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Activated Murine Endothelial Cells Have Reduced Immunogenicity for CD8$^+$ T Cells: A Mechanism of Immunoregulation?[1]

Federica M. Marelli-Berg,[2]* Diane Scott,† Istvan Bartok,† Emma Peek,* Julian Dyson,† and Robert I. Lechler*

The immunogenic properties of primary cultures of murine lung microvascular endothelial cells (EC) were analyzed. Resting endothelial cells were found to constitutively express low levels of MHC class I and CD80 molecules. IFN-γ treatment of EC resulted in a marked up-regulation of MHC class I, but no change was observed in the level of CD80 expression. No CD86 molecules were detectable under either condition. The ability of peptide-pulsed EC to induce the proliferation of either the HY-specific, H2-K^k-restricted CD8$^+$ T cell clone (C6) or C6 TCR-transgenic naive CD8$^+$ T cells was analyzed. Resting T cells were stimulated to divide by quiescent peptide-prepulsed EC, while peptide-pulsed, cytokine-activated EC lost the ability to induce T cell division. Furthermore, Ag presentation by cytokine-activated EC induced CD8$^+$ T cell hyporesponsiveness. The immunogenicity of activated EC could be restored by adding nonsaturating concentrations of anti-H2-K^k Ab in the presence of an optimal concentration of cognate peptide. This is consistent with the suggestion that the ratio of TCR engagement to costimulation determines the outcome of T cell recognition. In contrast, activated peptide-pulsed EC were killed more efficiently by fully differentiated effector CD8$^+$ T cells. Finally, evidence is provided that Ag recognition of EC can profoundly affect the transendothelial migration of CD8$^+$ T cells. Taken together, these results suggest that EC immunogenicity is regulated in a manner that contributes to peripheral tolerance. *The Journal of Immunology,* **2000, 165: 4182–4189.**

Due to their location at the interface between the bloodstream and the parenchymal sites of Ag presentation, endothelial cells (EC)[3] are likely to play a major role in the regulation of immune responses by migratory T lymphocytes. T cell migration into inflamed tissues involves sustained adhesive interactions with the microvascular endothelium, which expresses MHC molecules either constitutively or as a result of inflammation. Thus, Ag presentation by EC to migrating T cells is likely to occur during the required lymphocyte/EC binding interactions, leading to extravasation of Ag-specific T cells following priming in lymph nodes.

The majority of studies analyzing this issue have focused on Ag presentation by EC to CD4$^+$ T cells, and conflicting results have been reported in both the human and the murine system. The lack of proliferative responses following cognate recognition of EC by murine (1) and costimulation-dependent human (2) T cell clones has been reported. Most interestingly, in both systems the failure to induce T cell division was not accompanied by the induction of unresponsiveness. In other studies acetylcholine receptor-specific human T cell lines and murine OVA-specific, TCR-transgenic T cell lines were able to proliferate to Ag presented by EC (3, 4). Many studies have suggested that due to the limited ability of EC to deliver costimulatory signals, the consequences of this interaction are likely to correlate with the stringency of T cell requirements for B7-mediated costimulation (2, 4, 5).

It is likely that cognate interactions between T lymphocytes and EC may modulate T cell functions other than cell division. We have recently provided in vitro evidence that Ag presentation by EC may contribute to the development of the immune response by enhancing the recruitment of Ag-specific CD4$^+$ T cells into the tissue rather than by inducing T cell proliferation (6). Furthermore, these studies suggested that T cell division is incompatible with transendothelial migration.

CD8$^+$ CTL are major effectors of immune responses and carry out their function by migrating through postcapillary venules and infiltrating target tissues. The few studies that have analyzed the functional consequences of cognate interactions between EC and CD8$^+$ T cells have focused on the development of proliferative responses, and similar to what has been described for CD4$^+$ T cells, contradictory conclusions have been drawn concerning the outcome of such interactions. Some studies have described that EC can induce human and murine CD8$^+$ T lymphocyte differentiation (7–9), although with a low efficiency (7) compared with conventional APCs and with impaired ability to secrete IFN-γ following antigenic rechallenge (8). In contrast, other studies concluded that human EC are unable to stimulate a functional response by freshly purified peripheral blood CD8$^+$ T cells (10). These discrepancies may reflect differences in the stage of differentiation of the CD8$^+$ T cell population analyzed or that the stimulator EC were not activated by cytokines. No information is currently available on the effect that cognate recognition of EC may have on the migratory ability of CD8$^+$ T cells.

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[3] Abbreviations used in this paper: EC, endothelial cells; DC, dendritic cells; HY, male-specific minor histocompatibility Ag.

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In the present study we have investigated the effects of Ag presentation by primary cultures of murine lung EC to male-specific minor histocompatibility Ag (HY)-specific $CD8^+$ T cells at different stages of differentiation, namely naive, effector, and memory T cells. EC were used either in a resting state or were activated by cytokine treatment. In addition, the effect of cognate recognition of resting or activated EC on the migratory ability of $CD8^+$ T cells was analyzed. The results obtained are consistent with a role for EC Ag presentation in the induction and maintenance of peripheral tolerance and the prevention of autoimmune responses.

Materials and Methods

Mice

C6 TCR Tg mice, specific for the HY peptide epitope TENSGKDI presented by $H2\text{-}K^k$, were generated using a CD2 cassette (11) encoding the $\alpha 8$ and $\beta 11$ TCR chains from the HY-specific $CD8^+$ T cell clone C6 (12). CBA/Ca mice were purchased from Olac Harlan (Bicester, U.K.) and were used at 6–8 wk of age.

Peptides and Abs

The peptide TENSGKDI derived from the *Smcy* gene product was used as cognate Ag for the C6 TCR. The mAbs used for naive T cell purification as hybridoma supernatant were anti-$H2\text{-}A^k$ (10.2.16, TIB93, American Type Culture Collection, Manassas, VA), anti-$H2\text{-}E^k$ (14-4-4S, HB, American Type Culture Collection), anti-$H2\text{-}E^{k,\ d}/A^{b,\ d}$ (M5/114, HIB120, American Type Culture Collection), anti-CD4 (YTS 191) (13), and anti-CD44/Ly-24/Pgp-1 (142.5) (14). The following purified mAbs were used in functional assays: anti-CD80/B7-1 (16-10A1, PharMingen, San Diego, CA), anti-CD86/B7-2 (GL-1, PharMingen), and anti-$H2\text{-}K^k$ (HB25, American Type Culture Collection). Purified rat IgG, hamster IgG, and mouse IgG (PharMingen) were used as the negative control. The anti-clonotypic TCR Ab KTL2 (15) was used as the purified mAb.

