11). A recent study reporting a pathogenic role for Th17-polarized populations in autoimmune disease of the eye also indicated that their ability to produce IL-17 waned with continued stimulation (16). Consistent with our data, that study showed the induction of disease by a Th1 cell line in the complete absence of IL-17 production (16). Clearly there is a need for better

understanding of the stability of the Th17 phenotype in terms of production of IL-17, versus other cytokines thought to be more associated with a Th1 response. It may be that either cell can cause pathology under the appropriate conditions and this may not require the production of the Th1 or Th17 signature cytokines. For example, both cell types can produce significant amounts of TNF- α (Fig 4). Our data indicate that Th1 cells enter the CNS and modify conditions therein, making it both attractive and accessible to Th17 cells. Understanding the basis for the differential migratory potential and cytokine-stability of these polarized populations might allow the development of therapeutic strategies to modulate their distribution and function in vivo.

Methods

Mice, antigen and tissue culture medium

C57BL/6 mice (either CD45.2 CD90.2, CD45.1 CD90.2, or CD45.2 CD90.1), Tg4xCD45.1 mice and B10.PL mice were bred under specific pathogen-free conditions at the University of Edinburgh. Tg4 and B10.PLxRAG2^{-/-} mice were bred under specific pathogen-free conditions at the University of Bristol. Experiments received University of Edinburgh / University of Bristol ethical approval and were performed under UK legislation. pMOG (MEVGWYRSPFSRVVHLYRNGK) and MBP Ac1-9 (Ac-ASQKRPSQR) were synthesised by the Advanced Biotechnology Centre, Imperial College, London. Tissue culture medium was RPMI 1640 medium, supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, and 5 x 10⁻⁵ M 2-ME (all from Invitrogen Life Technologies, Paisley, UK) and 5% FCS (Sigma, Poole, UK)

Cell culture and passive EAE

C57BL/6 mice were immunized s.c. with 100 µg of pMOG in CFA. 10 days later, draining lymph node cells were recovered and cultured w with 10µg/ml pMOG for a total of 72h. For Th1-polarization, 25 ng/ml rIL-12 (R and D systems) and 25 ng/ml rIL-18 (MBL, Nagoya) were used. 0.5 ng/ml rIL-2 (R and D systems) was added at the start of Th1-polarizing cultures and the concentration of IL-2 was raised to 2.5 ng/ml after 48 h. For Th17-polarization, 20 ng/ml rIL-6, 20 ng/ml rIL-23 and 3 ng/ml rTGF-β (all from R and D systems) were used. After 72h, 4 x 10⁶ blasts were injected i.v.

FACS-sorted CD4⁺CD62L^{hi}CD25⁻ Tg4 TCR transgenic cells were cultured for 72h with irradiated B10.PL splenocytes and 10 µg/ml of MBP Ac1-9 using the Th1- and Th17-polarizing conditions described above. After 72h, 3 x 10⁶ blasts

were injected i.v. For transfer to B10.PLxRAG2^{-/-} recipients, naïve Tg4 splenic and lymph node populations were stimulated with 10 µg/ml Ac1-9 under either Th1-polarizing conditions (5ng/ml IL-12, and 10µg/ml anti-IL-4), or Th17-polarizing conditions (3ng/ml TGF-β, 20 ng/ml IL-6, 20 ng/ml IL-23 and 3 µg/ml anti-IFN-γ) for 5 days. IL-2 (20 U/ml for Th1-polarization and 10 U/ml for Th17-polarization) was included from day 3. After 5 days culture, 3x10⁶ blasts were injected i.v. On the day of transfer each mouse also received 200ng of pertussis toxin. Clinical signs of EAE were assessed as described previously (10).

FACS

All antibodies were from eBioscience, CA, except anti-CD4 PerCP (BD Pharmingen, Oxford, UK) anti-IL-17 APC (biolegend). Intracellular cytokine staining of peptide stimulated cells was performed as previously described (10). For staining at the end of polarizing cultures, 1 µg/ml PMA, 50 ng/ml ionomycin and 1 µl/ml brefeldin A was added for the last 4h of culture.

Analysis of cytokine secretion

Ag-induced cytokine production by lymph node cells (6 x 10⁵ / 200 µl well) was assessed by ELISA. Polarized T cells (2 x 10⁴ / 200 µl well) were extensively washed prior to restimulation with irradiated splenocytes (2 x 10⁵ / well) and a dose range of Ag as indicated. After 72h cytokine concentration was determined by ELISA or cytometric bead array using an inflammation array (BD Pharmingen, Oxford, UK) or Th1/Th2 10plex kit (Bender Medsystems, Vienna, Austria) according to the manufacturer's instructions.

