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T-cell seeding: neonatal transfer of anti-myelin basic protein T-cell lines renders Fischer rats susceptible later in life to the active induction of experimental autoimmune encephalitis

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Summary

Fischer strain rats resist active induction of experimental autoimmune encephalomyelitis (EAE) following immunization with guinea-pig myelin basic protein (MBP) in complete Freund's adjuvant (CFA). Nevertheless, we now report that an encephalitogenic $CD4^+$ anti-MBP T-cell line could be developed from actively immunized Fischer rats. Adoptive transfer of the activated line mediated acute EAE in adult Fischer rats, but not in 1-day-old rats. Moreover, we found that both resting and activated anti-MBP T cells injected 1 day post-natally rendered these rats susceptible later in life to the active induction of EAE by immunization with MBP/CFA. The actively induced EAE manifested the accelerated onset of a secondary, memory-type response. Resting anti-MBP T cells injected even up to 2 weeks post-natally produced no clinical signs but seeded 50–100% of the recipients for an active encephalitogenic immune response to MBP. An earlier T-cell injection (1–2 days) produced a higher incidence and stronger response. The transferred resting T cells entered the neonatal spleen and thymus and proliferated there but did not change the total anti-MBP precursor number in adults. Splenocytes harvested from rats that were injected neonatally but not exposed to MBP *in vivo* proliferated strongly and produced significant amounts of interferon-γ to MBP *in vitro*. Similar results were observed in rats injected with resting T-cell lines reactive to ovalbumin, suggesting that the neonatal injection of resting T cells specific for a self or for a foreign antigen can seed the immune system with the potential for an enhanced effector response to that antigen later in life.

Keywords: antigens/peptides/epitopes; EAE/MS; memory; rodent; Th1/Th2 cells

Introduction

Antigen specific T-cell lines are grown in consecutive cycles of 2–3 days of activation with their cognate antigen, followed by 4–7 days of maintenance in media supplemented with interleukin-2 or T-cell growth factor (TCGF) without antigen.[1] Adoptive transfer of self-reactive CD4 T-cell lines in an activated state has been shown to generate various antigen-specific autoimmune diseases in syngeneic recipients.[1,2] However, following attenuation by irradiation or chemical cross-linkers, injection of these activated self-reactive T cells can induce resistance to the specific autoimmune disease – a process termed T-cell vaccination.[2–5] Both the adoptive transfer of autoimmune disease and the induction of T-cell vaccination require that the transferred T cells be activated by specific antigen or by mitogen before injection;[4,6] the same lines of T cells that have entered a resting state can neither adoptively transfer an autoimmune disease nor vaccinate against it.[4,7] Resting and activated T cells differ in their

Abbreviations: BBP, bovine MBP; CFA, complete Freund's adjuvant; CFSE, carboxyfluorescein succinimidyl ester; c.p.m., counts per minute; DMEM, Dulbecco's modified Eagle's minimal essential medium; EAE, experimental autoimmune encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; HBP, human MBP; IFN, interferon; i.p., intraperitoneal; MBP, myelin basic protein; MHC, major histocompatibility complex; OVA, ovalbumin; PBS, phosphate-buffered saline; RBP, rat MBP; TCGF, T cell growth factor; TCV, T-cell vaccination.

phenotypes as well as in their functionality: activated T cells are large, form clumps when grown in culture, and express activation markers;[8] resting T cells are smaller, do not clump, and do not express activation markers.[4,7,8]

Rats of the Lewis strain are susceptible to experimental autoimmune encephalomyelitis (EAE) induced either by active immunization with myelin basic protein/complete Freund's adjuvant (MBP/CFA) or adoptively transferred by activated anti-MBP T-cell lines.[4,8] Although they bear the same RT-1[l] major histocompatibility complex (MHC) allele as Lewis rats,[9] rats of the Fischer strain resist attempts to induce EAE by immunization with myelin antigens in adjuvant.[10,11] While the above findings were obtained in experiments with adult Lewis or Fischer rats; neonatal rats have been found to behave somewhat differently.

Flugel and associates have reported that neonatal Lewis rats in the first 2 days of life resist EAE mediated by activated anti-MBP T-cell lines;[12,13] after 2 days of age, activated anti-MBP T cells mediated severe EAE.[14] Interestingly, neonatal Lewis rats that had been injected with activated anti-MBP T cells were still susceptible to active induction of EAE by MBP/CFA later in life, but the disease manifested an earlier onset,[13,15] suggesting a memory-type response.[14] Indeed, anti-MBP T cells that were transferred neonatally into Lewis rats persisted in various lymph node cells for over 2 years and maintained a memory phenotype with low levels of L-selectin and CD45RC, and high expression of CD44.[13]

The present study aimed to investigate the effects of adoptive transfer of anti-MBP T cells, either activated or resting, in Fischer strain rats, which, as adults, resist the active induction of EAE by MBP/CFA.[10,11] We raised autoimmune CD4 T-cell lines specific for MBP and foreign-antigen-reactive T cells specific for ovalbumin (OVA) and transferred these T cells into syngeneic Fischer recipient rats at various times after birth. We report here that activated anti-MBP T cells could mediate EAE in adult Fischer rats; resting T cells of the same line, in contrast, did not mediate EAE. However, transfer of the resting anti-MBP T cells into neonatal Fischer rats, even 14 days after birth, rendered the otherwise resistant recipients susceptible to actively induced EAE in adult life. Consequently, antigen-specific T cells introduced early in life can seed the immune system with the potential for an enhanced response to that specific antigen later in life.

Materials and methods

Rats

Fischer (F344) and Lewis rats (both RT1[l]) were obtained from Harlan Laboratories, Rehovot, Israel. Animals were maintained in a specific pathogen-free environment in the Weizmann Institute of Science Animal Facilities. Animal experiments have been reviewed and approved by the institutional review committee.