Preparation of Con A supernatant

Murine splenocytes (5×10^6/well) were seeded onto a 24-well plate and incubated with medium containing 2.5 μg/ml Con A lectin for 96 h. At this time the supernatant was harvested, filtered, aliquoted, and stored at $-20°C$.

EC culture

Murine microvascular EC were purified from lung tissue obtained from female CBA/Ca mice based on a technique described by Dong and colleagues (16, 17). Following isolation, EC were serially subcultured at 37°C with 5% CO_2 in DMEM (Life Technologies, Paisley, U.K.) supplemented with 2 mM glutamine (Life Technologies), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 10 mM HEPES (Life Technologies), 1% nonessential amino acids (Life Technologies), and 50 μM 2-ME (Life Technologies) with freshly added 20% heat-inactivated FCS (Globepharm, Esher, U.K.), 150 μg/ml EC growth supplement (Sigma, Poole, U.K.), and 12 U/ml heparin (CP Pharmaceuticals, Wrexham, U.K.) in 2% gelatin-coated (Sigma) tissue culture flasks (Greiner Labortechnik, Dursley, U.K.). At confluence the EC were detached from the culture flasks using trypsin/EDTA (Life Technologies) and passaged. The EC lineage of the cell populations obtained was confirmed by positive staining with anti-CD31 and anti-CD105 Abs (data not shown). For functional assays the EC were used between passages 4 and 6. To activate the EC, 10% supernatant from Con A-treated splenocytes or 400 U/ml murine IFN-γ (PeproTech, London, U.K.) was added to the tissue culture flask and incubated for 96 h.

Purification of naive TCR-Tg $CD8^+$ T cells

Spleen and pooled lymph node (both s.c. and mesenteric) cells were depleted of adherent cells by two 45-min rounds of adherence to plastic on tissue culture dishes at 37°C. The nonadherent cells were subsequently collected and incubated with a cocktail of purified mAbs (anti-$H2\text{-}A^k$, anti-$H2\text{-}E^k$, anti-$H2\text{-}E^{k,d}/A^{bd}$, anti-CD4, anti-CD44/Ly-24/Pgp-1) at saturating concentrations for 30 min at 4°C. The cells were then washed twice to remove excess Ab and further enriched by magnetic immunodepletion. Briefly, mAb-treated cells were incubated with magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) coated with goat anti-rat and sheep anti-mouse Ig for 15 min at 4°C, and bead/mAb-coated cells were removed by passage through a magnetic column (miniMAC system, Miltenyi Biotec). The purified cells were resuspended in medium ready for the proliferation assay, and accessory cell contamination was assessed by culture with 2 μg/ml Con A in a 48-h assay.

C6 T cell clone

The C6 T cell clone, specific for HY peptide epitope TENSGKDI presented by $H2\text{-}K^k$, was maintained in culture by stimulation every 2 wk with splenocytes from CBA/Ca male mice and rIL-2 (20 U/ml; Roche, Mannheim, Germany) in RPMI 1640 medium supplemented with 10% FCS (Globepharm), 2 mM glutamine (Life Technologies), 50 IU/ml penicillin (Life Technologies), 50 μg/ml streptomycin (Life Technologies), and 50 μM 2-ME (Life Technologies). For use in experiments, the T cells were purified by isolation on a Lympho-Sep (Harlan-Sera, Crawley, U.K.) gradient 15 days (proliferation and transmigration experiments) or 5 days (cytotoxicity assays) after restimulation and were washed twice by low speed centrifugation ($210 \times g$, 5 min) before use to exclude any contamination by accessory cells.

T cell proliferation assays

Naive TCR-Tg $CD8^+$ T cells (2×10^4 cells/well) or the C6 T cell clone (10^4 cells/well) were cultured in the presence of EC (2×10^4/well) treated with 30 Gy of γ-irradiation in round-bottom microtiter plates in a total volume of 200 μl. The stimulator cells were prepulsed overnight with peptide and then washed to remove any soluble peptide. Wells were pulsed with 1 μCi of [^{3}H]TdR (Amersham, Aylesbury, U.K.), after 72 h (naive TCR-Tg $CD8^+$ T cells) or 48 h (C6 T cell clone), and the cultures were harvested onto glass-fiber filters 18 h later. Proliferation was measured as [^{3}H]TdR incorporation by liquid scintillation spectroscopy.

Two-stage cultures for tolerance induction

Purified TCR-Tg naive $CD8^+$ T cells (10^6/well) were plated out in 24-well plates in the presence of allogeneic cytokine-treated EC (2×10^5/well) irradiated with 30 Gy of γ-irradiation or untreated irradiated EC (2×10^5/well). In some wells the EC had been pulsed with the cognate K^k/HY peptide overnight. After 3 days T cells were harvested and purified by centrifugation on a Lympho-Sep (Harlan-Sera) gradient. Recovered cells were then rested for a further 72 h in RPM1 1640 medium with supplements and 10% FCS in 24-well plates before being used in a rechallenge proliferation assay using peptide-prepulsed female CBA/Ca splenocytes (5×10^5/well).

T cell clones (3×10^5) were plated out in 24-well plates in the same conditions as those described for the naive $CD8^+$ T cells in a total volume of 500 μl in a 24-well plate. After overnight incubation in the same conditions as those described for the naive T cells, the cells were harvested, rested for an additional 72 h in RPM1 1640 medium with supplements and 10% FCS, and used in proliferation assays.

Cytokine assays

Triplicate samples of cell-free culture supernatants were collected after overnight incubation and were stored at $-70°C$. IL-4 and IFN-γ were detected using Quantikine cytokine ELISA kits (R&D Systems, Abingdon, U.K.) according to the manufacturer's directions. Plates were read using a THERMOmax ELISA Reader (Molecular Devices, Abingdon, U.K.). The concentrations of IL-4 and IFN-γ were calculated by quadratic curve fitting to known standards using Soft Max Pro 1.2.0 program (Molecular Devices).

