Targeted Overexpression of IL-18 Binding Protein at the Central Nervous System Overrides Flexibility in Functional Polarization of Antigen-Specific Th2 Cells

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Targeted Overexpression of IL-18 Binding Protein at the Central Nervous System Overrides Flexibility in Functional Polarization of Antigen-Specific Th2 Cells

Sagie Schif-Zuck,∗ Juergen Westermann,† Nir Netzer,∗ Yaniv Zohar,∗ Moran Meiron,∗ Gizi Wildbaum,§ and Nathan Karin2*†

The current study shows that functional polarization of Ag-specific CD4+ Th2 cells entering the CNS during the accelerating phase of experimental autoimmune encephalomyelitis is flexible and dependent on the cytokine milieu there. Thus, targeted cell/gene therapy by Ag-specific T cells overexpressing IL-18 binding protein overrides this flexibility and induces infectious spread of Th2 cells. Using a congeneric system, we show that at this time, Ag-specific Th2 cells accumulate at the CNS but then arrest of IL-4 production. A manipulation of targeted cell/gene delivery was then used to detect whether this function is dependent on the cytokine milieu there. Targeted overexpression of IL-18 binding protein, a natural inhibitor of IL-18, restored the ability of these Ag-specific Th2 cells to produce IL-4 and subsequently induce protective spread of Th2 polarization. These findings not only suggest a novel way of therapy, but also explain why shifting the balance of Ag-specific T cells toward Th2 suppresses ongoing experimental autoimmune encephalomyelitis, whereas a direct transfer of these cells is ineffective. The Journal of Immunology, 2005, 174: 4307–4315.

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§ Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; IL-18BP, IL-18 binding protein; MBP, myelin basic protein; LN, lymph node; EAN, experimental autoimmune neuritis; Tr1, T regulatory 1; Thnp, nonpolarized T cell.
affects the course of disease and the fate of other CD4+ Th2 cells entering this site.

Materials and Methods

Animals

Female Lewis rats ~6 wk old were purchased from Harlan Biotech Israel and maintained under special pathogen-free conditions in our animal facility (RT.7®). The congenic rat strain (LEW.7B/Won; RT.7®) was obtained from K. Woneige (Hannover School of Medicine, Hannover, Germany) (31). The LEW.7B strain is identical with the congenic strain originally sequenced (Sequenase version 2; USB) according to the manufacturer’s protocol. Each clone was then sequenced by standard 9-fluorenylmethoxy-carbonyl chemistry. Peptides were purified by HPLC. Sequence was confirmed by amino acid analysis and mass spectroscopy. Only peptides that were >95% pure were used in our study.

Active induction of EAE or experimental autoimmune neuritis (EAN)

MBPp68 – 86 or P2p57–81 was used at a concentration of 1 mg/ml to dissolve in PBS and emulsified with an equal volume of IFA supplemented with 4 mg/ml heat-killed Mycobacterium tuberculosis H37Ra in oil (Difco Laboratories). Rats were immunized s.c. in the hind footpads with 0.1 ml of the emulsion and monitored daily for clinical signs by an observer blind to the treatment protocol. EAE was scored as follows: 0, clinically normal; 1, flaccid tail; 2, hind limb paralysis; 3, front and hind limb paralysis; and 4, total body paralysis. EAN was scored as follows: 0, clinically normal; 1, flaccid tail; and 2, hind limb paralysis.

Selection of MBP- or P2-specific T cell lines

MBPp68 – 86 or P2p57–81 were selected according to the method developed by Ben-Nun et al. (4), with our minor modifications (34).

Induction of transferred EAE

Transferred EAE was induced by immunizing Lewis rats (i.p.) with 105 in vitro activated (day 3) MBPp68–86 or P2p57–81 cells.

Antibodies

Rabbit anti-rat IL-18 Abs and IgG fraction were generated as described (18).

RT-PCR analysis

RT-PCR analysis was used on brain samples according to the protocol we described elsewhere (18). IL-4, IL-10- and actin-specific oligonucleotide primers were designed based on their published sequences (National Center for Biotechnology Information accession no. NM_053374 and NM_031144, accordingly) as follows: rat IL-18BP sense, 5′-CATGAGA GCT-3′, antisense, 5′-TCATGGGGCCCCTGGGCCT CACTGTGGCTGTGC-3′; rat IL-4 sense, 5′-TCAA TACAACAGCCAGCCAGTTC-3′, antisense 5′-GCCGCGCTTTG TACAGCTGCCC ATGC-3′; and GADPH sense, 5′-CTGTTTACAGCCAGCCAGG-3′, antisense 5′-GCCGCGCTTTG TACAGCTGCCC ATGC-3′. The PCR products were cloned and their sequences were verified as described below.