Peptide synthesis

Peptides spanning the 18 500 molecular weight isoform of rat MBP[16] with a 10-amino-acid overlap were synthesized on an ABIMED AMS 422 multiple peptide synthesizer (ABIMED, Langenfeld, Germany), using the *a-N*-fluorenylmethoxycarbonyl strategy following the commercially available protocols of the company. The MBP 71–90 peptide: (SLPQKSQRSQDENPVVHF) corresponds to position 71–90 in the human MBP sequence[6] and has 95% identity to rat MBP (a serine to threonine exchange).

T-cell lines

T-cell lines specific for guinea-pig MBP (Sigma, Rehovot, Israel) were established and maintained using a standard protocol:[1] briefly, rats were immunized with antigen (at 1 mg/ml) emulsified 1 : 1 with CFA (4 mg/ml *Mycobacterium tuberculosis*, Difco, Detroit, MI). The lymphocytes from the draining lymph nodes of antigen-immunized rats were isolated on day 10–12 post-immunization. The lymphocytes were stimulated *in vitro* (at 5×10^6/ml) for 72 hr with 20 μg/ml of guinea-pig MBP or OVA (both Sigma). Stimulation medium contained Dulbecco's modified Eagle's minimal essential medium (DMEM), 1% syngeneic normal serum, 2 mM glutamine, combined antibiotics, 1 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol and 1% non-essential amino acids. Following a 3-day cycle of stimulation, the cells were collected and transferred to rest for 4–7 days in resting medium containing DMEM, 10% fetal calf serum, 2 mM glutamine, combined antibiotics, 1 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol, 1% non-essential amino acids and 10% TCGF medium. TCGF medium was prepared from the supernatants of rat splenocytes stimulated at 2×10^6 cells/ml for 2 days in resting medium (TCGF-free) supplemented with 2 μg/ml of concanavalin A.

In all the next cycles of stimulation, gamma-irradiated (50 Gy) syngeneic rat thymocytes (at 10^7 cells/ml) were used as antigen-presenting cells, which were added to the resting T cells (at 5×10^5 cells/ml) for 3 days of stimulation with 10 μg/ml antigen. Lines used were of similar stimulation cycles, to avoid changes occurring in line properties following repeated stimulations in culture.

The following $CD4^+$ T-cell lines were used:

LewMBP – Lewis rat T-cell lines recognizing the guinea-pig MBP protein.[6]

FischMBP – a Fischer rat T-cell line recognizing guinea-pig MBP protein.[10]

FischOVA – a Fischer rat T-cell line raised against OVA (Sigma).

Induction of active or adoptive EAE

For induction of adoptive EAE, rats were injected either intraperitoneally (i.p.) or intravenously with 4×10^6 to 5×10^6 MBP-activated T cells and followed for clinical signs of EAE.

For active induction of EAE, groups of five to seven rats (unless stated otherwise) were injected subcutaneously into the dorsum of both hind footpads with 50 μl MBP protein or MBP peptides emulsified 1 : 1 in CFA (mycobacterium tuberculosis at 4 mg/ml; Difco). Signs of EAE were scored as 0 = absence of clinical signs; 0·5 = loss of motor control in a portion of the tail; 1 = loss of motor control in the entire tail; 1·5 = hindquarter weakness; 2 = complete paralysis of hind legs; 3 = half body paralysis; 4 = paralysis in front and hind legs; 5 = total paralysis including neck movement; and 6 = death caused by EAE. A cumulative EAE score was calculated by the summation of the EAE scores of each individual rat during all the days of its disease.

T-cell proliferation assay

Antigens were dispensed in stimulation medium in quadruplicate to 96-well U-shaped plates. Splenocytes were added to each well at $2{\cdot}5 \times 10^5$ cells/well. After 2 days, the cultures were pulsed overnight with 25 μl pulsing medium containing phosphate-buffered saline (PBS) and methyl-[^{3}H]thymidine (5 Ci/mM; Amersham, Buckinghamshire, UK) at a ratio of 25 : 1, respectively. Cultures were harvested to a 96 GF/C Unifilter (Perkin Elmer, Waltham, MA) and Microscint-20 scintillation fluid (Perkin Elmer) was added. The plates were read using a Topcount microplate scintillation and luminescence counter (Packard, Ramsey, MN). The results are expressed as the mean counts/min (c.p.m.) with standard error bars.

Enzyme-linked immunosorbent assay cytokine assay

In the proliferation assay described above, 80–100 μl of supernatants from the cultures were collected after 2 days of stimulation, before labelling with radioactive thymidine, and used in enzyme-linked immunosorbent assays (ELISA) for detection of cytokines in the medium. Cytokine secretion was determined using the manufacturer's standard protocols (BD-OptEIA, Erembodegem, Belgium).

Transfer of T cells to newborns

Rats were injected 0–14 days post-partum with activated or resting T cells in PBS. Resting T cells were used 4–7 days following their transfer to resting medium. In some experiments, the T cells were irradiated (3000 rad) before injection. The T cells (1×10^6 to 3×10^6) were injected i.p. using a fine insulin syringe (Microfine-Plus 0·5 ml; Becton Dickinson, Franklin Lakes, NJ) in a volume of 50–100 μl.

CFSE labelling of T cells and detection by fluorescence-activated cell sorting

Resting T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Carlsbad, CA) according to the manufacturer's protocols. The labelled cells were injected i.p. to rats within 24 hr of birth. Spleens and thymuses were obtained and assayed using fluorescence-activated cell sorting for CFSE expression. To facilitate the CFSE analysis, the cells were also stained for CD4 expression using W3/25 ascites fluid, and indirectly stained using donkey anti-mouse Cy5 antibody (Jackson Laboratories, West Grove, PA). The percentage of CFSE-positive cells in the harvested spleens or thymuses was calculated by multiplying the overall percentage of CFSE-positive cells by the total cells found in the organ.