Cytotoxicity assays

EC killing by effector C6 T cells was assessed using a protocol described by Pardi and Bender (10). Resting or cytokine-treated EC were seeded onto 2% gelatin-coated flat-bottom 96-well plates (Greiner Labortechnik) at $1\text{–}2 \times 10^4$/well and incubated with 100 nM K^k/HY peptide or T cell culture medium overnight. EC were then labeled in the wells with 1–2 μCi of ^{51}Cr (Amersham) for 2 h. An Abelson virus-transformed pre-B cell line, derived from a mouse expressing $H2\text{-}K^k$, was incubated with peptide overnight and labeled with 50–100 μCi of ^{51}Cr for 2 h, then washed three times in medium and added to the plates at 10^4 cells/well. Effector $CD8^+$ T cells were harvested, purified, and added to the plates in the ratio indicated. Spontaneous release was measured from labeled target cells in medium alone, and maximal release was measured from labeled target cells lysed with 1% Triton-X 100. Following incubation at 37°C for 4 h, 100 μl of supernatant was harvested from each well, and ^{51}Cr release was measured using a Wallac gamma counter (Gaithersburg, MD). Specific lysis was calculated using the equation: % specific lysis = [(counts sample − counts spontaneous)/(counts maximal − counts spontaneous)] × 100.

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Lymphocyte transmigration assays

The transmigration experiments were conducted using EC monolayers grown on Transwell tissue culture well inserts (diameter, 24.5 mm; Costar, High Wycombe, U.K.) that contained polycarbonate membranes with a 3-μm pore size (Costar) as previously described (6, 18). EC (10^5) were seeded onto fibronectin-coated (50 μg/ml; Sigma) polycarbonate membranes overnight to form a monolayer. In some well inserts, 100 nM HY peptide was added for 16–18 h before the transmigration assay. Before the assay, the EC monolayers were gently washed with warm medium. Purified C6 CD8$^+$ T cells (7×10^5) in RPMI 1640 supplemented with 10% FCS were added to each insert and left to migrate through the monolayer; the well content was replaced with fresh medium. After 1 h the number of migrated T cells was determined by counting the lymphocytes present in the well medium. This was performed at different time points for the next 6 h. In these experiments results are expressed as a percentage of the value for transmigrated cells.

Flow cytofluorometric analysis

For cytofluorometric analysis, 5×10^5 cells were incubated with the indicated mAb at 4°C for 30 min. After washing twice in PBS with 2.5% FCS, the cells were incubated for an additional 30 min at 4°C with 100 μl of either a 1/100 dilution of fluoresceinated goat anti-rat Ig (PharMingen), a 1/100 dilution of fluoresceinated goat anti-hamster Ig (PharMingen), or a 1/64 dilution of fluoresceinated goat anti-mouse Ig (Sigma). After two additional washes, stained cells were analyzed using an EPICS Profile flow cytometer (Coulter, Luton, U.K.).

Results

Expression of immunogenic molecules by murine microvascular EC

Resting EC from primary cultures obtained from murine lung expressed low levels of H2-K^k molecules (Fig. 1*a*) compared with those seen on primary cultures of fibroblasts or renal cells isolated from the same animal (data not shown). In addition, murine EC constitutively expressed low levels of CD80 molecules (Fig. 1*b*), similar to what has been observed by others (4). This was not an organ- or strain-related characteristic, in that a similar pattern of expression was observed in a murine brain endothelioma line (gift from E. Dejana and A. Vecchi, Istituto Mario Negri, Milan, Italy), in primary cultures of EC isolated from murine hearts, and in primary cultures of lung microvascular EC isolated from BALB/c and C57BL/6 mouse strains (data not shown). The phenotypic changes induced in murine EC by incubation in medium containing 10% supernatant from Con A-treated splenocytes or murine IFN-γ (400 U/ml) for 72 h were also analyzed. Following this treatment, the expression of H2-K^k molecules was dramatically up-regulated (Fig. 1*c*), while the level of CD80 molecules remained unchanged (Fig. 1*d*). MHC class II molecules were also induced, however at significantly lower levels than those observed for MHC class I molecules (data not shown). The failure of EC to up-regulate CD80 molecules was not due to an effect mediated by IL-10 or TGF-β, as neutralizing Abs against both cytokines did not alter the pattern of H2-K^k/CD80 expression (data not shown). Similarly, the Con A lectin used to produce IFN-γ-enriched supernatant did not affect the expression of endothelial cell surface molecules. EC were incubated with 2.5 μg/ml Con A lectin for 72 h, as described for the production of splenocyte supernatant, and no changes were detected in the expression of any of the surface molecules tested (results not shown). Notably, neither CD86 molecules nor CD40 molecules were expressed by the EC under any circumstances (data not shown).

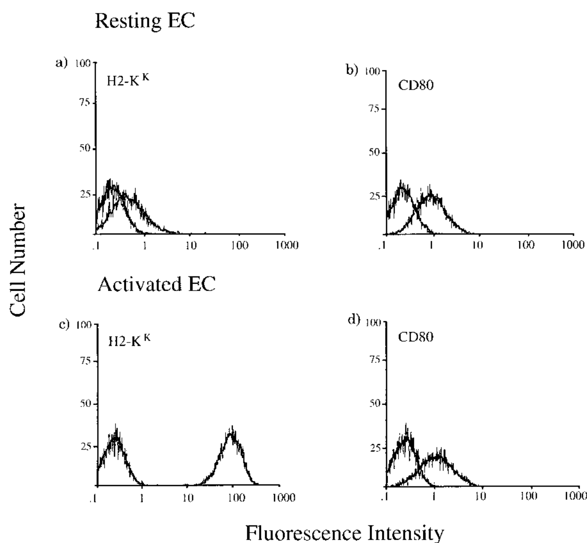

FIGURE 1. H2-K^k and CD80 expression by resting or cytokine-treated murine microvascular EC. EC were cultured either in the absence (*a* and *b*) or the presence of 400 U/ml IFN-γ for 72 h (*c* and *d*) and subsequently stained with Abs recognizing either H2-K^k (*a* and *c*) or CD80 (*b* and *d*) molecules. Isotype-matched irrelevant Abs were used as a control. Similar results were obtained when EC were treated with 10% supernatant from Con-A-stimulated splenocytes.

