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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.

A wide variety of strategies is used by viruses to evade immune-mediated clearance, and these strategies can be considered to belong to one of three major mechanisms: escape, resistance, and counterattack (1). Viruses escape immune recognition by disruption of Ag presentation pathways (2) and epitope mutation (3). Resistance is mediated by inhibiting apoptosis of virally infected cells. Counterattack comprises the killing of effector T cells (4). However, these strategies do not explain all examples of viral immune evasion. For instance, the persistence of latent EBV infection is not fully understood, although several evasion mechanisms have been described. The most obvious is reduced gene expression during latency. Another mechanism is escape of detection in Burkitt’s lymphoma, where the cells are unable to process endogenously expressed Ags for presentation on MHC class I due to the down-regulation of TAP-1 and TAP-2 (5). Unable to process endogenously expressed Ags for presentation on-effector T cells (4). However, these strategies do not explain all examples of viral immune evasion. For instance, the persistence of latent EBV infection is not fully understood, although several evasion mechanisms have been described. The most obvious is reduced gene expression during latency. Another mechanism is escape of detection in Burkitt’s lymphoma, where the cells are unable to process endogenously expressed Ags for presentation on MHC class I due to the down-regulation of TAP-1 and TAP-2 (5). In addition, Epstein-Barr nuclear Ag (EBNA)1 escapes MHC class I presentation due to its N-terminal Gly/Ala repeat domain (6). EBV may also evade immune responses by resistance to apoptosis through the viral Bcl-2 homolog BHRF-1 (8) or through induction of the anti-apoptotic protein A20 by latent membrane protein (LMP)1 (9). However, these mechanisms remain insufficient to explain how the virus avoids immune responses against EBV latent Ags.

EBV, a human gammaherpesvirus, is carried as a latent infection by >90% of adults, replicating in B cells and nasopharyngeal epithelial cells (10). The acute infection is controlled by a cytotoxic response predominantly against EBNA3A, -3B, and -3C (10), but in all cases, the virus enters a latent state in B cells (10). A restricted panel of genes, including LMP1, is expressed during latency and in some cases of EBV-associated malignancies such as Hodgkin’s disease and nasopharyngeal carcinoma (11, 12). LMP1 is a transmembrane protein comprising six hydrophobic transmembrane domains in the N-terminal half of the protein and a cytoplasmic tail that mediates cellular signaling cascades (10). The protein acts as a constitutively activated TNFR, transforming cells through activation of molecules including NF-κB and the anti-apoptotic protein A20 (13–16).

The role of different CD4+ Th cell subsets in regulating the nature and efficacy of immune responses is increasingly recognized (17–21). Initially, attention focused on the mutual antagonism between Th1 and Th2 cells, which produce IFN-γ and IL-4, respectively (22), but additional T regulatory (Tr) cell subpopulations with important roles in immunoregulation and tolerance have now been defined (18–21). In particular, production of the Th1 cytokine IL-10 can protect rodents against a number of immunemediated diseases (18, 19), while Th3 cell secretion of TGF-β prevents spontaneous autoimmunity (23) and mediates some forms of oral tolerance (24). Regulatory populations characterized by CD25 expression have also been isolated from rodents (25, 26) and more recently from human peripheral blood (27, 28), but in most reports, the suppressive effects of these cells are nonspecific and not dependent on cytokine production.

The effectiveness of Tr cells in controlling immune-mediated disease raises the prospect that microorganisms may exploit such regulation as a fourth major mechanism to evade immune clearance. There is already evidence that certain bacteria can stimulate Tr cells, with the demonstration of IL-10 responses by mice exposed to Bordetella pertussis (29) or Mycobacterium vaccae (30), and hepatitis C-specific, IL-10-secreting human CD4+ T cells have now been identified (31). Furthermore, the cytokines produced by human CD4+ T cells stimulated with autologous EBV-transformed lymphoblastoid cell lines include IL-10 (32), suggesting that EBV Ags might induce a Tr response.

Given that cells latently infected with EBV express LMP1, the question arises as to why this Ag fails to elicit effective cytotoxic immunity (33, 34). Indeed, cytotoxic T cells specific for LMP1 are notable for their relative absence from infected individuals (33), leading us to hypothesize that LMP1 stimulates a regulatory response. Therefore, the aim of the current work was to determine whether the apparent lack of immunogenicity of LMP1 is due to selective activation of T cells secreting cytokines such as IL-4 or IL-10, which would be expected to inhibit protective Th1 and cytotoxic responses.
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 (~10^6) 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 CS1–4 (Biomag; Polyscience, 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 gene (895.8) (38). Selected peptides were screened by HPLC and amino acid analysis. All peptides were used to stimulate cultures at 15 μg/ml, although, as in previous mapping studies (39), responses were similar over a wide range of concentrations (4–50 μg/ml).

The control recall Ag Mycobacterium tuberculosis purified protein derivative (PPD; Statens Serum Institut, Copenhagen, Denmark), the T cell mitogen Con A (Sigma-Aldrich, Poole, U.K.), and the primary Ag keyhole limpet hemocyanin (KLH; Calbiochem-Behring, San Diego, CA) were used to stimulate cultures at 10 μg/ml. PPD readily provokes recall T cell responses in vitro (40), because most U.K. citizens have been immunized with bacillus Calmette-Guérin. A further recall Ag, tetanus toxoid (TT; Aventis Pasteur, Lyon, France), was used at 6 μg/ml in some cultures.

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 1.25×10^6 cells/ml. Cellular proliferation was estimated from the incorporation of [3H]thymidine in triplicate 100-μl aliquots taken from the wells 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).

Inhibition of APC protein processing

To investigate the requirement for Ag processing in LMP1-induced IL-10 responses, the pH modulator chloroquine (Siga-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 10^6 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.
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 ($p = 2.2 \times 10^{-2}$; 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).

**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-\(\gamma\) 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-\(\gamma\)/H9253 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-\(\gamma\) responses to PPD by 41–99% (Fig. 7). In parallel experiments, the peptides also inhibited proliferative and IFN-\(\gamma\) 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-
LMP1 peptides.

A bystander Ag to adopt an anergic, IL-10-secreting phenotype. LMP1-mediated suppression deviates T cells specific for both hydrophobic and hydrophilic sequences (48). 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 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.csd.abdn.ac.uk/~gjlk/MHC-Thread). How-
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 IL-10-secreting 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 up-regulate APC activity (5, 52).

The stimulation of IL-10-secreting 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 avoids, immune detection may well be exploited by other pathogens (29, 31), particularly those with the ability to maintain chronic infections. This is especially likely for those viruses, such as CMV, that also encode a homolog of IL-10 with potent immunosuppressive effects (53), which may also induce a regulatory 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