Limiting dilution assay of cell frequency

Spleens of rats were harvested, teased into single-cell suspensions, and serially diluted from 2×10^5 to 0·5 cells/well. To each well, 5×10^4 irradiated (3000 rad), syngeneic thymocytes were added as antigen-presenting cells. At each dilution, six wells did not contain antigen and 18 wells were supplemented with 10 μg/ml antigen. A well was counted as positive only if visible clamping of cells was observed and the c.p.m. of the well was over three standard deviations over the mean of the wells without antigen (the correspondence between visual assessment and c.p.m. was over 90%). Linear regressions of percentage of responding cultures to cell numbers were calculated and used to determine cell frequency – the cell number corresponding to 37% antigen-responding cultures.

Results

Fischer rats resist active EAE but can generate an encephalitogenic anti-MBP T-cell line

Figure 1(a) confirms that adult Fischer rats do not develop EAE following immunization with MBP/CFA; in contrast, Fig. 1(b) illustrates the course of EAE developing in MHC identical, age-matched Lewis rats induced by immunization with MBP/CFA.

To test whether Fischer rats possess T cells capable of responding to MBP and whether EAE could be adoptively transferred by such T cells, we raised an anti-MBP CD4

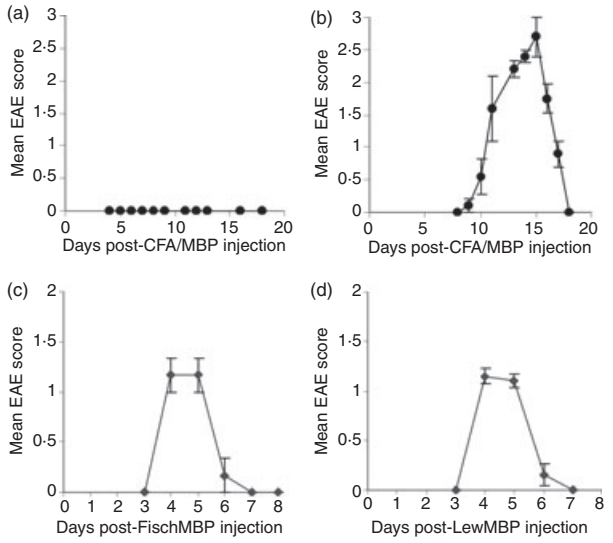

Figure 1. Active and adoptive experimental autoimmune encephalitis (EAE) in Lewis and Fischer rats: 6- to 8-week-old Fischer (a) or Lewis (b) rats were immunized with myelin basic protein/complete Freund's adjuvant (MBP/CFA); (c) Fischer rats were injected intravenously with 5×10^6 activated FischMBP T cells; (d) Lewis rats were injected with 5×10^6 activated LewMBP T cells. The rats were scored for EAE. These experiments were repeated two or more times with similar results.

T-cell line (termed FischMBP) from the lymph node cells of Fischer rats that had been immunized with MBP/CFA. All the lines raised were CD4, and expressed Th1 cytokines, such as interferon-γ (IFN-γ).

Figure 1(c) shows that the activated FischMBP line could mediate EAE in Fischer rats; this adoptive EAE was quite similar to the EAE adoptively transferred to Lewis strain rats by the same number of the activated Lewis anti-MBP T-cell line LewMBP (see Fig. 1d). As has been reported for pathogenic autoimmune T cells in other rats and mice,[4,7] the transfer of FischMBP T cells in a resting state could not mediate EAE (data not shown). Hence, Fischer rats can generate encephalitogenic T cells despite the fact that they resist actively induced EAE. A similar finding has been reported in PVG strain rats.[17]

Antigen specificities of the Fischer anti-MBP line

Figure 2 shows the antigen specificity of the FischMBP T-cell line. The proliferative response of FischMBP to guinea-pig MBP but not to OVA is shown in Fig. 2(a); conversely, Fig. 2(b) shows that the anti-OVA line Fisch-OVA responded to OVA but not to guinea-pig MBP.

To determine the peptide specificity of FischMBP, the line was stimulated with a panel of overlapping guinea-pig MBP peptides spanning the rat MBP protein.[16] Figure 2(c) shows that the FischMBP line proliferated vigorously to the peptide formed by amino acids 71–90, and also to a weaker epitope located in amino acids 81–100. Interestingly, this pattern of proliferation has been observed in Lewis anti-MBP encephalitogenic lines: immunization of Lewis rats with guinea-pig MBP was shown to induce two distinct encephalitogenic T-cell