Murine EC lose immunogenicity and become tolerogenic to CD8$^+$ T cells following cytokine-mediated activation

The ability of resting and activated EC to present Ag and induce proliferation by CD8$^+$ T cells was investigated. Ag-pulsed, MHC class I^{dim} resting EC were capable of inducing significant proliferative responses by both the C6 T cell clone (Fig. 2*a*) and naive C6 TCR-Tg T cells (Fig. 2*d*) of a magnitude similar to that observed following stimulation with peptide-prepulsed splenocytes (Fig. 2, *c* and *f*). T cell proliferation could be partially inhibited by Ab-mediated CD80 blockade, while isotype-matched control Ab had no effect. Similarly, an anti-CD86 mAb did not inhibit EC-induced T cell proliferation (data not shown).

In contrast, despite the marked up-regulation of H2-K^k molecules and the presence of CD80 molecules on their surface, Ag-pulsed cytokine-treated EC failed to induce proliferation by both the C6 T cell clone (Fig. 2*b*) and naive C6 TCR-Tg T cells (Fig. 2*e*). A proliferative response could be partly reconstituted by the addition of submitogenic doses of rIL-2. However, CD8$^+$ T cell proliferation in response to peptide-pulsed EC could not be restored by the addition of neutralizing Abs against IL-10 or TGF-β (data not shown). The possibility that in the presence of high TCR engagement, a limiting level of CD80 expression might favor CD152 ligation and thus inhibit proliferation (19) was ruled out by the failure to restore proliferation by the addition of an anti-CD152 Ab (data not shown).

Since it has been reported that proliferative hyporesponsiveness can be induced in CD8$^+$ T cells following cognate recognition in association with CD80-mediated costimulation (20), we set out to investigate the functional consequences of cognate interaction between CD8$^+$ T cells and EC. While failing to induce T cell division (Fig. 3, *a* and *f*), cognate recognition of either cytokine-treated or untreated EC induced substantial levels of IFN-γ production (Fig. 3, *b* and *g*), suggesting that IFN-γ secretion occurs independently of B7-mediated signals. These data are consistent with previous findings by Sancho and colleagues, who observed that IFN-γ production by CD8$^+$ T cells depends on interactions between ICAM-1 molecules on EC and LFA-1 on T cells and is enhanced by IL-15 (21). No IL-4 was detected in these cultures (data not shown).

To rule out the possibility that either EC- or T cell-derived molecules produced during Ag presentation by activated EC might alter T cell reactivity, the C6 T cell clone proliferation in response to peptide-pulsed splenocytes in the presence of 50% supernatant

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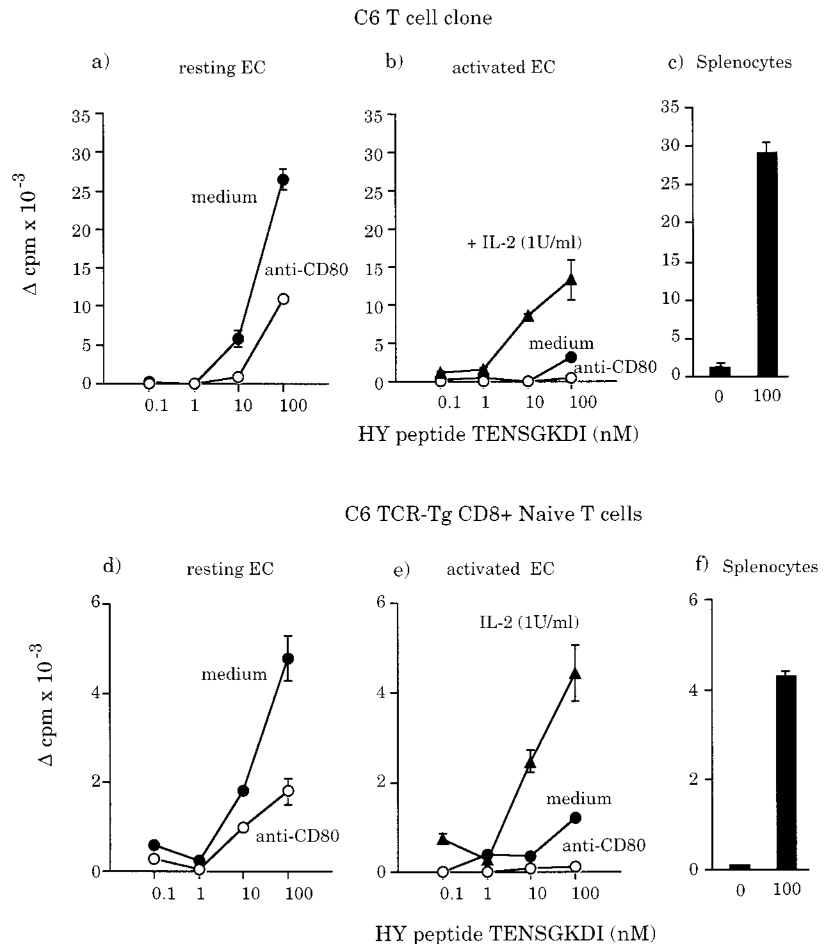

FIGURE 2. Only resting EC can induce proliferation by CD8$^+$ T cells. Resting or cytokine-treated EC were pulsed overnight with increasing amounts of K^k/HY peptide, as indicated on the horizontal axis of the graphs. The HY-specific T cell clone C6 (10^4/well) was cultured with 10^4/well peptide-prepulsed resting (*a*, ●) or cytokine-treated (*b*, ●) EC. In some wells an anti-CD80 Ab (○) or a low dose of rIL-2 (▲) was added. *c*, For comparison, proliferation to unpulsed or peptide-pulsed (100 nM) female splenocytes (5×10^5/well) is shown. *d* and *e*, A similar experiment performed using C6 TCR-transgenic naive T cells is shown. T cells (2×10^4/well) were cultured with 10^4/well peptide-prepulsed resting (*d*, ●) or cytokine-treated (*e*, ●) EC. In some wells an anti-CD80 Ab (○) or a low dose of rIL-2 (▲) was added. *f*, Proliferation by naive CD8$^+$ T cells to unpulsed or peptide-pulsed female splenocytes (5×10^5/well) is shown. The plates were incubated for 48 h (T cell clone) or 72 h (naive TCR-transgenic T cells), and [^{3}H]TdR was added for an additional 16 h. Results are expressed as the mean counts per minute for triplicate cultures $\times 10^{-3}$, corrected for background proliferation of both T cells and stimulators alone (Δ cpm). SEs are shown.

from cocultures of T cells with peptide-loaded or unloaded cytokine-activated EC was tested, but no effect on the T cell proliferation was observed (data not shown).