Cloning and sequencing of PCR products

The PCR products described above were cloned into a pUC57/T vector (T-cloning kit K1212; MBI Fermentas) and transformed to Escherichia coli according to the manufacturer’s protocol. Each clone was then sequenced (Sequenase version 2; USB) according to the manufacturer’s protocol.

Construction of the retroviral vectors

A 1337-bp EcoRI-Hpal fragment of the IRES-EGFP region from pIRE2-EGFP vector (BD Clontech) was introduced to the MoMuLV-derived vector pLXSN (BD Clontech) to construct the pLXSN-GFP vector. The IL-18BP EcoRI-Sall fragment from the pUC57/T vector was introduced to the pLXSN-IL-18BP-GFP vector. The retroviral vectors were transduced into the 293/E3 packaging cells with FuGENE6 transfection reagent (Roche Diagnostics) according to the manufacturer’s protocol.

Transfection of T lymphocyte with retrovirus

Retroviral gene transfer to T cells was done using the method developed by Flugel et al. (35, 36) with our minor modifications. Primary T lymphocytes from rats injected 9 days earlier with 1 mg of MBPp68–86 or P2p57–81 were cocultivated with 293/E3 packaging cells producing replication incompetent retroviruses. After 72 h, the lymphocytes were separated from the packaging cells with selection of 0.4 mg/ml G418 was started and maintained for the 2 wk of the culture period. Rats immunized s.c. 10 days earlier with 0.2 mg of MBPp68–86 or P2p57–81 were injected with 3 × 106 stably transfected lymphocytes.

Cytokine determination in primary cultures

Spleen cells or lymph node (LN) cells from EAE donors were stimulated in vitro (103 cells/ml) in 24-well plates (Nalgé Nunc International) with 100 μM MBPp68–86. After 72 h of stimulation, supernatants were assayed for the protein level of various cytokines using commercially available ELISA kits as specified; for IFN-γ, rat IFN-γ Eli-pair (lot EL-3200-01; Diaclon Research), for IL-4, rat IL-4 Eil-pair (lot EL-3004-01; Diaclon Research).

Isolation of CNS mononuclear cells

Cells were isolated according to protocol described previously by Katz-Levy et al. (37).

FACS analysis

FACS analysis was conducted according to the basic protocol we used previously (38). FITC-labeled mouse anti-rat CD45.2 (RT7B) (catalog no. 22124D; BD Pharmingen, CD Immunocytometry Systems) was used for gating of congenic CD4+ T cells. We found out that under our working conditions for FACS analysis, the CD4+ is a more convenient marker than the GFP. Intracellular staining of IL-4 and IFN-γ was done using a commercially available kit (LEUCOPERM, BUF9; Serotec) according to the manufacturer’s protocol. PE-labeled mouse anti-rat IFN-γ mAb (catalog no. 559499; BD Pharmingen, BD Immunocytometry Systems), FITC-labeled mouse anti-rat IFN-γ mAb (catalog no. 559498; BD Pharmingen, BD Immunocytometry Systems), and PE-labeled mouse anti-rat IL-4 mAb (catalog no. 555082; BD Pharmingen, BD Immunocytometry Systems) were used for direct staining. Brefeldin A was added to cells for the last 6 h as described previously by Openshaw et al. (39). Cells were analyzed using a FACS Calibur (BD Immunocytometry Systems). Data were collected for 10,000 events and analyzed using a CellQuest software program (BD Immunocytometry Systems).