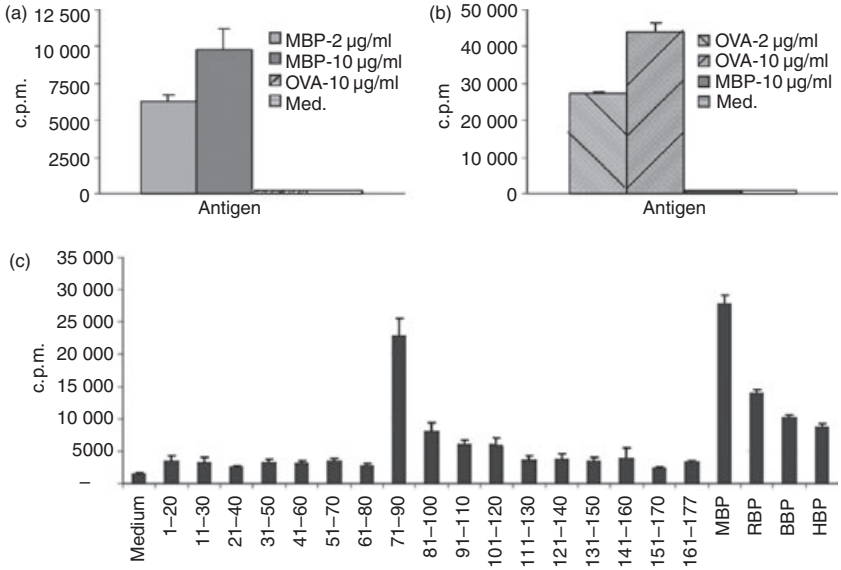

Figure 2. Antigen-specificity of the FischOVA and the FischMBP lines: the FischMBP (a) and the FischOVA (b) lines were tested in proliferation assays with guinea-pig myelin basic protein (MBP) or ovalbumin (OVA). (c) The FischMBP line was tested using a panel of overlapping rat MBP peptides, and to the following MBP molecules: guinea-pig MBP (MBP), rat MBP (RBP), bovine MBP (BBP) or human MBP (HBP). Two days after incubation, cells were pulsed with [H^3]thymidine. The results represent the average counts per minute (c.p.m.) of triplicate or quadruplicate wells with standard error bars. Statistics for (c): analysis of variance + Tukey–Kramer $P < 0{\cdot}01$ for peptides 71–90, 81–100, MBP, RBP, BBP and HBP, versus all other peptides or medium. Peptides 91–110 and 101–120 not different from peptide 81–100. The experiments were repeated at least twice with similar results.

populations, one responding strongly to the immunodominant 72–89 epitope of rat MBP, and the other responding more weakly to an epitope defined by residues 87–99.[18]

The FischMBP line proliferated most vigorously to guinea-pig MBP, the antigen against which it was raised, but also to the rat MBP, which it probably recognizes in the context of the rat central nervous system to mediate EAE. The line showed cross-reactivity also to bovine MBP and to human MBP. These results indicate that although Fischer rats resist active EAE, they can generate anti-MBP pathogenic T-cell lines that manifest a peptide specificity similar to that seen in the MHC-identical, EAE-susceptible Lewis strain.[6,11]

Neonatal transfer of activated anti-MBP T cells renders the Fischer strain susceptible to active EAE

It has been reported that the immune system in the first days following birth is in a formative state and can be readily manipulated.[19,20] To study the effect of adoptive transfer of anti-MBP T cells into neonates, we injected activated FischMBP T cells into newborn Fischer rats within 1 day following birth. Similar to the results of experiments reported in Lewis rats,[12,13] these anti-MBP T cells did not mediate clinically observable EAE in the recipients (data not shown) subsequent to the transfer. The recipients also did not exhibit any motor deficit or any observable clinical signs as adults.

Upon reaching the age of 8 weeks, we immunized these recipient rats with MBP/CFA and found that the rats developed accelerated EAE starting on day 6, peaking on day 8 and declining by day 9 after immunization (Fig. 3); this course of disease contrasts with the typical primary course of actively induced EAE in Lewis rats, which usually begins after day 8 or 9 (Fig. 1b). Fischer rats that had been injected neonatally with activated FischOVA T cells or with activated FischMBP T cells that were irradiated remained resistant to the induction of active EAE. Hence, activated FischMBP T cells mediate adoptive EAE in adult rats, but not in newborn rats within 1 day following birth. The injected newborns, however, acquire susceptibility to the induction of active EAE later in life.

Resting FischMBP T cells, as we reported above, do not mediate EAE in adult Fischer rats; do they produce any effects on newborn Fischer rats?

Early transfer of resting anti-MBP T cells renders Fischer rats susceptible to active induction of EAE

We injected 10^6 or 3×10^6 resting FischMBP T cells or 10^6 resting FischOVA T cells into Fischer rats within 24 hr of birth. As observed following the injection of activated FischMBP T cells, the rats did not manifest adoptive EAE (not shown) nor did they exhibit any clinical

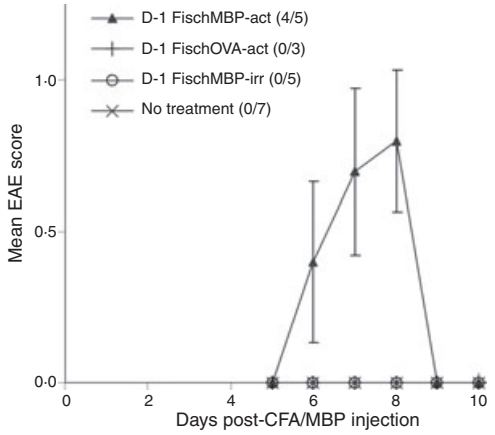

Figure 3. The effects on active induction of experimental autoimmune encephalitis (EAE) of activated or resting T cells transferred neonatally: Fischer rats were injected within 24 hr of birth with 1×10^6 activated FischMBP T cells or with activated and irradiated (irr.) FischMBP T cells, with 1×10^6 activated FischOVA T cells (FischOVA), or were left untreated (Naïve). Eight weeks following the transfer, the rats were immunized with myelin basic protein/complete Freund's adjuvant (MBP/CFA) and signs of EAE were scored. Statistics: the activated FischMBP group was significantly different from the naïve group on days 7 and 8 (Wilcoxon, $P < 0{\cdot}05$).

sign as adults. Eight weeks later, the rats were immunized with MBP/CFA to test their acquired susceptibility to active EAE. Figure 4(a) shows that 80–100% of the rats that had received resting FischMBP T cells as newborns now developed active EAE starting on day 5, peaking on day 6, and waning by day 9. This accelerated pattern of EAE development resembled the active EAE seen following neonatal inoculation with activated FischMBP T cells (Fig. 3) or to some extent, that seen following an adoptive disease (see Fig. 1c,d). The observed effect was specific; no active EAE was seen following active immunization with MBP/CFA in naïve Fischer rats or in those that had received FischOVA T cells (Fig. 4a). The neonatally transferred rats also did not exhibit any clinical sign as adults.