Real-time PCR

RNA was extracted after 72h culture in the presence or absence of polarizing cytokines. Primer sequences used for ROR- γ t and T-bet have been published previously (13). Expression was normalised to HPRT and expressed as fold induction over cells stimulated under neutral conditions.

Statistical analysis

Statistical analysis of results was performed using the Mann-Whitney U test and the two tailed Student's *t* test.

Abbreviations

CNS; central nervous system, EAE; experimental autoimmune encephalomyelitis, MBP; myelin basic protein, MOG; myelin oligodendrocyte glycoprotein

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Figure Legends

Fig 1

Polyclonal Th1, but not Th17, effectors initially activated in vivo induce disease upon passive transfer

Draining lymph node cells from pMOG-immunized mice were restimulated for 72 h with pMOG under Th1 or Th17 polarizing conditions. a) the proportion of CD4+ T cells capable of producing IFN- γ (black bars) IL-17 (open bars) or IFN- γ and IL-17 (gray bars) after three days culture in the presence of pMOG and the indicated recombinant cytokines. B) EAE score in recipients of Th1 (closed circles), Th17 (open circles) or Th1 and Th17 (open squares) polarized cells. Data (n=5 to 7 mice per group) are from one of three experiments giving consistent results. C) EAE score in recipients of Th1 (closed circles), IL-6+TGF- β polarised cells (open circles), or Th1 and IL-6+TGF- β polarised cells ((open squares) (n=3 to 6 mice per group).

Fig 2

Th1 but not Th17 polarized effectors enter the CNS and initiate inflammation.

Polyclonal Th1 (CD45.1 CD90.2) and Th17 (CD45.2 CD90.1) polarized cells generated from the DLN of pMOG-immunized mice were transferred to CD45.2 CD90.2 hosts alone (Th1, Th17) or in combination at a 1:1 ratio (Th1:Th17). The distribution of donor cells on day 16 after transfer was assessed by FACS analysis. a) the number of Th1 (closed bars) and Th17 (open bars) polarized cells recovered from the spleen of recipient mice. b) total number of mononuclear cells recovered from the CNS of recipient mice. c) total number of CD4+ T cells recovered from the CNS (donor and host cells combined). d)

number of donor Th1 (closed bars) and Th17 (open bars) recovered from the CNS. Individual splenic populations (e,f), or pooled CNS mononuclear cells (g,h), sampled from recipient mice were stimulated overnight with pMOG and cytokine production by donor cells originally polarized to Th1 cells (closed bars) and Th17 cells (open bars) was assayed by intracellular cytokine staining IFN- γ (e,g) and IL-17 (f,h). Bars represent the mean \pm SE of 5-7 recipient mice per group. In the co-transferred groups shown in panels e-h, the populations analyzed for cytokine production are identified as **bold underlined**.

Fig 3

Th17-polarized cells from MBP-reactive TCR transgenic mice can induce mild/delayed EAE, correlating with the in vivo appearance of IFN γ -producing cells

Th1- and Th17-polarized populations were generated from naïve Tg4 mice and transferred into B10.PL mice (a-c), or B10.PLxRAG2^{-/-} mice (d-f). a,d) intracellular cytokine staining of Th1-polarized (top panel) and Th17 polarized (lower panel) Tg4 cells prior to transfer. b) clinical course of EAE induced by transfer of Th1 (upper panel) and Th17 (lower panel) cells n=5. c) Ac1-9 stimulated IFN- γ and IL-17 production by Th1 (upper panel) and Th17 (lower panel) donor cells recovered from the CNS as determined by ICCS. Graphs show the mean \pm SD of cells recovered from 3 surviving Th1-transferred and 5 Th17-transferred mice. e) Th1 (closed symbols), or Th17 (open symbols) polarized Tg4 T cells (as shown in d) were transferred to B10.PLxRAG^{-/-} recipients (3 to 4 mice per group). f) Ac1-9-stimulated cytokine production by CD4⁺ T cells recovered from the CNS of a representative B10.PLxRAG2^{-/-} recipient with a disease score of 3. Data are from two of seven experiments giving consistent results.

Fig 4

Th1-polarized cells are phenotypically stable

a-f) Th1-polarized (closed circles) and Th17-polarized (open circles) Tg4 cells were washed extensively and restimulated with graded concentrations of Ac1-9. Cytokine production after 72hrs culture was assessed by ELISA (a,b) or cytometric bead array (c-f). g) IFN- γ and IL-10 production by polyclonal Th1- and Th17-polarized cells derived from pMOG-primed mice as determined by ELISA. h) signature cytokine production versus foxp3 expression in populations of Th1 (left panel) and Th17 (right panel) Tg4 cells sampled on the day of transfer. i) Expression of T-bet (filled bars) and ROR- γ t (open bars) at 72h (expressed as fold induction over cells stimulated under neutral conditions).