Real-time PCR analysis

Real-time PCR analysis was done according to Ref. 40, with minor modifications. Briefly, total RNA was extracted from frozen rat spinal cords using Trizol reagent (Invitrogen Life Technologies) according to manufacturer’s instructions. Total RNA (2 μg) was reverse transcribed into cDNA and amplified using TaqMan Reverse Transcription Reagents (Applied Biosystems). Real-time quantitative PCR was performed using the ABI Prism 7000 Sequence Detector (Applied Biosystems). To validate the amount and quality of source RNA, transcript levels of a housekeeping gene (GAPDH) were also measured. Published cDNA sequences for rat IL-4 (GenBank accession no. NM_201270) and GAPDH (GenBank accession no. NM_017008) were also measured for primer and probe construction with the assistance of the computer program Primer Express (Applied Biosystems). The following primers and probes were used for relative quantification of targeted gene expression: for IL-4, forward primer 5′-TCAA CACCTTTGAAACCAGGTC-3′, reverse primer 5′-TGATTTCAGACCGCT GCAC-3′, and probe 5′-CATCTGAGGCTCTCCAGGGTGC-3′; for GAPDH, forward primer 5′-CCATCACCATCTTCCAGGAG-3′, reverse primer 5′-TCACCATCACATCAACACGTCC-3′, and probe 5′-GG CCTGTCGTCACCAACACTGGCCG-3′. The reaction was conducted with 1 μl of cDNA in a 25–50 μl final volume mixture containing primers and probe at optimized concentrations (400 and 200 nM, respectively) and 1/10 TaqMan Master Mix (PerkinElmer). PCR-retained conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles with 15 s at 95°C and 60 s at 60°C in each cycle. Negative controls (no template cDNA) were performed on each PCR plate. For each sample, target transcript values were normalized in comparison to the housekeeping gene.
Relative quantification and calculation of the confidential range was performed using the comparative \( \Delta \Delta C_t \) method. All amplifications were conducted at least in duplicates.

**Histopathology**

Frozen sections of the same area of the lumbar region of the spinal cord were stained with H&E. Each section was evaluated without knowledge of the treatment status of the animal. The following scale was used: 0, no perivascular lesions; 1, 1–5 perivascular lesions per section with minimal parenchymal infiltration; 2, 5–10 perivascular lesions per section with parenchymal infiltration; and 3, >10 perivascular lesions per section with extensive parenchymal infiltration. The mean histological score ± SE was calculated for each treatment group (representative photomicrographs are shown in Fig. 5D).

**Statistical analysis**

Significance of differences was examined using the Student's t test. Values \((p < 0.05)\) were considered significant. Mann Whitney sum of ranks test was used to evaluate significance of differences in mean of maximal clinical score. Values \((p < 0.05)\) were considered significant.

**Results**

**Suppression of ongoing EAE by neutralizing IL-18 is associated with selection of Ag-specific Th2 cells that do not transfer disease resistance**

We have previously shown that repeated administrations of anti-IL-18 Abs, beginning before the onset of EAE, drives the in vivo balance of CD4° T cells into Th2 phenotype and suppresses the disease (18). Subsequently, we show in this study that even if these Abs are administered, for the first time, after the onset disease, they are also capable of suppressing the disease (Fig. 1A), mean maximal score of 1.33 ± 0.24 vs 3 ± 0.3 in control EAE rats, \(p < 0.001\). Once again, suppression of disease was associated with an alteration of the in vivo polarization of Ag-specific T cells into Th2 (18). Nevertheless, CD4° Th2 lines selected from these protected rats (in the presence of anti-IL-18 Abs) could never transfer the beneficial effect of anti-IL-18 therapy (Fig. 1D).

To investigate the role of Th2 cells in the regulation of disease we have used a congenic system (32, 41). The basic idea includes selection of CD4° T cell lines from congenic Lewis rats (RT7B) expressing the CD45.2 isofrom on all CD4° cells (CD45.2°) and transferring them into RT7A “normal” Lewis rats. In addition to their biological properties (i.e., the ability to transfer disease to RT7A rats or suppress an ongoing disease in these rats) the fate of these cells could be followed by a mAb to CD45.2 isoform (32, 41). At first, we have verified that CD45.2°'s are also susceptible to active induction of EAE, and that MBPp68–86-specific CD4° T cell lines selected from these EAE rats can adoptively transfer the disease to CD45.2° and CD45.2° Lewis rats. Then, MBPp68–86-specific Th1 and Th2 line were selected from CD45.2° donors. The Th2 line was selected in cultured medium supplemented with IL-4 and neutralizing Abs to IL-12 + IL-18 + IFN-\(\gamma\) (Fig. 1Ba). At the same time an MBP-specific Th1 line was selected in cultured medium supplemented with IL-12 + IL-18 + IFN-\(\gamma\) and neutralizing Abs to IFN-\(\gamma\) (Fig. 1Bb). After four consecutive selection cycles, ~90% of CD4° T cells selected under pro-Th2 conditions displayed a Th2 cytokine profile (Fig. 1Ba). Additional selection cycles did not further increase the number of IL-4°IL-13°IFN-\(\gamma\)-\(\gamma\)-producing CD4° T cells. Likewise, ~80% of our Ag-specific encephalitogenic Th1 cells indeed displayed a Th1 phenotype (Fig. 1Bb). Again, further selection cycles did not increase their relative number. It has been shown by others that even at the single clone level committed T cells (Th2) do not manifest a coherent cytokine profile, but rather display a balance comprised from IFN-\(\gamma\)-polarized Th2 cells and a small population of IFN-\(\gamma\)-IL-4-polarized T cells (42, 43).

