Analysis of Immune Activation and Clinical Events in Acute Infectious Mononucleosis

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The symptoms of infectious mononucleosis (IM) are thought to be caused by T cell activation and cytokine production. Surface lymphocyte activation marker (SLAM)–associated protein (SAP) regulates lymphocyte activation via signals from cell-surface CD244 (2B4) and SLAM (CD150). We followed T cell activation via this SAP/SLAM/CD244 pathway in IM and analyzed whether the results were associated with clinical severity. At diagnosis, SAP, SLAM, and CD244 were significantly up-regulated on CD4 and CD8 T cells; expression decreased during IM, but CD244 and SLAM levels remained higher on CD8 cells 40 days later. There were significantly more lymphocytes expressing CD8 and CD244/CD8 in patients with severe sore throat. The expression of CD8 alone and CD244 on CD8 cells correlated with increased virus load. We suggest that T cells expressing CD244 and SLAM are responsible for the clinical features of IM but that the control of activation is maintained by parallel increased expression of SAP.

Infectious mononucleosis (IM) is one of the most common causes of prolonged illness in university students in the developed world, with 5%–10% affected annually [1]. Typical clinical features of IM include fever, lymphadenopathy, sore throat, and prolonged fatigue, but the severity and duration of symptoms are highly variable, with rare but well-recognized chronic and fatal outcomes [2].

IM is believed to be an immunopathological disease, and it is characterized by dramatic antigen-driven clonal expansions of CD8 T lymphocytes in response to primary Epstein-Barr virus (EBV) infection [2, 3]. The activated CD8 lymphocytes, which can account for >50% of the total circulating lymphocyte population [3], release cytokines that are predominantly of a Th1 type (interferon [IFN]–γ and interleukin-2) [4–6] and are thought to induce the clinical features.

EBV is an oncogenic herpesvirus with which >90% of the adult population globally has been infected. After primary infection, the virus establishes life-long persistence in host B lymphocytes [7]. Most people undergo asymptomatic early childhood infection [8]; however, if EBV is first encountered as a young adult, IM results in 45%–56% of cases [9–11].

The mechanisms underlying the variable clinical response to primary EBV infection remain unclear. Although it has been suggested that IM is caused by an exaggerated cellular immune response to primary infection, a link between the severity of clinical illness and the degree of immune activation has not been established. Furthermore, there is no animal model in which to investigate this hypothesis.

Fatal IM is generally associated with the inherited X-linked lymphoproliferative syndrome (XLP) [12]. Primary EBV infection in affected male infants results in
overwhelming cytotoxic T cell activation and death either from fulminating IM with multiorgan failure or EBV-driven lymphoma [13]. Affected children have normal responses to other herpesvirus infections, including varicella-zoster virus (VZV), and no consistent immune dysfunction before encountering EBV.

The genetic defect underlying XLP has recently been elucidated. The abnormal gene product, signaling lymphocyte activation molecule (SLAM)–associated protein (SAP) [14] or src homology-2 (SH2D1A) [15–16], is a small intracytoplasmic protein with an SH2 domain and is most abundantly expressed in T lymphocytes and NK cells [16–17]. In vitro, SAP inhibits signal-transduction pathways that are initiated by at least 4 lymphocyte cell-surface receptors: SLAM/CD150, 2B4/CD244, CD84, and LY9 [18–21]. SLAM is expressed on CD4 and CD8 T lymphocytes and B lymphocytes, and it is rapidly up-regulated on these cells after activation [22]. The activation of SLAM directs the immune response toward a Th1 phenotype with increased secretion of IFN-γ [23].

CD244 is expressed on all NK cells, γδ T cells, and monocytes, on 30%–70% of resting CD8 T cells, and on up to 5% of CD4 T cells but not on B cells [24–26]. The in vitro triggering of CD244 on NK cells results in increased cytotoxicity and cytokine secretion, including IFN-γ [25]. This role has been demonstrated in vivo by the excessive production of IFN-γ by CD4 and CD8 T cells after lymphocytic choriomeningitis virus infection in SAP-deficient mice [27, 28], and this may provide important clues about the molecular mechanisms of immune activation in IM.

