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Regulatory T Cells Secreting IL-10 Dominate the Immune Response to EBV Latent Membrane Protein 1


Viruses exploit a number of strategies to evade immune recognition. In this study, we describe a novel mechanism by which EBV, rather than avoiding detection, subverts the immune response by stimulating regulatory T cells that secrete IL-10. Human PBMC from all EBV-seropositive, but not -seronegative, donors responded to both purified latent membrane protein 1 and the corresponding immunodominant peptides with high levels of IL-10 secretion by CD4+ T cells. These IL-10 responses, characteristic of T regulatory 1 cells, inhibited T cell proliferation and IFN-γ secretion induced by both mitogen and recall Ag. It was confirmed that the inhibition was IL-10 dependent by the use of neutralizing Ab. The deviation of the immune response toward suppression is likely to be important in maintaining latency and EBV-associated tumors. The Journal of Immunology, 2003, 170: 6183–6189.
Materials and Methods

Donors

Blood samples were obtained by venipuncture from a group of healthy volunteers. The donors were classified as EBV seropositive or seronegative by an ELISA for serum anti-EBNA1 IgG, with negative results confirmed by immunofluorescence staining for IgG and IgM antiviral capsid Ab.

Ags and mitogen

LMP1 was immunopurified using a modification of techniques previously described for other membrane Ags (35). EBV-transformed B cells (~10⁶) were lysed and centrifuged to remove insoluble debris, and the supernatant was incubated with the anti-LMP1 Abs CS1–4 (Novocastra Laboratories, Newcastle, U.K.). Immune complexes were isolated on magnetic beads coated with goat anti-mouse Ab (Biomag; Polysciences, Warrington, PA). LMP1 was added to cultures at an estimated concentration of 50 μg/ml, still complexed to the beads, because APC efficiently take up and process proteins bound to an insoluble matrix (35, 36). In all experiments, control cultures were stimulated with magnetic beads coated with CS1–4 Ab alone. SDS-PAGE analysis and Western blotting of LMP1 prepared from biotin-labeled B cells (37) revealed a major band consistent with the migration of LMP1 (59 kDa) (Fig. 1).

A panel of 76 20-mer peptides, with 15-aa overlaps, was synthesized (Department of Biochemistry, University of Birmingham, Birmingham, U.K.; or University of Bristol, Bristol, U.K.), spanning the entire length of the 63-kDa EBV LMP1, as determined from the prototype B cell-derived U.K.; or University of Bristol, Bristol, U.K.), spanning the entire length of the prototype B cell-derived

Cell proliferation and cytokine assays

As described previously (39), PBMC were separated from fresh blood samples by density gradient centrifugation and cultured in 1-ml volumes at a concentration of 1.25 × 10⁶ cells/ml. Cellular proliferation was estimated from the incorporation of [3H]thymidine in triplicate 100-μl aliquots taken 5 days after Ag stimulation, when recall T cell responses are maximal (40). Proliferation results are presented as the mean cpm ± SD of the triplicate samples or as stimulation index, expressing the ratio of mean cpm in stimulated vs unstimulated control cultures. A stimulation index of >3 with a cpm of >1000 is interpreted as representing a significant positive response (41). Production of the Th cytokines IFN-γ, IL-4, and IL-10 was assessed in duplicate 100-μl aliquots taken 5 days after stimulation of the cultures, using a sensitive cellular ELISA (41). Cytokine responses over twice the production in unstimulated cultures were considered positive (41).

Characterization of responding cells

The phenotypes of cultured cells that proliferate or secrete cytokine in response to Ag were determined by flow cytometry. Aliquots of PBMC were taken from responding cultures and stained with anti-CD3 PE-Texas Red-x and anti-CD4 FITC, with anti-CD25 PE-cyanin 5.1 (all Beckman Coulter, High Wycombe, U.K.) in some experiments. Activated cells in proliferating cultures were identified using anti-CD71-PE or anti-CD69-PE (both Beckman Coulter). Cells synthesizing IL-10 were labeled by incubation with anti-IL-10-PE (BD PharMingen, Oxford, U.K.) after incubation with brefeldin A. Stained cells were analyzed using an EPICS XL cytometer (Beckman Coulter) and Expo version 2 analysis software (Applied Cytometry Systems, Sheffield, U.K.).

Inhibition of APC protein processing

To investigate the requirement for Ag processing in LMP1-induced IL-10 responses, the pH modulator chloroquine (Sigma-Aldrich) was used to inhibit processing for the life span of APC in our cultures (42). Autologous PBMC were irradiated, before being incubated for 10 min with 100 μM chloroquine in PBS at 37°C. Still in the presence of 100 μM chloroquine, the cells were then pulsed with LMP1 or IL-10-inducing LMP1 peptide for 3 h, before being washed three times. They were then used as a source of APC in proliferation and cytokine assays at 10⁶ cells/ml.