Under our working conditions, both lines were low IL-10-producing cells (ELISA, levels below 250 pg/ml). At first, each of these lines was determined for its ability to adoptively transfer EAE to CD45.2° rats (Fig. 1C). A single administration of 5 × 10° cells from the Th1 line induced EAE in all recipients (6/6). None of the six rats administered with the same dose of MBPp68–86-specific Th2 cells showed any clinical signs of disease (Fig. 1C), even when 2 × 10° Th2 cells were transferred per rat. We also could not observe any clear histological infiltrates in sections obtained from these animals. Then the ability of the Th2 cells to inhibit active EAE was also determined (Fig. 1D). Our MBP-specific Th2 line, administered at the onset of disease, could not affect its severity. These results are compatible with previous observations made by us (19) and by others (26) regarding the ability of Th2 cells to transfer EAE. A subsequent experiment done under the same condition was then conducted to follow the migratory properties and fate of these cells.

**Ag-specific Th2 cells selectively accumulate in the CNS during the accelerating phase of EAE but loose their ability to produce IL-4**

MBP-specific CD45.2° lines that were polarized into Th1 or Th2 (Fig. 1B) were administered to CD45.2° EAE rats during the accelerating phase of disease (1 day after the onset). In the first set of experiments, we analyzed the cytokine profile produced by
these cells at the target organ (CNS) 48 h later. Fig. 2 represents one of three experiments done under the same protocol, with similar results. Predictably, CD45.2+ Th1 cells administered during the accelerating phase of disease maintained their IFN-γhighIL-4low cytokine profile at the target organ (Fig. 2, j and h). It is likely that this population includes Ag-specific T cells that were already polarized before their administration, and perhaps some cells that displayed an IFN-γhighIL-4low phenotype before their administration (Fig. 1B). Nevertheless, during the accelerating phase of disease, neither these cells, nor the IFN-γhighIL-4low congenic cells that were administered, redirected their polarization at the inflamed autoimmune site into Th2. By contrast, Th2 cells administered during the accelerating phase of disease did not maintain their cytokine profile. Surprisingly, at the target organ they developed a cytokine profile comparable to that of IFN-γhighIL-4low nonpolarized T cells or Th1 cells (Fig. 2, c and e). Moreover, the cytokine production by the endogenous CD4+ T cells (dominance of IFN-γ) was comparable irrespective whether Th1 or Th2 cells were injected (Fig. 2, b, d, g, and i). Once again, we cannot exclude the possibility that the progeny of some, or even all, of the IFN-γ-producing congenic cells that were isolated from the CNS, is from the IFN-γhighIL-4low nonpolarized T cells in the line (13%). Nevertheless, our results show, for the first time, that under the inflammatory milieu at the CNS, during the accelerating phase of disease, Th2 cells do not maintain their IL-4-producing phenotype and Ag-specific nonpolarized T cells are not being driven to the Th2 commitment. This may explain, in part, their incompetence to suppress EAE in adoptive transfer experiments (Fig. 1D).