Figure 4(b) shows that the effect of resting FischMBP T cells on the active induction of EAE in adult life was limited to inoculation relatively early in life: the severity index (mean cumulative EAE) and the relative incidence of actively induced EAE at 6–8 weeks of age declined when the T cells were injected later than the second day of life. Note, however, that about 50% of the rats injected up to 14 days of age were still susceptible to some degree to the later induction active EAE. This effect of resting FischMBP T cells was lost if the cells were injected into rats at 6 weeks of age (not shown). The degree of susceptibility to actively induced EAE appeared to be influenced by the age at which the rats had received the resting T cells.

The temporal pattern of active EAE inducible by MBP/CFA following resting T-cell inoculation (not shown) was

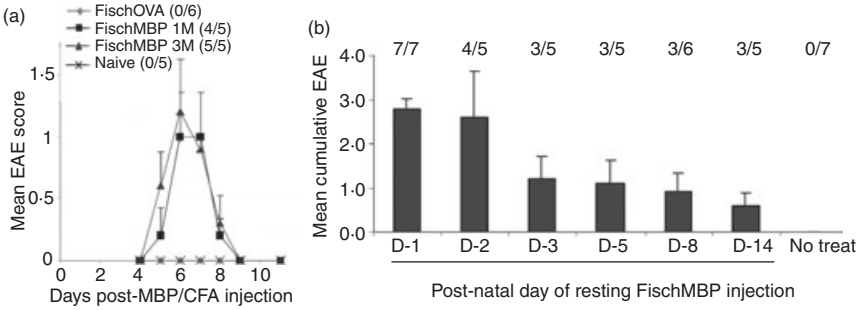

Figure 4. Time-dependency of the effect of neonatal T-cell transfer on experimental autoimmune encephalitis (EAE): Fischer rats were injected within 24 hr of birth (a) with resting 1×10^6 FischMBP T cells (FischMBP 1M) or with 3×10^6 FischMBP T cells (FischMBP 3M). As controls, other groups of neonates were injected with 1×10^6 FischOVA T cells (FischOVA), or were left untreated (Naïve). Six to eight weeks following the transfer, the rats were immunized with myelin basic protein/complete Freund's adjuvant (MBP/CFA) and signs of EAE were scored (b). Statistics: the resting FischMBP-1M group is significantly different from the Naïve and FischOVA-NT on days 6 and 7. The resting FischMBP-3M group is significantly different from Naïve and FischOVA-NT on days 5, 6 and 7 (Wilcoxon $P < 0{\cdot}05$). (b) Groups of five to seven female Fischer rats were transferred at 1, 2, 3, 5, 8 and 14 days post-partum with 10^6 resting FischMBP, or were left untreated. Upon reaching at least 6 weeks of age, the rats were immunized with MBP/CFA and scored for EAE. Statistics: all the groups were different from the non-treated control group (Wilcoxon $P < 0{\cdot}05$ to $P < 0{\cdot}001$)

accelerated and similar to that seen following adoptive EAE mediated by activated FischMBP T cells (see Fig. 3a). What then, happens to the resting T cells in the recipient rats that can explain their acquired susceptibility to active EAE?

Neonatally transferred resting T cells enter the neonatal spleen and the thymus and proliferate there

To examine the localization and possible expansion of transferred T cells following neonatal transfer, we labelled resting FischMBP T cells with CFSE and injected them 1-day post-partum. The frequency and extent of proliferation, expressed by two-fold loss of the CFSE fluorescence, was measured in spleens (Fig. 5a) and thymuses (Fig. 5b) 1, 3 and 9 days after cell transfer. The frequency ($\times 10^{-5}$) of CFSE-labelled cells (double positive for CD4 and for CFSE) was determined for each sample. The total estimated number of CFSE-labelled cells (total cells) was calculated by multiplying the overall frequency of CFSE-labelled cells by the total cells counted in the organ. The most frequent number of T-cell divisions (frequent divisions) and the maximal number of T-cell divisions (maximal divisions) were determined from the histograms of CFSE fluorescence intensities.

Figure 5(a) shows that the injected T cells entered the spleen and divided there. Most of the labelled cells in the spleen divided twice, 3 days after transfer, and approximately five times, 9 days after transfer. The total number of labelled cells residing in the spleen increased from approximately 400 cells 1 day after transfer to approximately 2000 cells 9 days after transfer, although the overall frequency remained generally similar to the frequency observed on day 1 (approximately 3×10^{-5}).

(a) day	CFSE spleen	Freq. ($\times 10^{-5}$)	Total cells	Frequent division	Maximal division
1		2·6	400	0	1
3		7·2	1750	2	5
9		3·5	2300	5	7

(b) day	CFSE thymus	Freq. ($\times 10^{-5}$)	Total cells	Frequent division	Maximal division
1		0·8	150	0	1
3		1·0	290	1	2
9		4·2	3860	4 + 5	6

Figure 5. Cell division of transferred FischMBP T cells in spleens and thymus: FischMBP T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) and injected neonatally into rats. Spleens (a) and thymuses (b) were harvested on days 1, 3 and 9. Cell division was estimated based on CFSE fluorescence intensity. Histograms display CFSE fluorescence (horizontal axis) of cells gated on cell size and granularity (forward to side scatter) of lymphocytes. At least 10^6 cells were assayed. No labelled cells were detected in control spleens or thymuses of uninjected rats (not shown). The frequency of CFSE-labelled cells (frequency $\times 10^{-5}$) and numbers of total cells in each organ (total cells) are shown, along with the number of the most frequent division (frequent division), and the maximal number of cell divisions (maximal division).