Results

LMP1 induces high levels of IL-10 secretion by PBMC from EBV-seropositive but not -seronegative donors

PBMC from 10 EBV-seropositive donors were tested for the ability to respond to purified LMP1 with either Th cytokine secretion

![FIGURE 1. SDS-PAGE analysis and Western blotting of LMP1 preparation. Representative results obtained from SDS-PAGE analysis and Western blotting of LMP1 preparation. Lane A shows SDS-PAGE molecular mass markers; lane B, control SDS-PAGE analysis of the anti-LMP1 Abs (CS1–4); lane C, SDS-PAGE analysis of the LMP1 preparation precipitated with CS1–4. Protein bands in lanes A–C were stained with Coomassie blue. Lane D illustrates a Western blot of LMP1 precipitated with CS1–4 from biotin-labeled EBV-transformed B cells and developed with chemiluminescent staining.](http://www.jimmunol.org/)

![FIGURE 2. Cytokine and proliferative responses to purified LMP1 by PBMC from healthy EBV-seropositive and -seronegative donors. PBMC from two representative seropositive donors (n = 10) and two seronegative donors (n = 2) were stimulated with purified LMP1, the T cell mitogen Con A, or the recall Ag PPD. Proliferative, IFN-γ, and IL-10 responses are shown.](http://www.jimmunol.org/)
or proliferation. In all seropositive donors, IL-10 was the predominant cytokine measured, with no significant proliferative, IFN-γ, or IL-4 responses. Fig. 2 shows representative results obtained from two seropositive donors. To confirm that the observed responses resulted from previous EBV infection, PBMC from two EBV-seronegative donors were tested for responsiveness to the purified LMP1. It can also be seen from Fig. 2 that, in these donors, the LMP1 failed to elicit either IL-10 secretion or significant proliferative and IFN-γ responses. The results in both donor groups are specific to LMP1, because the T cell mitogen Con A and the control recall Ag PPD induced responses dominated by proliferation and IFN-γ production, regardless of EBV serological status.

PBMC from EBV-seropositive donors respond strongly to multiple LMP1 peptides by secreting IL-10

To further characterize the immune response to LMP1, epitopes that induced IL-10 secretion were mapped by screening PBMC from 20 EBV-seropositive healthy donors with a panel of synthetic 20-mer peptides spanning the entire sequence of LMP1. Representative results obtained from one donor (Fig. 3) demonstrate that multiple LMP1 peptides induced secretion of high concentrations of IL-10. In contrast, only three peptides induced proliferation, five peptides induced IFN-γ, two peptides induced IL-4, and all the latter responses were weak. Similar patterns of responsiveness were found in a total of 20 seropositive donors (summarized in Fig. 4). Strikingly, certain peptides commonly elicited IL-10 responses in different donors ($p = 2.2 \times 10^{-3}$; Poisson heterogeneity test), with, for example, peptide 4 (aa 16–35) inducing IL-10 in 70% of the individuals.

PBMC from four EBV-seronegative donors were also screened with the panel of LMP1 peptides. Reactivity was rare in this group, with totals of only nine IL-10, one IFN-γ, one proliferative, and no IL-4 responses. Moreover, all these responses were relatively weak (data not shown).

**FIGURE 3.** Cytokine and proliferative responses of PBMC from EBV-seropositive donor to the panel of LMP1 peptides. Representative results obtained from one donor ($n = 20$) are shown for the cytokine ELISA (IL-10, IL-4, and IFN-γ) and the proliferation assay. The broken line on each chart shows the minimum level considered to be a positive response.

**FIGURE 4.** Summary of responses to LMP1 peptides by PBMC from the seropositive donor panel. The graph shows the percentage of EBV-seropositive donors ($n = 20$) whose PBMC responded to each LMP1 peptide with cytokine secretion (IL-10, IL-4, and IFN-γ) or proliferation. The results were demonstrated to be reproducible by retesting all of the 18 available donors.

Cells responding to LMP1 and LMP1 peptides with IL-10 secretion are CD3+CD4+

The phenotype of the cells responsible for the IL-10 production was determined by flow cytometry, comparing peptide-stimulated and unstimulated cultures from four seropositive individuals. Most IL-10-producing cells bore the CD3 marker for T cells (mean = 83.9%; SD = 9.4%), and of these, the majority were of the CD4+ helper phenotype (mean = 90.6%; SD = 8.9%) (Fig. 5). The lack of IFN-γ secretion in response to LMP1 is unusual; thus, additional experiments that analyzed the IFN-γ secretion of LMP1-stimulated cells at an earlier time point (12 h poststimulation) were conducted to ensure that any early IFN-γ secretion was not missed. No significant IFN-γ secretion was detected from such cultures, nor did any of the LMP1-stimulated T cells display the heterogeneous IL-10 and IFN-γ secretion described by Picker et al. (Ref. 43; results not shown). Activated cells, as judged by expression of CD69 and CD71, in the rare cultures proliferating in response to peptides were also CD4+ (data not shown).
IL-10-secreting Tr1 cells specific for LMP1 suppress bystander proliferative and IFN-γ responses