A subsequent set of experiments was conducted to follow the kinetics of accumulation of our CD45.2+ congenic Th2 cell line. Fig. 3A summarizes the results of one of six independent experiments with a very similar pattern of results. The congenic T cell line was injected to EAE rats ~24 h after the onset of disease and was monitored at the CNS 24, 48, and 72 h later. Fig. 3 shows that these cells could be identified, at a very low level, in the CNS within 24 h (Fig. 3A, 0.1%). Their number accelerated within the next 24 h (25-folds of increase in relative ratio), but not later on. It is likely that this rapid increase results from an intensive propagation of these cells in response to the target Ag. The notable reduction (~50%) in the relative number of congenic cells later on could be due to a continuing influx of endogenous cells to the CNS along the progression of disease. Analysis of the kinetics of accumulation of our CD45.2+ Th1 line revealed a similar pattern of results, implying that regardless their basic subtype, these Ag-specific T cells accumulate at the autoimmune site. A subsequent set of experiments was conducted to analyze the location of these congenic cells while comparing their relative ratio in the CNS, spleen, inguinal LNs, and cervical LNs that drain the CNS (Fig. 3B). We show that the congenic Th2 cells accumulated over time (48 h) in the CNS (3%), to a lesser degree in the draining LN

**FIGURE 2.** MBP-specific CD4+ Th2 cells redirect their cytokine profile at the target organ active EAE was induced in CD45.2− rats. Just after the onset of disease (day 10) these rats were administered (i.v.) with the MBP-specific Th2 (a–e) or Th1 (f–j) congenic (CD45.2+) CD4+ T cell lines described in Fig. 1B. Forty-eight hours later, spinal cords were removed. CD45.2− and CD45.2+ obtained from the spinal cords were subjected to intracellular staining of IL-4 and IFN-γ. Representative data of one of three independent experiments are presented in this figure.

**FIGURE 3.** During the accelerating phase of disease, Ag-specific T cells selectively accumulate in the CNS and then in the LNs that drain it. Congenic (CD45.2+) Th2 cells and Th1 cells (data not shown) were transferred (5 × 10⁶/rat) to CD45.2− recipients at the onset of EAE (day 10). At different time points (24, 48, and 72 h post T cell transfer), spinal cords were subjected to FACS analysis of CD45.2+ of all CD4+ T cells. A, The level of CD45.2+ of all CD4+ T cells at the CNS at different time points. In another experiment, done under the same experimental conditions (B), animals were sacrificed 48 h after cell transfer, and spinal cords, spleens, inguinal LN, and cervical LN were subjected to FACS analysis comparing the ratio of CD45.2+ of all CD4+ T cells in these organs. Similar results were obtained using our CD45.2+ Th1 line (data not shown). Representative data of one of three independent experiments are presented in this figure.
Targeted overexpression of IL-18BP at the autoimmune site reduces CNS damage and clinically improves EAE in an Ag-specific fashion.

We have then determined the possibility that at the autoimmune site, IL-4 production by Th2 cells is dependent on the inflammatory milieu there. Therefore, we looked for a novel way of altering this milieu in a targeted manner. IL-18BP is a natural inhibitor of IL-18 (33). We thought to overexpress it in MBP-specific CD4+ T cells that would be used as a vehicle for targeted cell/gene therapy. At first, we cloned rat IL-18BP. The gene product was determined for its expression (Western blot), and for its biological activity, i.e., the ability to inhibit IFN-γ production in cultured primary T cells responding to their target MBP (data not shown). Thereafter, IL-18BP was stably transduced, together with GFP, into anti-MBPp68–86 primary T cells that were then stimulated by MBPp68–86 under either Th2- or Th1-polarizing conditions. The overexpression of IL-18BP and GFP by the transduced T cells was verified by RT-PCR (Fig. 4A). We also observed that the Th2 lines maintained their IL-4highIFN-γlow profile whether IL-18BP was expressed or not (Fig. 4B). In addition, transduction of GFP alone did not alter the cytokine profile of Th1 cells (Fig. 4B). All lines produced very low levels of IL-10 and TGF-β (below 250 pg/ml IL-10, TGF-β ND). Before being used in adoptive transfer experiments, we have verified that the transduction rate of the retroviral vectors was >80% and that there is no packaging cell contamination in the injected lines. The biological properties of the IL-18BP produced by these transduced cells were verified by an in vitro assay as follows. The IL-18BP-encoding construct was ligated into

(cervical, 1.8%), but not in the spleen and in unrelated LNs (0.2% and 0.4%, respectively). Similar data that have been obtained in six independent experiments and the results were comparable, independent of whether Ag-specific Th2 cells or Th1 cells were used for transfer. This also motivated us to use this system for targeted delivery of IL-18BP as described below.

Targeted overexpression of IL-18BP at the autoimmune site overcomes CNS damage and clinically improves EAE in an Ag-specific fashion.