To assess whether the lymphocyte activation pathway controlled by SAP is involved in the regulation of the immune response in IM, we investigated the expression of SAP, SLAM, and CD244 in subsets of peripheral blood mononuclear cells (PBMCs) from a cohort of patients with acute IM. To gain insight into whether the changes are specific to primary EBV infection, we compared the results from patients with IM with those from adults with primary VZV infection (chicken pox).

Our data show that primary EBV infection results in significant up-regulation of SAP, SLAM, and CD244. Moreover, the severity of sore throat at the time of diagnosis is significantly linked to the extent of immune activation in IM, and the expression of

### Table 1. Summary of clinical details for 26 patients with infectious mononucleosis.

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<th>Patient no.</th>
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<th>Fever</th>
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**NOTE.** F, female; M, male; NK, not known.

\(^a\) Defined as housebound at the worst point during illness.

\(^b\) No. of copies of Epstein-Barr virus/10\(^6\) peripheral blood mononuclear cells at the time of diagnosis.
both CD8 alone and CD244 on CD8 cells correlates with increased virus load. Subsequent prolonged expression of CD244 and SLAM on CD4 and CD8 cells may explain the extended clinical course of IM. We also show that the up-regulation of this pathway occurs during primary VZV infection, although activation is more marked in IM than in VZV infection.

SUBJECTS, MATERIALS, AND METHODS

Clinical Cohort and Follow-Up

Subjects with IM. All participants with IM were recruited at Edinburgh University Health Service and gave written, informed consent. The study had approval from the Lothian Research Ethics Committee. The diagnostic criterion for IM was a positive EBV viral capsid antigen IgM test result. To obtain a standardized diagnosis date for the analysis, the date on which blood was obtained for EBV serological testing was recorded as the date of diagnosis. Because of limits on the amount of blood obtained, separate groups of patients were used in the different analyses. Clinical data were available for 26 patients (age range, 18–26 years; sex, 14 female and 12 male; table 1), and full blood counts (FBCs) at diagnosis were available from 21 patients.

Clinical assessments. Patients with IM were examined, and blood samples were obtained on 2–4 occasions. The first interview was performed as soon as possible after diagnosis (mean, 9 days), and patients were asked to report their most severe symptoms. The severity of sore throat was assessed as mild (able to swallow a normal diet at the worst point of illness) or severe (unable to swallow a normal diet). Where possible, patients were monitored at 2 weeks and 1 and 2 months or until they had returned to the preillness activity level. All patients were recalled at 6 months. FBCs were carried out at each visit by use of a Coulter counter (Coulter), and, when these values were used in the analyses, they were compared with the established control values for the counter (see Results).

Subjects with primary VZV infection. Four adults (2 men and 2 women; age range, 28–40 years) with laboratory-proven primary VZV infection, 3 of whom required hospital admission and treatment with acyclovir, were studied. Blood for the study was obtained before the administration of antiviral therapy.

Control subjects. Blood was obtained from 13 healthy volunteers (age range, 22–30 years; 3 men and 10 women) and used for control experiments. Additional control data were available from UK blood donors (n = 14); however, FBCs were not available.

Flow-Cytometric Analysis

PBMCs were separated from whole blood by density-gradient centrifugation and either used fresh or cryopreserved for later use. PBMCs were stained with antibodies to SLAM (A12 and IgG1; Sheffield University Hybridoma Unit), CD244 (Immunootech), CD3, CD8, CD56, CD19, or isotype control antibodies (BD Pharmingen), according to the manufacturers’ instructions.

After 2 washes in PBS, the stained cells were analyzed on a FACScalibur machine (Becton Dickinson). Cells were gated on forward and side scatter, and dead cells were identified by staining for TO-PRO 3 (Molecular Probes). Samples were analyzed by either 2- or 3-color staining plus TO-PRO3. Unstained cells and those stained with appropriate isotype controls were used as negative controls. The data were processed by use of CELLQuest software (Becton Dickinson).