Similar results were observed in the thymus (Fig. 5b). Most CFSE-labelled T cells found in the thymus divided once, 3 days after transfer, and four to five times, 9 days after transfer. The total number of cells residing in the thymus increased from approximately 150 cells, 1 day after transfer, to approximately 4000 cells, 9 days after transfer. In contrast to the stable frequency in the spleen, the relative frequency of CFSE-labelled T cells in the thymus increased from $7{\cdot}8 \times 10^{-6}$ to $4{\cdot}2 \times 10^{-5}$ during 9 days. Both in the spleens and the thymuses, the dividing CFSE-labelled cells increased in number, but did not take over the lymphoid organ. In both organs, the cells reached frequencies that appear to be in the frequency range of memory T cells (10^{-4} to 10^{-6}).[21]

Neonatal transfer of resting T cells does not alter the frequency of anti-MBP T cells in adult rats but changes the functional response to MBP

We carried out limiting dilution assays to learn whether the transfer of resting anti-MBP T cells into neonates altered the frequency of anti-MBP-specific T cells detectable later in adult life. Figure 6(a) shows that the transfer of FischMBP T cells into neonates did not affect the overall frequency of T cells capable of proliferating in response to MBP in adults. The frequency of anti-MBP T cells remained similar to that observed in naïve rats – approximately 1/6000 splenocytes in the assay shown.

We also tested whether neonatal transfer of anti-MBP T cells might change the cytokine response to MBP in adults. We collected spleen cells from naïve adult rats and from those that had received resting FischMBP T cells neonatally; none of the rats had been immunized *in vivo* to MBP. Figure 6(b) shows that the splenocytes of the rats neonatally transferred with FischMBP T cells exhibited a strong dose-dependent IFN-γ secretion in response to MBP, but not to the irrelevant OVA protein or to medium alone. The naïve splenocytes secreted IFN-γ only in response to concanavalin A. The results show that, although the frequency of anti-MBP T cells did not change, the T cells persisting till adulthood were associated with a change in the IFN-γ cytokine response to MBP.

EAE following adoptive transfer of encephalitogenic T cells is inhibited following recovery from active induction of EAE

The spontaneous recovery from EAE actively induced in the susceptible Lewis rat by MBP/CFA is associated with acquired resistance to EAE mediated later by adoptive transfer of anti-MBP T cells.[22] As we have shown here, otherwise resistant Fischer rats can be rendered susceptible to actively induced EAE by MBP/CFA following neonatal injection of resting anti-MBP T cells (Fig. 4). We therefore wished to learn whether the line-treated Fischer rats, like Lewis rats, could acquire resistance to adoptively transferred EAE following their recovery from active EAE. Figure 7 shows that this was case. The neonatally line-treated Fischer rats that had recovered from active EAE following MBP/CFA were almost completely resistant to EAE induced by the activated FischMBP line administered on day 16 (red). In contrast, Fischer rats were susceptible to EAE induced by activated FischMBP T cells if they were previously untreated (green), or were previously

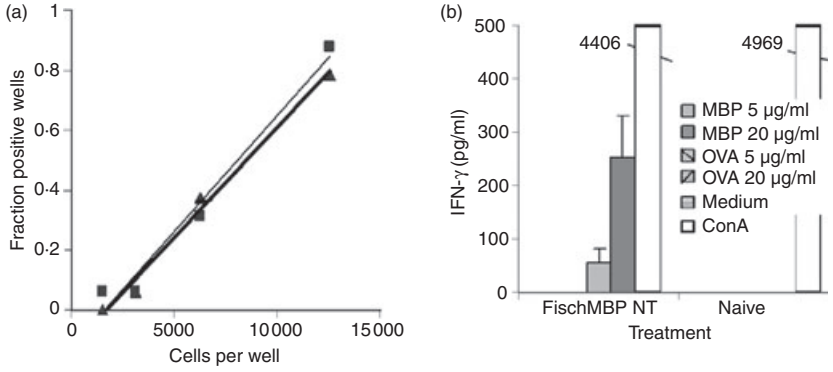

Figure 6. Precursor frequency of anti-myelin basic protein (MBP) T cells and interferon release in response to MBP following neonatal administration of resting anti-MBP T cells: (a) limiting dilution assay: female Fischer rats were either neonatally transferred with 10^6 resting FischMBP T cells (squares) or were left untreated (triangles). Upon reaching 10 weeks of age, the spleens were assayed for the frequency of responding cells using a limiting dilution assay and linear regressions were calculated [naïve rats, heavy line; neonatally transferred (NT) rats, delicate line; $R^2 > 0.97$ for either regression). Frequencies for Naïve and NT were 1/6600 and 1/6290 respectively (a statistically insignificant difference). The assay was repeated twice with similar results. (b) Enzyme-linked immunosorbent assay for interferon-γ (IFN-γ) secretion in splenocytes: 8 weeks after neonatal transfer, the spleens were harvested, and the cells were incubated with antigen. The results represent the average IFN-γ secretion detected in quadruplicate wells depicted with standard error bars.