We have then determined the possibility that at the autoimmune site, IL-4 production by Th2 cells is dependent on the inflammatory milieu there. Therefore, we looked for a novel way of altering this milieu in a targeted manner. IL-18BP is a natural inhibitor of IL-18 (33). We thought to overexpress it in MBP-specific CD4+ T cells that would be used as a vehicle for targeted cell/gene therapy. At first, we cloned rat IL-18BP. The gene product was determined for its expression (Western blot), and for its biological activity, i.e., the ability to inhibit IFN-γ production in cultured primary T cells responding to their target MBP (data not shown). Thereafter, IL-18BP was stably transduced, together with GFP, into anti-MBPp68–86 primary T cells that were then stimulated by MBPp68–86 under either Th2- or Th1-polarizing conditions. The overexpression of IL-18BP and GFP by the transduced T cells was verified by RT-PCR (Fig. 4A). We also observed that the Th2 lines maintained their IL-4highIFN-γlow profile whether IL-18BP was expressed or not (Fig. 4B). In addition, transduction of GFP alone did not alter the cytokine profile of Th1 cells (Fig. 4B). All lines produced very low levels of IL-10 and TGF-β (below 250 pg/ml IL-10, TGF-β ND). Before being used in adoptive transfer experiments, we have verified that the transduction rate of the retroviral vectors was >80% and that there is no packaging cell contamination in the injected lines. The biological properties of the IL-18BP produced by these transduced cells were verified by an in vitro assay as follows. The IL-18BP-encoding construct was ligated into
sion of IL-18BP and GFP were monitored in lumbar CNS of rats of the peripheral nervous system named EAN (Fig. 5), in comparison to an autoimmune disease GFP, or GFP alone were then determined for their competence to suppress EAE (Fig. 5A), mean maximal score 0.833 ± 0.18 vs 3 ± 0.28, p < 0.01) within 48 h, whereas those administered with the MBP-specific Th2 line continued to manifest severe EAE. Histological evaluation showed a marked reduction in the inflammatory process in rats subjected to IL-18BP-targeted cell therapy (Fig. 5D). Whereas control EAE rats displayed an inflammatory process with more than five perivascular lesions per section and extensive parenchymal infiltration (Fig. 5D), animals treated with MBPp68–86-specific T cells overexpressing IL-18BP revealed only one to two perivascular lesions per section and minimal parenchymal infiltration. In additional experiments conducted under the same conditions, rats were repeatedly administered with IL-18BP-overexpressing T cells and then with anti-IL-4-neutralizing Abs (BD Pharmingen, BD Biosciences) that were administered at a dose of 100 μg/rat every other day. Under our working conditions these Abs could not override the therapeutic effect of the targeted cells therapy.

We also determined whether the observed therapeutic effect is Ag-specific. CD4⁺ Th2 cells and Th2 cells overexpressing IL-18BP were selected in response to P2p57–81, a major target Ag in EAN. These cells were analyzed for their ability to treat ongoing EAE (Fig. 5A). It should be noted that lines selected against MBPp68–86 have no cross-reactivity with those selected against P2p57–81 (44). After injecting of the usual number of cells (5 × 10⁶/rat), anti-P2p57–81 CD4⁺ T cells overexpressing IL-18BP did not accumulate in the CNS (<0.2% cells at all times). Furthermore, they had no effect on the manifestation of EAE (Fig. 5A). In addition, the anti-MBPp68–86 line overexpressing IL-18BP could not suppress EAN (Fig. 5B). Thus, targeted cell therapy using Ag-specific T cells overexpressing IL-18BP is disease-specific.

As a complementary set of experiments, we have overexpressed IL-18BP in our polarized Th1 line (Fig. 1B). In vivo administration of these cells provided very similar results to those described above (data not shown).

Targeted overexpression of IL-18BP at the autoimmune site maintains IL-4 production of Th2 cells after entry into the CNS

We have then studied how the targeted overexpression of IL-18BP is influencing the cytokine production of Th2 cells in the CNS. Therefore, the congeneric (CD45.2⁺) Th2 line (~90% of cells producing only IL-4; Fig. 1B) was injected into rats at the onset of disease either alone (control) or together with MBP-specific T cells overexpressing IL-18BP (CD45.2⁺). FACS analysis of the control
group showed that during the accelerating phase of disease (48 h after administration) in the absence of IL-18BP-producing T cells <0.5% of the injected CD45.2+ T cells recorded at the CNS continued to produce IL-4 only (Fig. 6A). However, in the presence of Ag-specific T cells overexpressing IL-18BP, ∼40% of the injected CD45.2+ T cells recorded at the CNS continued to produce IL-4 only (Fig. 6B). These results represent one of three experiments done under similar conditions with similar results.