Figure 1

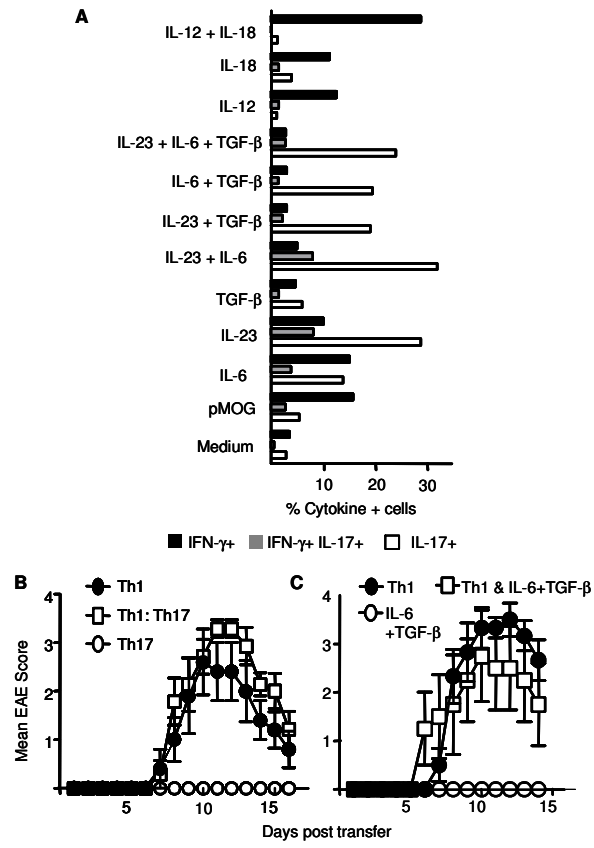


Figure 2

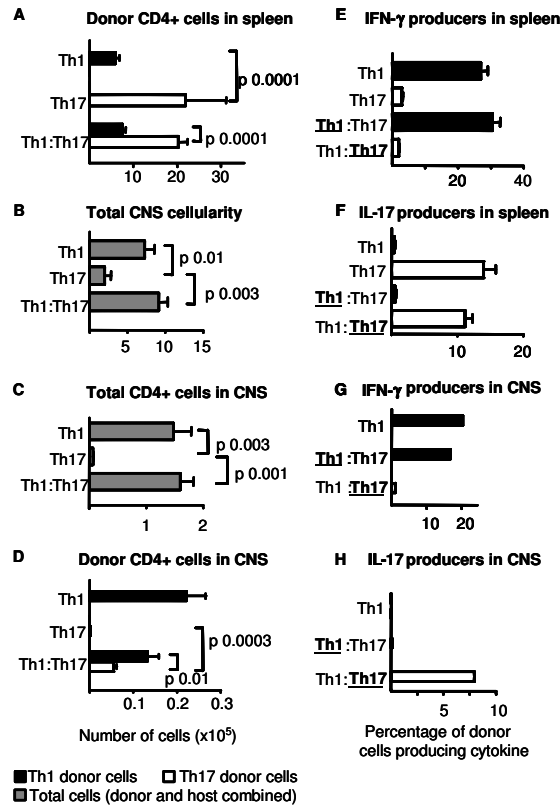


Figure 3

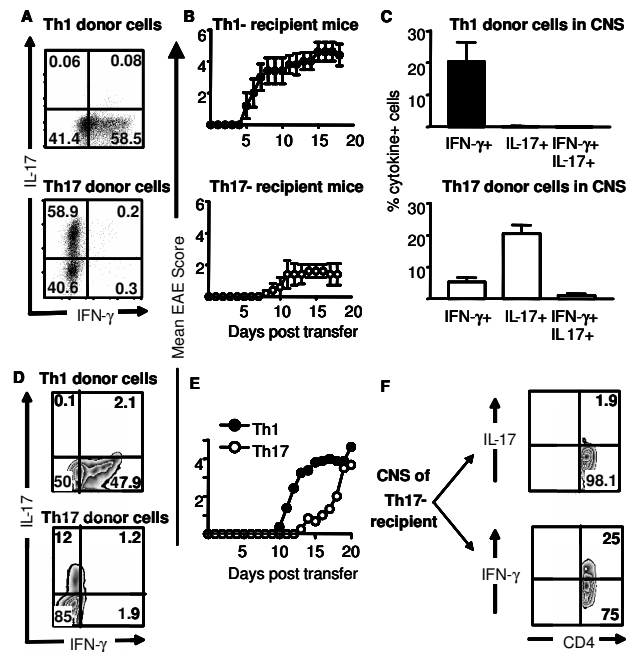


Figure 4