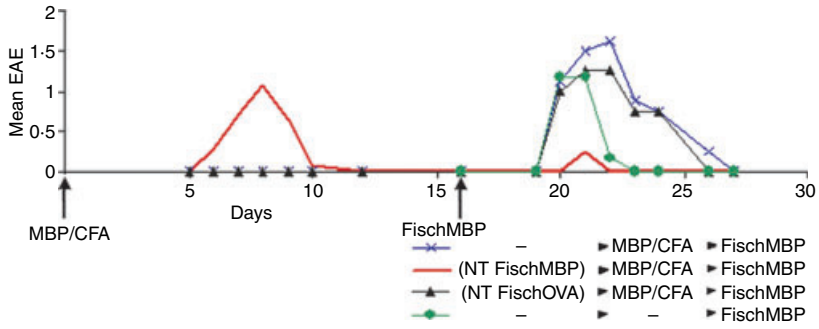

Figure 7. Fischer rats acquire resistance to FischMBP-mediated experimental autoimmune encephalitis (EAE) following recovery from active EAE: Fischer neonates were injected either with 10^6 resting FischMBP T cells and then injected (day 0) with myelin basic protein/complete Freund's adjuvant (MBP/CFA) [(NT FischMBP) > MBP/CFA > FischMBP] or transferred with 10^6 resting FischOVA T cells and then injected with CFA/MBP [(NT FischOVA) > MBP/CFA > FischMBP], or were injected with CFA/MBP [MBP/CFA > FischMBP] or were left untreated [FischMBP]. Six days after the disease had waned (day 16) in the rats that developed EAE in the [(NT FischMBP) > CFA/MBP > FischMBP] group, all the groups were injected intravenously with 4×10^6 activated FischMBP T cells. The rats were scored for EAE. Statistics: the [(NT FischMBP) > MBP/CFA > FischMBP] group was significantly different from the [FischMBP] group on day 20, from the [(NT FischOVA) > MBP/CFA > FischMBP] group on days 22 and 23, and from the [MBP/CFA > FischMBP] group on days 20 through 24. (Wilcoxon, $P < 0{\cdot}05$ at each day).

immunized with MBP/CFA, with or without neonatal transfer of FischOVA T cells (blue and black, respectively). These findings indicate that the active EAE following neonatal transfer with resting FischMBP T cells is able to induce the down-regulation of adoptive EAE mediated by activated FischMBP; neonatal transfer of resting FischMBP does not abrogate the capability to acquire resistance to activated FischMBP T cells.

Fischer rats recovered from actively induced EAE manifest down-regulated responses to MBP similar to naïve rats; neonatal transfer of anti-OVA T cells prime for enhanced responses to OVA

Figure 8 shows the IFN-γ (Fig. 8a) and proliferative responses (Fig. 8b) to MBP and to OVA of rats that had been neonatally injected with FischMBP or FischOVA and then immunized to MBP/CFA; this assay was conducted after the recipients of FischMBP T cells had recovered from actively induced EAE in the wake of the MBP/CFA. It can be seen that the rats that had recovered from active EAE manifested the low IFN-γ and low proliferation to MBP, equivalent to that manifested by untreated rats immunized to CFA/MBP. In contrast, neonatal transfer with FischOVA T cells markedly up-regulated the *in vitro* proliferative and IFN-γ responses to OVA. Note that these rats responding to OVA had not been immunized actively to OVA *in vivo*. Consequently, recovery from active EAE down-regulates the T-cell responses to MBP *in vitro*. In contrast, neonatal treatment with FischOVA T cells leads to an enhanced T-cell response to OVA *in vitro* even in the absence of *in vivo* immunization. The implanted anti-OVA T cells later respond to OVA, as if it were a memory-type response.

Discussion

Ever since it was discovered that anti-MBP lines and clones of T cells could adoptively transfer EAE,[1,4] it was repeatedly confirmed in EAE and other models that, to be functional *in vivo*, the transferred T cells had to be in an activated state.[4,6] Moreover, T-cell vaccination against autoimmune disease using attenuated autoimmune T cells also requires that the vaccines be prepared using T cells in an activated state.[2–5] These *in vivo* functions of T-cell lines could not be mediated or induced by the same T-cell clones when in a resting state.[4,7] It is not yet fully known by which mechanisms the functionality of transferred T cells is determined by the activated state of the cells.[7]

We now report that antigen-specific T cells in the resting state can also function in recipient animals: the resting cells do not cause EAE directly, but, as we show here, resting T cells obtained from Fischer rats can endow the otherwise resistant rats with susceptibility to EAE induced by active immunization with MBP/CFA (Figs 3 and 4). The window of opportunity for this function was found to be most receptive when the recipient rats received the resting T cells within the first 2 days of life; effectiveness declined thereafter, but about half of the recipient rats injected up to 14 days of age with the resting T cells were still able later to develop active EAE (Fig. 4b). At 6 weeks of age, however, the injection of resting T cells had no effect on the natural resistance of Fischer rats to active EAE (not shown). The reasons for this dependence on early age are unknown, but may be related to the observation that the successful implantation of syngeneic T cells in adults can be enhanced by manipulations that reduce the numbers of resident recipient cells.[23] It has