The lack of proliferation following Ag presentation by activated EC was accompanied by the induction of proliferative hyporesponsiveness to subsequent antigenic rechallenge in both the C6 T cell clone and naive C6 TCR-Tg T cells (Fig. 3, *c* and *h*, respectively) that have been cocultured overnight with activated Ag-pulsed EC and rested for an additional 72 h to allow the recovery of Ag receptor sensitivity failed to proliferate in response to Ag-pulsed splenocytes. Both the T cell clone and the naive T cells cocultured with resting peptide-pulsed EC (Fig. 3, *c* and *h*, respectively) remained capable of responding to an antigenic rechallenge delivered by Ag-loaded splenocytes. The observation that Ag-hyporesponsive T cells still proliferated vigorously in response to rIL-2, suggesting that anergy induction was the predominant mechanism accounting for the loss of responsiveness (Fig. 3, *d* and *i*). Consistent with this interpretation, no induction of IL-4 secretion was observed in the rechallenge cultures (data not shown). Interestingly, IFN-γ production by the C6 T cell clone (Fig. 3*e*) upon antigenic rechallenge appeared unaffected by Ag recognition on cytokine-treated EC, while it was greatly diminished following cognate interactions between naive T cells and activated EC (Fig. 3*j*). Diminished IFN-γ secretion has been previously reported by a study analyzing the functional characteristics of allospecific CTL generated from naive T cells in the presence of EC as APC and exogenous IL-2 in the human system (8).

The responsiveness of T cells incubated with EC in the absence of Ag was unaffected (data not shown).

Modulation of signal 1 restores proliferative responses to activated EC

A possible explanation for the results described above is that the failure to up-regulate CD80 molecules following cytokine treatment resulted in the delivery of insufficient costimulation relative to the increase of H2-K^k molecule expression. This, in turn, may lead to partial activation and the induction of hyporesponsiveness. To assess this possibility, we analyzed the degree of modulation of TCR expression following cognate recognition of resting or activated EC as an indicator of the extent of TCR engagement. In Fig. 4 TCR expression by the C6 CD8$^+$ T cell clone 12 h after cognate recognition of resting and activated EC pulsed with optimal doses of HY peptide is shown. This time point was chosen based on previous studies that had established that the C6 TCR was maximally down-regulated by 12 h following antigenic stimulation (11). As a control, TCR expression by T cells incubated in medium alone is shown. TCR engagement was greater following Ag presentation by IFN-γ-treated, H2-K$^{k\text{-bright}}$ EC, as suggested by the more marked TCR down-regulation (Fig. 4, *a* and *b*).

In parallel, proliferation of C6 CD8$^+$ T cells in response to peptide-pulsed resting (Fig. 4*c*) or cytokine-activated (Fig. 4*d*) EC was measured. As previously observed, despite the higher TCR engagement a minimal T cell response to peptide presented by IFN-γ-treated EC was detected at a high dose of peptide compared with that induced by a similar concentration of Ag presented by resting EC. The addition of a suboptimal dose of anti-H2-K^k mAb resulted in the partial inhibition (Fig. 4*c*) of proliferation induced by the resting EC, while the same amount of blockade partially

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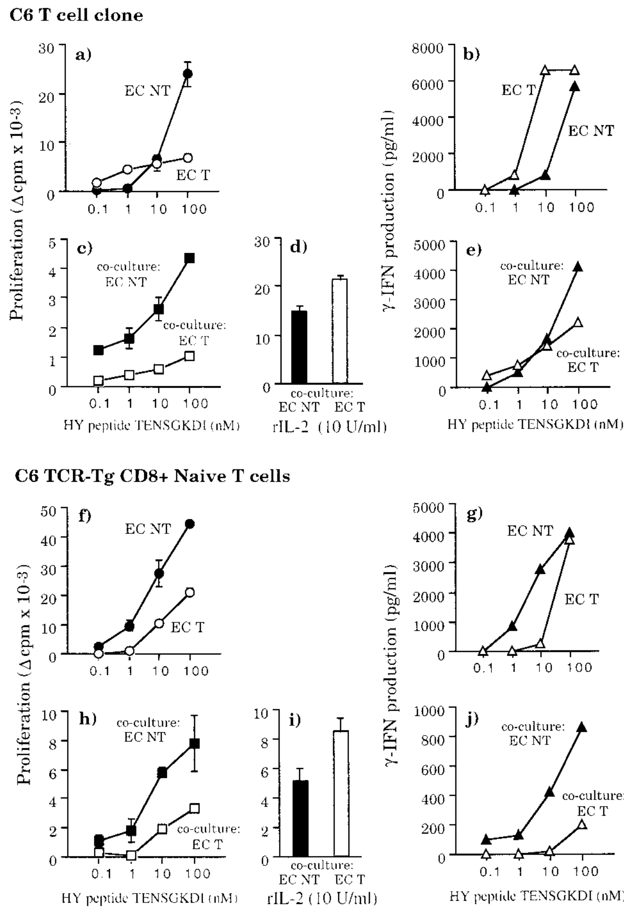