CD4+ T cells biased toward IL-10 secretion, termed Tr1 cells, play an important role in immunoregulation (18, 19) and have been shown to inhibit inflammatory responses (18, 20). As the responses to LMP1 and the peptide panel were predominantly mediated by Tr1 cells, we established whether they were capable of mediating suppression. In all 10 seropositive donors tested, the addition of LMP1 strongly inhibited proliferative and IFN-γ responses to the T cell mitogen Con A, the recall Ag PPD, and the primary Ag KLH by 56–99% (Fig. 6). The IL-10 responses to LMP1, and the associated inhibition, were dependent on the donor having been infected with EBV, because no such effects were seen when PBMC were obtained from two control seronegative volunteers (Fig. 6). Results similar to those obtained with purified LMP1 protein were found when IL-10-inducing LMP1 peptides were added to PBMC cultures from five seropositive donors (comprising a subset of donors tested with whole LMP1), with suppression of proliferative and IFN-γ responses to PPD by 41–99% (Fig. 7). In parallel experiments, the peptides also inhibited proliferative and IFN-γ responses to the mitogen Con A or primary Ag KLH (results not shown). Fig. 7 also demonstrates that the inhibitory effect is dependent on IL-10, because when LMP1-derived peptides that did not elicit this cytokine were added to PPD-stimulated cultures, no suppression was seen. Furthermore, in cultures treated with anti-IL-10 Ab, the LMP1 peptide-mediated suppression was reversed by up to 71% (Fig. 7).

Inhibition of Ag processing prevents IL-10 secretion induced by LMP1 protein, but not synthetic LMP1 peptides

The induction of IL-10 secretion from CD4+ T cells by the LMP1 peptides suggests that whole LMP1 also induces such responses after the protein has been processed and presented as antigenic peptide fragments by the APC. However, molecules from other pathogens have been shown to induce IL-10, not after processing, but by direct interaction with innate pattern recognition receptors (29, 44, 45).

To investigate the requirement for processing in LMP1-mediated suppression, Ag loading was inhibited by the addition of chloroquine. PBMC cultures, with chloroquine-treated or control APC, were stimulated with purified LMP1 or IL-10-inducing LMP1 pep-
FIGURE 8. Ag processing is required for IL-10 secretion induced by purified LMP1. IL-10 responses are shown when PBMC from an EBV-seropositive donor were stimulated with purified LMP1 or IL-10-inducing LMP1 peptides in the presence or absence of the processing inhibitor chloroquine. ■, Control cultures lacking chloroquine; □, those with chloroquine.

The results (Fig. 8) show that inhibition of Ag processing prevents IL-10 secretion induced by purified LMP1, but not by the LMP1 peptides.

FIGURE 9. LMP1-mediated suppression deviates T cells specific for a bystander Ag to adopt an anergic, IL-10-secreting phenotype. A, The proliferative, IFN-γ, and IL-10 responses when PBMC from an EBV-seropositive donor were first stimulated in culture with the mitogen Con A, or the recall Ags PPD or TT in the presence or absence of suppression by LMP1. Cells from the cultures were rested for 7 days, washed to remove the Ags, added to fresh irradiated autologous PBMC as a source of APC, and restimulated with Con A, PPD, or TT (B). Representative results are shown from one EBV-seropositive donor (n = 4).

Specificity and persistence of LMP1-mediated suppression in vitro

The PBMC of seropositive donors were incubated with PPD and LMP1 (which inhibited the proliferative and IFN-γ responses), washed to remove Ags, rested, and then restimulated. Representative results (n = 4) from one donor (Fig. 9) demonstrate that these cells, unlike those from control cultures that had not been suppressed with LMP1, were unable to proliferate and produce IFN-γ, but did secrete IL-10 when restimulated with PPD. This loss of Th1 responsiveness was Ag specific, because the cells obtained from LMP1 suppressed, PPD-stimulated cultures retained the ability to proliferate and produce IFN-γ against the mitogen, ConA, or another recall Ag, TT. Thus, the Tr1 responses to LMP1 deviate T cells recognizing a bystander Ag to adopt an anergic, IL-10-secreting phenotype. This induction of persistent and specific anergy to a bystander Ag was also demonstrated with the second recall Ag, TT (Fig. 9).