Cytospin Preparations and Immunohistochemistry

Cytospin preparations were made from fresh PBMCs, on polystyrene-coated slides (BDH), and were fixed in acetone. Slides were incubated with anti–SAP rabbit polyclonal antibody (Santa Cruz Biotechnology), followed by goat anti–rabbit antibody (DAKO) and detection with biotin-avidin complex (Vector) and 3,3′ diaminobenzidine tetrahydrochloride (DAKO). Slides were counterstained with hematoxylin and mounted in Ultra-mount (DAKO); 200 cells were counted in random fields on each slide.

Virus Load Quantification

DNA was extracted by use of the Easy-DNA kit (Invitrogen). The EBV-DNA load was determined by quantitative competitive polymerase chain reaction exactly as described by Stevens et al. [29]. The amplification reaction contained 50 mmol KCl/L, 1.5 mmol MgCl2/L, 10 mmol Tris 9/L (pH 8.5), 25 pmol of each primer (1 of which was labeled with biotin), and 1 U of Taq. Cycling conditions were 4 min at 95°C; 40 cycles at 95°C, 55°C, and 72°C for 1 min each; and 3 min at 72°C. Products were captured on a streptavidin-coated plate and probed with digoxigenin-labeled wild-type and internal standard probes. Optical density was measured and used to calculate the number of copies.

Statistical Analysis

The Mann-Whitney U test was used to test for differences in the medians of quantitative variables (e.g., CD8 cell counts between patients and control subjects). Spearman’s rank correlation test was used to examine associations between 2 quantitative variables. All significance tests were 2-tailed.

RESULTS

Analysis of Samples from Patients with Acute IM

Significant increases in CD3, CD8, and NK (CD3+CD56+) cells in PBMCs during acute IM. We analyzed lymphocyte subsets from 26 patients at the time of diagnosis with acute IM (see table 1). Data were compared with results from healthy control
Total lymphocyte counts and CD3, CD8, and NK cell counts were all significantly elevated at the time of diagnosis with IM ($P<.01$, for all subsets). The median value for total lymphocyte counts was 7.19 x 10^6 cells/mL for patients with IM and 1.67 x 10^6 cells/mL for control subjects; for CD3 counts, the median was 5.07 x 10^6 cells/mL for patients and 1.00 x 10^6 cells/mL for control subjects; and for CD8 counts, the median was 4.40 x 10^6 cells/mL for patients and 0.48 x 10^6 cells/mL for control subjects. NK cell counts in patients with IM were also significantly elevated, compared with those of control subjects: the median for patients was 1.31 x 10^6 cells/mL, and that for control subjects was 0.24 x 10^6 cells/mL. Neither CD4 nor B cell counts were significantly altered (figure 1A–1F).

**Significant activation of the SAP/SLAM/CD244 activation pathway during acute IM.** Next, we analyzed the expression of the lymphocyte activation molecules CD244 and SLAM in the same 26 patients (see above). At the time of diagnosis with acute IM, CD3, CD4, and CD8 T cell subsets expressing CD244 were all significantly elevated ($P<.01$, for all subsets). The median number of CD3+ cells expressing CD244 was higher in patients (3.95 x 10^6 cells/mL) than in control subjects (0.27 x 10^6 cells/mL). The median values for CD4+/CD244+ cells was 0.2 x 10^6 cells/mL for patients and 0.04 x 10^6 cells/mL for control subjects; the median value for CD8+/CD244+ cells was also markedly higher (3.17 x 10^6 cells/mL for patients vs. 0.22 x 10^6 cells/mL for control subjects; figure 2A and 2B). The percentages of CD3 (median, 77%), CD4 (median, 30%), and CD8 (median, 82%) cells expressing CD244 in patients with IM were also increased over the control median values of 14%, 9%, and 51% for CD3, CD4, and CD8, respectively ($P<.001$, for all data sets). As has been reported elsewhere, CD244 was not expressed on B cells and was constitutively expressed on NK cells [12].