FIGURE 3. Cognate recognition of cytokine-treated EC induces hyporesponsiveness in CD8 T cells in the absence of cytokine gene reprogramming. The functional consequences of cognate recognition of resting or cytokine-activated EC by CD8^{+} T cells were assessed in a two-step culture system. First, the C6 T cell clone and C6 TCR-transgenic naive CD8^{+} T cells (10^6/well) were cultured overnight with Ag-pulsed, resting or IFN-γ-treated EC (10^5/well), prepulsed with 100 nM of the HY peptide in 24-well plates. The functional activation of T cells in these culture conditions was assessed in parallel in a proliferation assay in which the HY-specific T cell clone C6 (10^4/well; *a*) or C6 TCR-transgenic naive T cells (2×10^4/well; *f*) were cultured with either resting (EC NT, ●) or cytokine-treated (EC T, ○) EC pulsed overnight with increasing amounts of K^k/HY peptide. Proliferation was measured as described in Fig. 2. *b* and *g*, After 24-h culture, supernatant was harvested, and IFN-γ production in the cultures containing either resting (EC NT, ▲) or cytokine-treated (EC T, △) EC was measured. After overnight incubation T cells were collected, washed, and rested for an additional 72 h in medium alone. The T cell clone (10^4/well; *c*) and naive CD8^{+} T cells (2×10^4/well; *g*) previously cocultured with either peptide-loaded resting (EC NT, ■) or activated (EC T, □) EC were then rechallenged with peptide-prepulsed splenocytes (5×10^5/well) in a proliferation assay. T cells responsiveness to IL-2 (10 U/ml) was assessed in parallel (*d* and *i*, ■ and □ representing cocultures with resting or activated EC, respectively). Proliferation was measured and reported as described in Fig. 2. *e* and *j*, After 24-h culture, supernantant was harvested, and IFN-γ production in the cultures containing either resting (EC NT, ▲) or cytokine-treated (EC T, △) EC was measured.

reconstituted the response to activated EC (Fig. 4*d*, see enhancement part of the curve). No difference was seen in the presence of an isotype-matched control mAb (data not shown).

Similarly, a prediction of our interpretation is that decreasing the amount of peptide bound to MHC class I molecules on the EC should reconstitute T cell proliferation once an optimal ratio of MHC:peptide complexes/costimulation is achieved. Indeed, as shown in Fig. 4*d* (see inhibition part of the curve), increased proliferation in response to the cytokine-treated EC could be induced by loading them with low doses of antigenic peptide (between 0.005 and 1 nM, depending on the experiment). These proliferative responses were lower than those induced by resting EC (Fig. 4*c*) but were similarly inhibited by the anti-MHC class I Ab (Fig. 4*d*, inhibition part of the curve). Consistent with our hypothesis, these results suggest that partial blockade of TCR engagement restores a ratio of signal 1 to costimulation compatible with the induction of T cell division by cytokine-activated Ag-presenting EC.

The cytotoxic activity of CD8^{+} T cells is costimulation independent

Although costimulatory signals are required to induce CD8^{+} T cell expansion, it is likely that less stringent conditions are needed once the T cells have differentiated into cytotoxic effector cells. The ability of effector CD8^{+} T lymphocytes to kill resting and activated EC was investigated using cytotoxicity assays. For these experiments the C6 T cell clone was used approximately 5 days after restimulation, when the T cells exhibit their strongest cytotoxic activity. Peptide-pulsed resting EC were lysed by effector CD8^{+} T cells with low efficiency, while substantial killing of cytokine-treated EC was observed (Fig. 5, *a* and *b*). The addition of an anti-CD80-blocking Ab did not affect cytotoxic activity under either condition, indicating that CD80-mediated signals are not required for this effector function.

Cognate recognition of EC delivers a stop signal to migrating CD8^{+} T cells

We have previously reported that Ag recognition on EC can profoundly affect transendothelial migration of CD4^{+} T cells (6). The effect of cognate recognition on the migratory ability of the C6 CD8^{+} T cell clone was investigated using a transendothelial migration assay. T cells were purified on a Ficoll gradient and were seeded onto an endothelial cell monolayer grown on a Transwell insert, and the number of transmigrated T cells was monitored for the following 6 h. T cells were used at least 2 wk following antigenic restimulation, when lytic activity against EC was undetectable (data not shown). As shown in Fig. 6, a few T cells migrated through the resting EC monolayer (*a*), while cytokine-treated EC could support the migration of up to 80% of the total T cell input (*b*), probably due to the up-regulation of adhesion molecules. When antigenic peptide was loaded onto the EC monolayer, T cell transendothelial migration was profoundly decreased under both conditions, suggesting that cognate recognition of EC delivers a stop signal to migrating CD8^{+} T cells. For comparison, transendothelial migration of an irrelevant alloreactive CD4^{+} T cell clone (2E4, specific for the amino acid-substituted NOD H2-A molecule NOD^{asp}) through resting and activated EC in the presence or the absence of the C6 T cell cognate peptide is shown in Fig. 5, *c* and *d*. C6 TCR-transgenic naive T cells did not migrate though EC monolayers under any circumstances (data not shown).

Discussion

In this study the functional consequences of Ag presentation by EC to CD8^{+} T cells were analyzed in a murine model. The results obtained suggest that EC, which express CD80 molecules constitutively, lose their immunogenic properties and become tolerogenic to naive and resting memory CD8^{+} T cells following activation with IFN-γ. In contrast, lysis of EC by effector CD8^{+} T cells was enhanced by cytokine-mediated activation of the EC. The experiments were performed using IFN-γ-treated EC because the focus of our study was on the consequence of recognition of antigenic peptide bound to MHC molecules, which are up-regulated