In a subsequent experiment, done under similar conditions, the congenic Th2 line (CD45.2+, ∼90% of cells producing only IL-4; Fig. 1B) was injected into rats at the onset of disease. Forty-eight hours later, endogenous T cells (CD45.2−), obtained from the CNS, spleen, and cervical LNs were analyzed for intracellular expression of IL-4 vs IFN-γ. Targeted cell therapy using high IL-18BP-expressing T cells that rapidly suppressed EAE in an organ-specific manner (Fig. 5) resulted in a significant increase in high IL-4-producing T cells in the CNS (Fig. 7aC, 15% CD4+IL-4highIFN-γlow and 10% CD4+IL-4highIFN-γhigh), and cervical LNs (Fig. 7aF, 18% CD4+IL-4highIFN-γlow and 5% CD4+IL-4highIFN-γhigh) that drain the CNS, but not in the spleen (Fig. 7aD). Rats administered with nontransformed Th2 line did not display this type of infectious spread of IL-4 production by endogenous CD4+ T cells (Fig. 7a, B, E, and H) and subsequently were not protected from EAE (Fig. 5). Similar results were obtained in three independent experiments conducted under comparable conditions. Finally, samples from the spinal cord of these animals (three per group) were also subjected to real time PCR analysis for the transcription of IL-4. Our results (Fig. 7b) show that rats administered with the congenic line overexpressing IL-18BP transcribe significantly higher levels of IL-4 (p < 0.001) compared with those administered with the control Th2 line.

Discussion

The development and progression of an autoimmune condition can be viewed as dynamic balance between the function of Ag-specific effector T cells and regulatory mechanisms that control their function, in particular by the induction of active suppression or apoptosis. During EAE, and possibly MS, Ag-specific effector Th1 cells enter the CNS and thereafter undergo Fas-FasL-mediated apoptosis (35, 38, 45–47). This may explain, in part, why the ongoing form of disease requires a continuing entry of effector T cells to commence the inflammatory process, and subsequently why blockade of T cell entry to the CNS suppresses ongoing EAE (48, 49) and MS (50). It may also explain why shifting the in vivo balance toward Th2, that increases the ratio of infiltrating nonpathogenic CD4+ T cells to the autoimmune site, ameliorates the severity of disease (18, 19). Additionally, high levels of the Th2 cytokine IL-4 and IL-13 at the CNS are thought to directly suppress the inflammatory activity of macrophages (51, 52) that are key players in the inflammatory process in EAE and other inflammatory autoimmune diseases (8, 53, 54). We have previously shown that Abs to IL-4 could reverse Th2-mediated tolerance induced by systemic administration of soluble peptide therapy (55). In this study, the same approach could not reverse tolerance. One possible explanation could be that in contrast to the systemic approach presented there, in this study the major events of T cell polarization occur at the target autoimmune organ and therefore systemic administration of neutralizing Abs to IL-4 is ineffective.

Finally, binding of IL-12 and IL-18 to their target receptors activates a Stat4-T-bet pathway that polarizes Th1, whereas early binding of IL-4 to its target receptor on these cells activates a Stat6-GATA-3-c-Maf pathway, or Stat5 (56) pathway that polarizes Th2 (57–61). It is therefore plausible that high levels of IL-4 at an autoimmune site would drive the polarization of invading T cells into Th2 and induce infectious spread of T cell tolerance. All of the above may well explain why shifting the in vivo balance from Th1 to Th2 could effectively suppress EAE (17–19), but cannot explain why a direct administration of Ag-specific Th2 cells during an ongoing disease had no beneficial effect on its manifestations (19, 26) (see also Fig. 1).