At the time of diagnosis with IM, CD3, CD8, and NK lymphocyte subsets expressing SLAM were all significantly elevated ($P<.01$, for all subsets). The median number of CD3 cells expressing SLAM was 2.17 x 10^6 cells/mL for patients, compared with 0.06 x 10^6 cells/mL for control subjects; for CD8/SLAM double-positive cells, the value for patients was 1.86 x 10^6/mL, compared with 0.06 x 10^6 cells/mL for control subjects (figure 2A); and, for CD56/SLAM double-positive cells, the

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**Figure 1.** Total lymphocyte counts (A) and expression of CD3 (B), CD4 (C), CD19 (D), CD8 (E), and CD56 (F) in peripheral blood mononuclear cells at the time of diagnosis with infectious mononucleosis (IM) and at the first follow-up visit, compared with those in healthy control subjects.
median count for patients was $0.2 \times 10^6$ cells/mL, compared with $0.04 \times 10^6$ cells/mL for control subjects. In patients with IM, the percentage of CD8 cells (median, 59%) expressing SLAM was also significantly different from that in control subjects (median, 41%,) ($P < .01$).

In patients with IM, the number of CD4 cells expressing SLAM was not significantly elevated (median, $0.21 \times 10^6$ vs. $0.09 \times 10^6$ cells/mL in control subjects) (figure 2B), but the percentage of CD4 cells expressing SLAM, and thus an activated phenotype, was significantly increased (median, 60% vs. 31% in control subjects) ($P < .05$).

The expression of SAP in PBMCs from 16 patients with acute IM and 17 healthy control subjects was analyzed by immunohistochemical testing. At the time of diagnosis, the level of the expression of SAP was high (54%–100%), compared with that in control subjects (2%–74%), and the median level of expression was significantly different from that in control subjects (85% vs. 33%) ($P < .001$) (table 2). Because the expression of SAP increases during acute IM, we investigated the relationship between it and the cell-surface components of the pathway, CD244 and SLAM. In 9 control subjects, we did not find a significant relationship between the percentage of cells expressing SAP and either the percentage of CD8 cells or the percentage of CD8 cells expressing either CD244 or SLAM in PBMCs. However, in the 11 patients with acute IM whose samples were obtained at the time of diagnosis, there was a significant correlation between the percentage of CD8 cells expressing CD244 and the percentage of SAP-positive cells in PBMCs ($P < .01$). As in the control data, we found no relationship between the expression of SAP and percentage of either CD8 cells or CD8 cells expressing SLAM.

Longitudinal Analysis of IM Immune Response

We performed a longitudinal study of a group of 7 patients (1, 4, 5, 9, 10, 11, and 12 in table 1). The median length of illness in this group was 58 days, with a range of 26–70 days after diagnosis.

Rapid decrease in lymphocytosis during acute IM, despite ongoing clinical symptoms. At the time of diagnosis, 86% of patients with IM had increased lymphocyte counts. In contrast, at the first review 3–16 days later, and despite features of ongoing clinical illness, all of the total lymphocyte counts and all of the CD3 and CD8 subsets had returned to within the range established for control subjects, and, in all subjects, they remained within this range for the duration of the illness (figure 1).

Activated phenotype of CD8 cells during clinical illness. At the time of the first follow-up, the total numbers of CD3 and CD8 cells expressing CD244 decreased in each patient. However, the median percentages of CD3, CD4, and CD8 cell subsets expressing CD244 remained significantly higher than those for control subjects ($P < .05$). The percentage of CD3 cells expressing CD244 was 40% in patients and 12% for control subjects, the percentage of CD8 cells expressing CD244 was 64% for patients and 50% for control subjects, and the percentage for CD4+/CD244+ cells was 14% for patients and 8% for control subjects. Both the number of CD8 cells expressing SLAM (median, $0.32 \times 10^6$ cells/mL) and the percentage of cells expressing SLAM (median, 59%) at the first follow-up visit were significantly higher than those for control subjects ($P <
Figure 3. Flow-cytometric analysis of lymphocytes, showing expression of CD244/CD8 in a single patient (10) at different time points during acute infectious mononucleosis (IM). A, At diagnosis, virus load (VL) = 3036; B, 16 days after diagnosis, VL = 78; C, 42 days after diagnosis, VL = 1; and D, 6 months after diagnosis, VL = 241. VL is given as no. of Epstein-Barr virus genomes/10⁶ peripheral blood mononuclear cells. FITC, fluorescein isothiocyanate.