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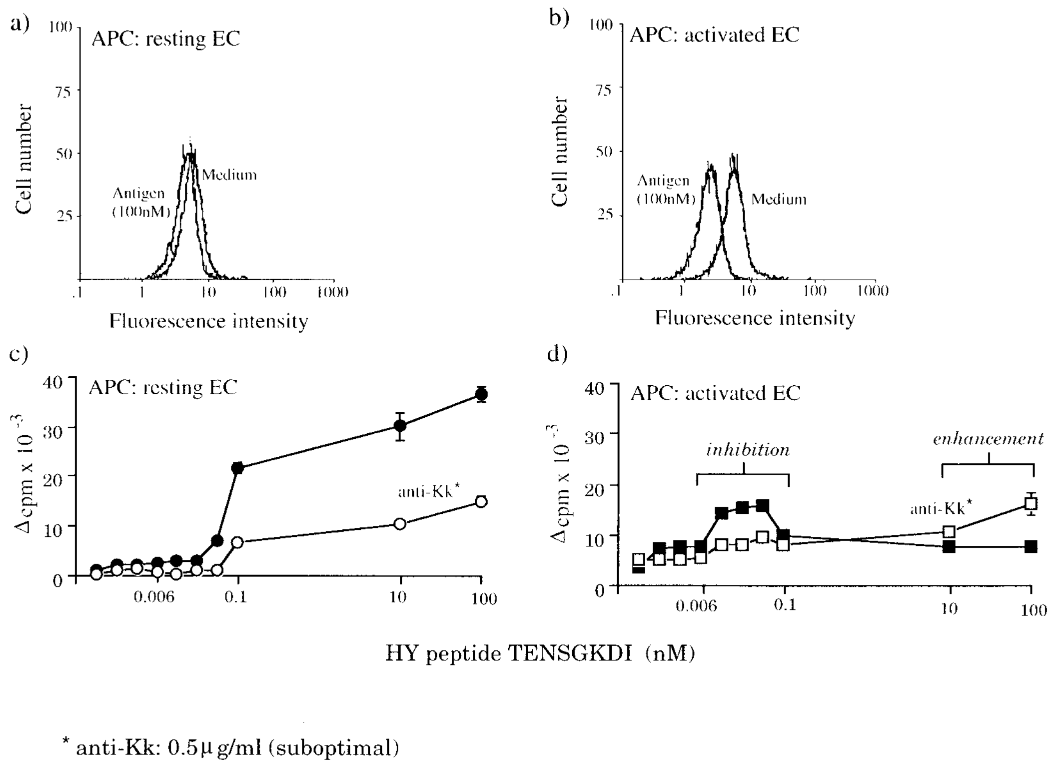

FIGURE 4. Effect of MHC class I blockade during resting and inflammatory conditions. C6 T cells were incubated with resting (*a*) or cytokine-treated (*b*) EC prepulsed with 100 nM HY peptide (indicated as Ag in the graphs) or in medium alone (indicated as Medium in the graphs) for 12 h, and the down-regulation of the TCR expression following exposure to Ag was monitored by staining T cells with the anti-clonotypic Ab KTL 2. In parallel, the proliferative responses of the C6 T cell clone (10^4/well) to resting (■, *c*) or activated EC (■, *d*; 10^4/well) pulsed with increasing amounts of the HY peptide are shown. In some wells a suboptimal dose (0.5 μg/ml) of an anti-H2-K^k mAb was added (*c*, ○ for resting Ag-presenting EC; *d*, □ for activated Ag-presenting EC). The dual effect (inhibition/enhancement) of the anti-H2-K^k mAb on T cell responses in the cultures containing activated EC is highlighted within the graph. Proliferation was assessed and reported as described in Fig. 2.

on EC by IFN-γ. In addition, all the experiments reported here were reproduced using EC-treated with mitogen-induced cytokine pools to resemble more closely physiological EC stimulation during inflammatory conditions.

The recent definition of human EC as semiprofessional APC (5) appears to apply also to murine microvascular EC. In agreement with a recent study we found that murine EC express low levels of CD80 molecules on their surface (4), unlike their human counterpart. Endothelial CD80 molecules appear to be functional, in that the EC can bind CTLA-4-Ig (4) and induce proliferation by $CD8^+$ T cells following Ag presentation by quiescent EC. However, in contrast with conventional APC, such as dendritic cells (DC), CD80 was not up-regulated and was not accompanied by the expression of CD86 molecules following cytokine-mediated activation. This may result in an imbalance between the extent of TCR engagement, which increases, and CD28 engagement, which remains unchanged, and may lead to the induction of hyporesponsiveness that we observed following cognate recognition of cytokine-treated EC. This hypothesis is supported by the observation that proliferation in response to activated EC could be recovered either by modulation of TCR engagement by the addition of an anti-H2-K^k mAb or by titrating down the amount of antigenic peptide bound to MHC molecules on activated EC, although it is technically impossible to rule out the interference of cytokine-induced molecules other than CD80 (e.g., VCAM-1 up-regulation) in the present system. In support of our interpretation is the observation that the surface phenotype of cytokine-activated EC may resemble that of immature DC (high MHC molecule expression and low B7 molecule expression), which have been shown to induce hyporesponsiveness in vitro (22–24) and tolerance in vivo (25, 26). The unfavorable signal 1 to signal 2 ratio observed on cytokine-activated EC may also explain the recent observation that murine lung microvascular IFN-γ-treated EC can sustain proliferative responses by memory $CD4^+$ T cells, but not naive $CD4^+$ T cells, which have more stringent costimulation requirements (4, 5).

The functional consequence of this interaction appears to be the induction of T cell anergy, rather than cytokine-gene reprogramming leading to immune deviation. Indeed, the proliferative hyporesponsiveness was accompanied by either unaffected or diminished IFN-γ secretion in the absence of induction of other cytokines such as IL-4, IL-10, or TGF-β and by increased reactivity to exogenous IL-2.

The tolerogenic properties of murine EC constitute a major difference from their human counterpart, which, despite being poor APC, do not induce tolerance (1, 2, 8) in responder T cells. These observations may be accounted for by the profound differences in the array of immunogenic molecules displayed on the EC surface, such as CD80, which is absent on human EC (27), and CD40, which is expressed by human EC (28, 29). These differences have to be borne in mind when extrapolating observations made in murine experimental models to the human system, such as the high rate of success of therapeutic strategies aimed at inducing allospecific tolerance to vascularized tissue graft in murine models.

It has been suggested that the induction of anergy in $CD4^+$ T cells in vivo is dependent on the delivery of dominant CD152-mediated signals, and that these conditions arise in the presence of a limiting amount of costimulation (19), particularly when CD80

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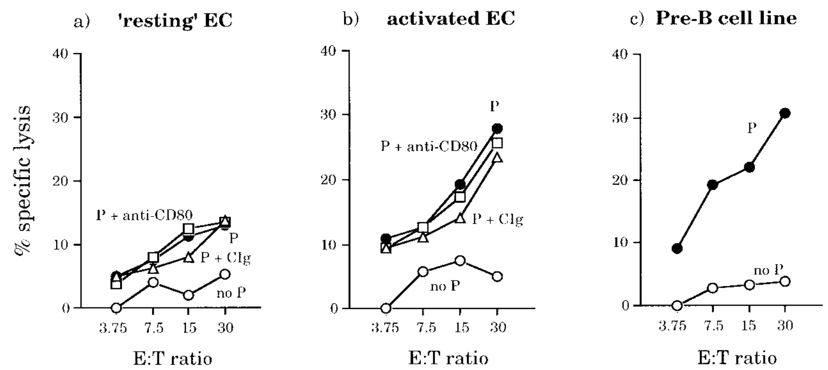