Discussion

This study was prompted by the lack of a protective immune response against LMP1 in individuals with latent EBV infection. The main conclusion from our data is that LMP1 is recognized by the immune system, but that this response is dominated by the induction of high levels of IL-10 secretion by cells with a Tr1 phenotype (19). Furthermore, this IL-10 response was able to suppress both proliferative and IFN-γ responses against other Ags and polyclonal stimuli, and therefore would be expected to inhibit the development of protective Th1 and cytotoxic immunity against EBV (46).

Our demonstration of Tr1 activation provides a mechanism for the previously reported observation that rLMP1 inhibits immune functions including mitogen, Ag, and CD3/CD28-stimulated T cell activation; NK cell cytotoxicity; and Ag-induced IFN-γ secretion (47). One peptide from LMP1, included within the sequence of peptide 7 (aa 31–50) from our panel, was reported (47) to replicate these inhibitory properties, and is identified in this study as one of many effective Tr1 IL-10 inducers.

There are several possible explanations for the propensity of LMP1 to elicit IL-10 production by Tr1 cells. First, this ability may be an intrinsic property of the protein, analogous to the effects of B. pertussis and malarial products via innate immune receptors (29, 44, 45). However, the finding that the regulatory response to LMP1 is limited to EBV-seropositive, but not -seronegative, donors argues strongly against this as a sole explanation. Further evidence against this hypothesis is provided by the demonstration that purified LMP1 requires Ag processing to induce IL-10 production in vitro, and that multiple LMP1 peptides can stimulate Tr1 responses. Mapping of the epitopes that induce IL-10 raises a second possibility, that they share structural features that predispose to specific immune recognition by Tr cells. The study demonstrated that the epitopes cluster within the highly hydrophobic, N-terminal half of LMP1, an area rich in binding motifs for many MHC class II molecules (http://imtech.res.in/raghava/propred/index.html; http://www.csl.abdn.ac.uk/~gjk/MHC-Thread). However, hydrophobicity alone is unlikely to explain the ability of the dominant LMP1 peptides to induce IL-10, because human Tr1 cells specific for both hydrophobic and hydrophilic sequences from other Ags have been identified (48).

An alternative hypothesis is that the initiation and maintenance of a suppressive response to LMP1 results from IL-10 conditioning. Activation of CD4+ T cells in the presence of IL-10 is known to generate Tr1 cells (18). EBV is one of the viruses that encodes
a homolog of this cytokine, viral IL-10 (vIL-10), which is expressed during lytic cycle infection (49). Thus, during the development of the immune response to EBV, the presence of vIL-10 may mediate the differentiation of LMP1-specific Th cells to favor 10-secrating Tr1 cells. If true, the question then arises: why should LMP1 be susceptible to deviation by vIL-10, because other latent proteins have been shown to elicit Th1 responses in vitro (50, 51)? For example, EBNA1 and, less commonly, EBNA3C and LMP2 can stimulate proliferation and IFN-γ production by cultured T cells (50, 51). In this context, it may be relevant that LMP1 is one of only two latent proteins expressed in the lytic cycle when vIL-10 is produced. More speculatively, LMP1 may be preferentially presented to T cells, given that the Ag contains a cluster of many MHC class II binding motifs, and has the ability to upregulate APC activity (5, 52).

The stimulation of IL-10-secrating Tr1 cells by LMP1 was capable of inhibiting proliferative and IFN-γ responses against recall Ags and mitogen. These observations suggest that LMP1 is able to inhibit Th1 responses to other coexpressed EBV proteins, which may be important in latent infection and associated tumors. However, regulation need not predominate, because LMP1-expressing B cells retain the ability to stimulate Th1 or cytotoxic cells specific for other Ags, and the balance may depend on factors such as the frequency of LMP1-specific Tr cells. We also observed that Tr1 cells stimulated by LMP1 in vitro not only inhibit but also anergize bystander responses, but it is unclear whether this property is relevant in vivo, given that responsive effector T cells specific for other EBV Ags can be obtained from infected donors. This apparent paradox highlights the complex balance between all pathogen evasion mechanisms and the evolution of host countermeasures. Thus, Tr1 responses are one part of an equilibrium in EBV latency, whereby the host is unable to clear the infection yet resists uncontrolled EBV-driven lymphoproliferation.

Numerous methods used by pathogens to avoid clearance by the immune system have been described, and are dependent on escape, resistance, or counterattack (1). In addition, the induction of a Tr1 response to EBV LMP1 represents a novel means of immune evasion. A similar regulatory mechanism that subverts, rather than resists, or counterattacks (1). In addition, the induction of a Tr1 type immune response. The design of strategies to overcome such Tr1 responses should provide an innovative approach to the development of vaccines to prevent or treat EBV-associated tumors. Conversely, it may be possible to exploit therapeutically such specific induction of bystander anergy to inhibit pathogenic responses in immune-mediated diseases.

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References