Extent of Immune Activation at Diagnosis and Severity of Sore Throat and Increased Virus Load

We recorded the severity of sore throat in 26 patients and classified this as either mild (14 subjects) or severe (12 subjects) (table 1). The median percentage and number of lymphocytes expressing CD3, CD8, CD244/CD3, and CD244/CD8 at the time of diagnosis were all significantly higher in patients with severe, compared with mild, sore throat ($P < .05$) (figure 4). The median total lymphocyte counts were higher in patients with severe sore throat; however, these changes were not significant. Other immune parameters investigated, including the expression of CD4 cells and SLAM in T cell subsets at the time of diagnosis, were not significantly linked to the severity of sore throat.

Other clinical features of IM were more difficult to relate to immune parameters, because either almost all patients experienced the symptom (e.g., lymphadenopathy) or very few did (e.g., rash). However, we found significant correlations between self-reported fever and the percentage of peripheral blood CD8+ T cells, rash and the percentage of peripheral blood CD3+ cells, and fatigue (assessed as the inability to leave home) and the percentage of peripheral blood CD8+ and CD244+ T cells (data not shown). Of note, there was no significant difference in the length of illness before diagnosis in those with mild versus those with severe sore throat.

Virus loads were available for 23 patients with IM (range, 0–49,249 EBV genomes/10⁶ PBMCs). Increased virus load cor-
Figure 4. CD8 T cell lymphocytosis in patients with acute infectious mononucleosis (IM) who had either severe or mild sore throat. A, Significantly higher median no. of lymphocytes expressing CD8 in patients with severe (n = 11) than in those with mild (n = 10) sore throat (P < .05). The median value for the severe group was 5.18 × 10⁶ cells/mL; for the mild group, it was 2.89 × 10⁶ cells/mL. Bars show the median values. B, Significantly higher median no. of lymphocytes expressing CD244/CD8 in patients with severe (n = 10) than in those with mild (n = 10) sore throat (P < .05). The median value for the severe group was 6.46 × 10⁶ cells/mL; for the mild group, it was 2.20 × 10⁶ cells/mL. Bars show median values.

related directly with increased percentages of both CD3 (P < .05) and CD8 (P < .05) cells in PBMCs at the time of diagnosis with IM and with the increased expression of CD244 on CD8 cells (P < .05) (figure 5). In addition, the median virus load was significantly higher in patients with severe sore throat (6868 EBV genomes/10⁶ PBMCs) than those with mild sore throat (2722 EBV genomes/10⁶ PBMCs) (P < .05) (figure 6).

DISCUSSION

IM is caused by delayed primary EBV infection and is thought to be immunopathological in nature, because of an exaggerated CD8 T cell response to viral antigens expressed on infected B lymphocytes. However, the mechanisms that underlie this intense T cell activation and its relationship to the clinical symptoms of IM are not clearly understood. Because the absence of a functional SAP protein in XLP leads to fatal IM, the level of control of immune activation by SAP is likely to be critical in determining the outcome of primary EBV infection in healthy individuals. The aim of the present study was to determine whether the SAP/SLAM/CD244 pathway is activated in IM, and, if so, how this activation relates to the clinical symptoms.

In agreement with the investigators of earlier studies, we found lymphocytosis in 85% of patients with acute IM at the time of diagnosis, which mainly consisted of CD8 T cells [30, 31], although numbers of NK cells were also significantly higher [31]. In addition, several studies have documented the expression of T cell activation markers such as HLA-DR and CD25 on CD8 T cells in peripheral blood during IM [30, 31]. We have demonstrated that these CD8 T cells also show up-regulation of CD244 on CD8 cells in IM, which is consistent with the observation that CD244 is significantly up-regulated on T lymphocytes during acute IM and VZV infection, on both CD3 and CD8 subsets, but the level of activation is more marked during IM.