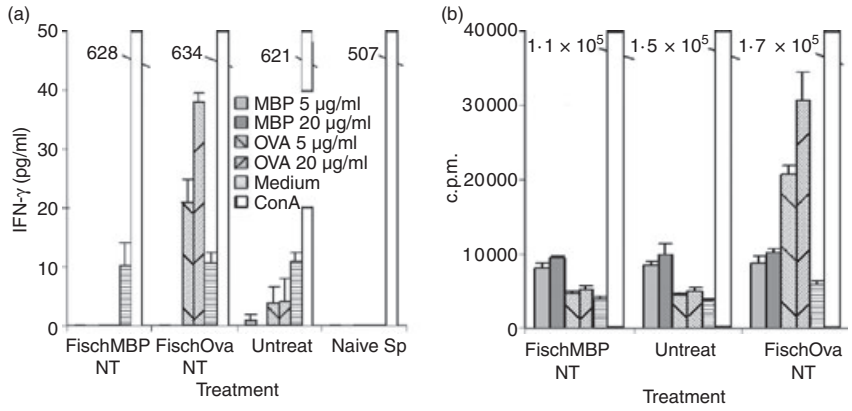

Figure 8. T-cell proliferative and interferon-γ (IFN-γ) responses following recovery from experimental autoimmune encephalitis (EAE): the spleen cells of the groups of rats described in the legend to Fig. 7 were assayed for (a) IFN-γ secretion or (b) proliferation: Fischer rats were injected neonatally with either FischMBP or FischOVA T cells or were left untreated. After 8 weeks the rats, including the untreated group, were immunized with myelin basic protein/complete Freund's adjuvant (MBP/CFA), and 2 weeks later the draining popliteal lymph nodes were collected separately from one to two rats per group and the cells were incubated with MBP or ovalbumin (OVA). The group labelled 'Naïve' was not injected with MBP/CFA, and was used as negative control. The proliferation culture supernatants were collected and assayed for IFN-γ and interleukin-10 (not depicted). There was no detectable interleukin-10. The results represent the average IFN-γ secretion of quadruplicate wells depicted with standard error bars. Statistics: IFN-γ secretion – the FischOVA transferred group IFN-γ secretion to OVA 20 μg/ml was significantly higher than medium (two-tailed Student's *t*-test, $P < 0{\cdot}0005$). Proliferation – the counts per minute (c.p.m.) to MBP at both concentrations is significantly higher than medium in all the groups (Dunnet, $P < 0{\cdot}05$) and similar between all groups. The c.p.m. to OVA, (both concentrations), is different from MBP, (both concentrations), and from medium in the FischOVA neonatally transferred group (Tukey–Kramer, $P < 0{\cdot}05$).

been proposed that transferred cells need cytokine-defined 'space' to be accepted.[23,24] This space for transferred T cells may be naturally available in lymphopenic adults or in the neonatal immune system, considered physiologically lymphopenic.[25]

The transferred resting T cells were observed to enter the spleen and the thymus and to multiply and persist in these organs (Fig. 5). Earlier studies in adult Lewis rats showed that activated encephalitogenic T cells migrated to the thymus and persisted there even after the rats had recovered from EAE mediated by the injected T-cell line.[26] The anti-MBP T cells did not enter the thymus when they were injected in a resting state.[26,27] Hence, the return of T cells to the thymus may depend both on the age of the recipient and the state of the T-cell activation. The function in the T cells returning to the thymus is not known.[27,28] Considering the central role of the thymus in selection of the T-cell repertoire, it is conceivable that the returning T cells might play a role in stimulating the differentiation of anti-idiotypic[22] or anti-ergotypic[29] regulatory T cells.

An interesting finding in the present study was that, although they proliferated, the transferred T cells did not increase the precursor frequency of anti-MBP T cells above the frequency detectable in naïve, untreated Fischer rats (Fig. 6). Some mechanisms, especially the competition for limited self-ligands, were reported to regulate the numbers of antigen-specific T-cell clones in lymphopenic hosts.[30,31] Nevertheless, the entry of the transferred T cells into the lymphoid organs was associated with qualitative changes in the nature of the immune response to the specific antigen: transfer of FischMBP T cells led to enhanced IFN-γ production in the *in vitro* response to MBP in the recipient rats that had not been immunized to MBP *in vivo* (Fig. 6b). Moreover, the EAE induced by subsequent immunization with MBP/CFA manifested the accelerated onset (Figs 3 and 4a) associated with a memory response.[13,32] In addition, the FischOVA line administered neonatally to Fischer rat pups enhanced the response to OVA detected *in vitro* by proliferation and secretion of IFN-γ (Fig. 8) without prior immunization to OVA. The neonatal transfer of antigen-specific T cells led to a change in the quality of the T-cell responses to both self and foreign antigens; the neonatally transferred cells seemed to endow the recipients with a memory immune-response phenotype.

We have observed a similar phenomenon in Wistar Furth rats: active immunization to MBP/CFA induces EAE in only about 10% of Wistar Furth rats,[33] but neonatal transfer of resting anti-MBP T cells could seed about 90–100% of the rats for actively induced EAE induced later by MBP/CFA immunization (not shown).

Qin *et al.* studied the transfer of activated anti-MBP T cells into neonatal Lewis rats, susceptible to actively induced EAE.[32] Although our study focused on resting anti-MBP T cells and resistant Fischer rats, it is interesting to compare the results. Qin *et al.* found that the activated Lewis T cells mediated adoptive EAE only if the

recipient Lewis rats were older than 2 days at the time of transfer; neonatal transfer of the activated anti-MBP T cells, however, led to an accelerated, memory-type response when the rats were immunized later to MBP/CFA. Moreover, the recipient rats were unable to develop an anti-idiotypic regulatory response to the line of anti-MBP T cells that was neonatally transferred to them; the rats were susceptible to multiple bouts of adoptive EAE mediated by the neonatally transferred line, but not by a different anti-MBP line.[32] Our study in Fischer rats found that the neonatal administration of resting anti-MBP T cells could also lead to accelerated EAE in response to MBP/CFA, but, in contrast to the Lewis rats, the recipient Fischer rats could still acquire the ability to down-regulate a future bout of EAE (Fig. 7). Consequently, Lewis and Fischer rats, despite bearing the same MHC alleles, manifest different mechanisms of autoimmune regulation.

We would like to use the term T-cell seeding to describe the phenomenon reported here in which the injection of resting, antigen-specific T cells endows the recipient with a potential for an enhanced effector T-cell response to a subsequent encounter with the specific antigen without inducing any apparent clinical effects immediately following transfer. The mechanisms responsible for the effects mediated by T-cell seeding are still an experimental question. A practical question is whether T-cell seeding can be exploited to endow recipients with desirable T-cell effector responses to infectious agents or to tumour cells. We are currently investigating the potential of T-cell seeding in cancer immunotherapy.

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Disclosures

The authors declare no conflict of interest or financial interests.

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