A previous study on the subject demonstrated that overexpression of IL-4 in the CNS could ameliorate EAE (30). A major difference between this study, and those that failed in suppressing the disease by an adoptive transfer administration of Ag-specific Th2 cells is that in this study, neither the production of IL-4, nor the regulation of proliferative response of the IL-4-producing cells, was naturally controlled and affected by the milieu at the autoimmune site. After all, these were Ag-specific hybridoma T cells engineered to overexpress IL-4 under the control of a CMV promoter (30). Notably, the direct administration of other types of Ag-specific regulatory T cells, such as Tr1 or Th3, could rapidly and effectively suppress an ongoing EAE (9, 14) even if cells were not immortalized, and the production of their regulatory cytokines has been controlled in a natural manner (9, 14). The present study aims at resolving this discrepancy. We show that after transfer of Th2 cells, they reach the CNS. Furthermore, Th2 cells selectively accumulate in the CNS and to a lesser extent in the draining LN (Fig. 3) probably due to proliferation in response to their Ag presented at those sites (14). However, within the CNS, under proinflammatory conditions, they arrest IL-4 production (Fig. 2), and subsequently cannot direct IL-4-mediated functions such as suppression of macrophage inflammatory functions. They are also incapable of providing the appropriate conditions for infectious spread of Th2 polarization (Fig. 7), an IL-4-dependent feature (57).

Together, this may explain why administration of Ag-specific Th2 cells does not improve the clinical outcome of EAE (Fig. 5). Interestingly, a notable portion of congenic cells from our Th2 line that were isolated from the CNS displayed a high IFN-γ-producing phenotype (Figs. 2 and 6). One possibility is that Ag-specific Th2 cells that were selected in vitro redirect their commitment under the inflammatory milieu at the CNS. It should be noted that others (62) and us (Fig. 1) could not achieve a similar effect under in vitro conditions. That is, after several in vitro selection cycles a defined Th2 population of CD4+ T cells (even at a clonal level) reached a defined balance between IFN-γlowIL-4high-Th2 and a notable portion of IFN-γlowIL-4low-nopolarized T cells (Thnp), that could not be redirected into IFN-γhigh-producing Th1 cells (42, 43, 62), probably because even the Thnp cells that persisted several selection cycles under pro Th2 conditions engaged commitment to the Th2 type. This strongly suggests that either novel factors or types of regulatory cells, yet to be identified, participate in the in vivo regulation of T cell polarization, which is apparently different from the selection cycles in a culture dish. It is not clear whether the progeny of the congenic IFN-γ-producing Th1 cells collected from the CNS (Figs. 2 and 6) are from the IFN-γlowIL-4high-Th2 cells or the IFN-γlowIL-4low-nopolarized T cells in the line (3%). Therefore, we cannot conclude that at the autoimmune site Th2 cells are indeed converted to Th1. Nevertheless, our results show, for the first time that under the inflammatory milieu at the CNS during the accelerating phase of disease, Th2 cells do not maintain their IL-4-producing phenotype, and that Ag-specific nonpolarized T cells are not being driven to the Th2 commitment. This may explain, in part, their incompetence to suppress EAE in adoptive transfer experiments (Fig. 1D). We also show that this process is dependent on the inflammatory milieu at the CNS, and that alteration of this milieu not only enables maintaining the cytokine characteristic of polarized T cells, but also their ability to induce infectious spread of Th2 polarization. Practically, the issue of flexibility in T cell
polarization could be very important for clinical trials involving an administration of Ag-specific polarized T cells, particularly because polarization of human memory CD4+ T cells could be flexible (63).

Finally, we show that the functional depolarization of Th2 cells in the CNS and the draining LN could be prevented by targeted expression of the IL-18BP (Fig. 7). Our laboratory was the first to demonstrate the pivotal role of IL-18 in the regulation of T cell-mediated autoimmunity (18). We showed that neutralizing IL-18 suppresses EAE (18), an observation that has later extended to different autoimmune diseases such as experimentally induced rheumatoid arthritis (64), inflammatory bowel disease (65), and myasthenia gravis (66). Interestingly, neutralizing IL-18 suppressed experimentally induced arthritis even in IFN-γ−/− mice (67), suggesting that its effect is beyond enhancing IFN-γ production (68). Five years ago, Novick et al. (33) identified IL-18BP as the natural inhibitor of IL-18. This gene product is an important potential candidate for neutralizing IL-18 in autoimmune diseases (69, 70). It is possible that in addition to neutralizing IL-18, the IL-18BP directly affects IL-4 production by T cells. This possibility has yet to be determined. This may strongly imply for IL-18BP-based therapy as a promising way to treating MS and possibly other autoimmune diseases.

Disclosures

The authors have no financial conflict of interest.

References

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