Figure 5. Expression of CD244 on CD8 cells at the time of diagnosis of infectious mononucleosis (IM) and peripheral blood mononuclear cell (PBMC) Epstein-Barr virus (EBV) genome copies (virus load). A significant correlation was shown between the percentage of PBMCs expressing CD244/CD8 and EBV genomes/10⁶ PBMCs (P < .05).
regulation of the SLAM/CD244 activation pathway. The results of earlier studies have shown that the activated T cells in IM secrete a variety of inflammatory cytokines [4, 5], which are generally thought to be responsible for the clinical symptoms of IM. Our results indicate that the activation of CD4 and CD8 cells through the SLAM/CD244 pathway is involved in this process. The change in the activation status of CD4 cells in particular may be pivotal in directing the cytokine profile seen in IM by their ability to regulate the Th1/Th2 balance [32]. Of interest, even during an early stage of the disease, we found that the expression of SAP was markedly increased in PBMCs, thus maintaining control of the activation process. This contrasts with the situation in XLP, where the loss of SAP function allows uncontrolled T cell activation and an overproduction of inflammatory cytokines, usually with fatal consequences.

Next, we were interested to see whether T cell activation could be linked to clinical events and the duration of illness. Our results show that the prolonged clinical features are not simply a reflection of increased T cell numbers, because the lymphocytosis present at the time of diagnosis decreased rapidly. However, T cells maintained expression of the activation markers SLAM and CD244 for prolonged periods, which suggests continued production of cytokines.

Sore throat with enlarged tonsils is one of the distinguishing characteristics of IM and is present in association with fever and lymphadenopathy in most acute cases [2]. We graded the degree of sore throat at the time of diagnosis as either mild or severe and found that patients with severe sore throat had a significantly higher number of CD8 T cells expressing CD244 and higher virus loads. This result, in addition to our findings of positive correlations between immune parameters and rash, fever, and fatigue, supports the theory of immune activation as the cause of IM.

Because defective SAP function in XLP confers a specific inability to control EBV infection [14–16, 33], we were interested to find out whether the activation of the SAP/SLAM/CD244 pathway was in any way a typical feature of primary EBV infection. We therefore analyzed the expression of these molecules in PBMCs from adults with primary VZV infection. We found that the expression of CD244 was significantly enhanced in patients with VZV, compared with healthy control subjects, but that the changes were significantly more pronounced in IM, despite patients with VZV being more unwell, with 3 of 4 patients requiring hospital admission (no patients with IM required hospital admission). One reason for this difference may be that VZV is directly cytopathic [34], whereas EBV infection causes cell proliferation and induces the expression and activation of costimulatory molecules, which may enhance immune stimulation [35].

Our results suggest that the variability in clinical features of IM directly relates to the level of T cell activation, which, in turn, relates to the number of EBV-infected B cells in the circulation (virus load). A favored explanation for the age-related variability in clinical manifestations of primary EBV infection is the difference in magnitude of viral dose between that acquired by a child through salivary contact and that acquired by a young adult during sexual activity [36, 37]. In this model, a high multiplicity of infection would target more B cells and allow for a high virus load early in the disease process and a consequent high level of stimulation of specific T cells. Our data lend some support to this theory, although they are in contrast to those of a recent report that showed comparable virus loads in acute IM and asymptomatic primary EBV infection [38]. However, the same report showed limited T cell expansions in those undergoing asymptomatic primary infection compared with IM, and this corroborates our finding that more-severe disease is linked to a more marked immune response. If SAP is pivotal to the control of T cell activation in IM, then another possible explanation for the variation in clinical response to primary EBV infection is that the expression of SAP differs between infants and young adults. In this scenario, higher levels of SAP expression in early childhood would exert tighter control over T cell activation and thereby allow for subclinical seroconversion. Further studies are required to test this hypothesis.

Acknowledgments

We thank the Edinburgh University students who enrolled in the study and the staff of the University Health Centre for their collaboration; J. M. Middeldorp (Department of Pathology, Free University Hospital, Amsterdam, The Netherlands) for the bacterial DNA-based vector with modified DNA sequences that we used in the quantitative polymerase chain reaction for Epstein-Barr virus detection; and Francis Rae and Cathy Simpson for valuable technical support. Blood samples from patients with varicella-
zoster virus were kindly provided by the staff of the Regional Infectious Disease Unit, Edinburgh.

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