FIGURE 5. EC lysis by the C6 T cell clone is more efficient following cytokine treatment. EC lysis by the T cells was assessed in a 4-h ^{51}Cr release assay. ^{51}Cr-labeled, peptide-pulsed (100 nM; ●) resting (*a*) or cytokine-treated (*b*) EC (10^4/well) were incubated with increasing numbers of C6 T cells. In some wells an anti-CD80 mAb (10 μg/ml; □) or an isotype-matched irrelevant mAb (10 μg/ml; △) was added. After 4 h at 37°C, supernatant was collected, and gamma emission from each sample was counted using a Wallac gamma counter. As a control, lysis of EC that had not been prepulsed with cognate peptide is shown in each panel (○). *c*, The lysis of an Abelson virus-transformed B cell line either pulsed with HY peptide (●) or unpulsed (○) is shown for comparison.

molecules, which have a slower off-rate from CD152, are the predominant costimulatory molecules (30, 31). However, it is unlikely that CD152-mediated signaling plays a role in our system, because CD152 blockade did not restore activated EC immunogenicity. Finally, an alternative explanation for our observations is that in conditions of high TCR engagement, CD86-mediated signals are required for the induction of CD8$^+$ T cell proliferation. The induction of anergy in CD8$^+$ T cells has been described following transient proliferation in the presence of optimal CD80-mediated costimulation but in the absence of CD86-derived signals (20).

Finally, our data suggest that cognate recognition of EC appears to deliver a stop signal to migrating CD8$^+$ T cells, in line with previous results showing that TCR ligation delivers a stop signal to ICAM-1-triggered T cell locomotion on planar phospholipid bilayers (32). These observations suggest that TCR triggering by the EC may down-modulate migration, although this effect is likely to be combined with that elicited by chemokine gradients in vivo. Further studies are required to estimate which effect may prevail under these circumstances, both in vitro and in vivo.

The results we have obtained in this system may have revealed a novel pattern of regulation of APC function by cytokines and are consistent with an immunoregulatory role of EC as APC. Although EC are immunogenic in their resting state, it is unlikely that they initiate or support proliferative responses by CD8$^+$ T cells, because T cells do not engage in sustained contacts with resting EC due to the high flow rate and the absence of adhesive interactions. In addition, due to the low expression of MHC class I molecules, T cell/EC cognate interactions would require high avidity T cells. This also appears to be an extremely unlikely event, given that MHC class I molecules are generally loaded with self-peptides, and self-reactive peripheral T cells that have escaped thymic selection have low avidity (33, 34). However, if T cell recognition of Ag presented by resting EC were to occur, this would have an inhibitory effect on migration into the underlying tissue, as suggested by our transendothelial migration experiments, and possibly slow down T cell recruitment into the tissue.

Most importantly, EC activation during inflammation would render them tolerogenic to CD8$^+$ T cells by both the induction of nonresponsiveness and the blockade of transendothelial migration. As a consequence, only T cells that do not find their cognate peptide displayed by EC or that have reached their effector stage following activation would be able to cross the endothelial barrier unaffected. It is conceivable that these two criteria selectively apply to CD8$^+$ T cells that are specific for non-self Ags or that have recently been activated in the local lymph nodes and re-enter the circulation once they are fully differentiated into effector CD8$^+$ T cells (35). It has been shown recently that bone marrow-derived APC, such as DC, are required for the initiation of anti-viral immune responses, and that alternative MHC class I pathways that lead to the presentation of peptides derived from exogenous Ag are restricted to these cells (36). Thus, following priming by DC in the local lymph node, recirculating activated virus-specific effector CD8$^+$ T cells are unlikely to find their cognate peptides displayed by EC, so they would not be rendered unresponsive, nor would their migration into the tissue be halted.

EC damage would instead occur when EC are recognized and lysed by DC-primed effector CD8$^+$ T cells, as in the event of vascularized allotransplantation or as a by-product of priming against a viral infection. However, the latter event would normally be self-limiting due to the lack of CD4-mediated help necessary for further CD8$^+$ T cell expansion and differentiation into effector cells, in that self-reactive CD4$^+$ T cells are likely to re-encounter

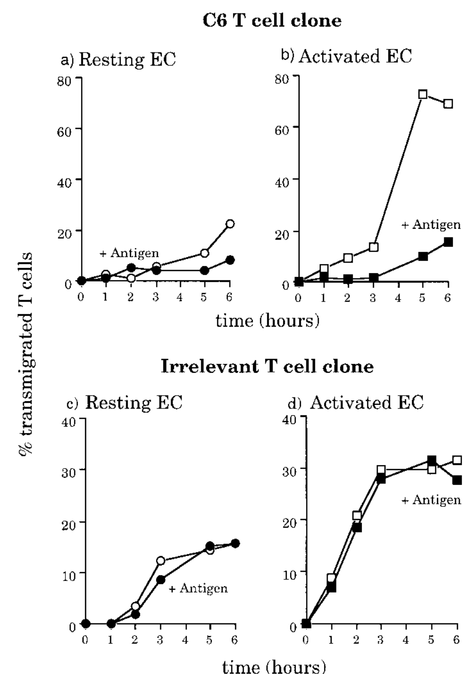

FIGURE 6. Ag presentation by EC halts transendothelial migration of CD8$^+$ T cells. The C6 T cell clone (*a* and *b*) or an irrelevant T cell clone (*c* and *d*; 5×10^5/well) was seeded onto resting (*a* and *c*) or cytokine-activated (*b* and *d*) EC monolayers with (filled symbols, indicated by + Ag) or without (empty symbols) Ag pulsing (100 nM). EC monolayers were gently washed 16 h after the addition of Ag to avoid possible interference by unbound peptide. T cell migration was monitored for the following 6 h and is expressed as a percentage of migrated T cells at the specified time points.

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cognate Ag on costimulation-deficient APC and thus be rendered hyporesponsive (31, 37).

The present report supports the idea that the ratio of TCR engagement and delivery of costimulation determines APC immunogenicity and is consistent with a role for EC in the induction and maintenance of peripheral tolerance.

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