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Relative Ability of Different Bovine Leukocyte Populations To Support Active Replication of Rinderpest Virus

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Bovine peripheral blood mononuclear cells (PBMC) were infected with the pathogenic Saudi isolate of rinderpest virus (RPV) in order to identify the cell subpopulation(s) susceptible to active replication of this virus. Flow cytometry analysis, using a monoclonal antibody recognizing the H glycoprotein of RPV, showed that monocytes were the main subpopulation in which the virus replicated, whereas <2% of lymphocytes expressed viral antigen. The activation of PBMC with concanavalin A before infection resulted in an increase in the capacity of lymphocytes to support RPV replication; >90% of $CD4^+$ and $CD8^+$ T lymphocytes expressed viral antigen at 3 days postinfection, although ≤40% of γ/δ T cells were productively infected. B-lymphocyte activation with pokeweed mitogen also resulted in increased replication of this virus in these cells, involving up to 40% of B lymphocytes. An enhancement of lymphocyte susceptibility to infection and active replication by RPV was observed upon coculture of RPV-infected PBMC on bovine endothelial cells. Such enhancement was most marked with the B-cell and $CD4^+$ T-cell subpopulations. Contact between lymphocytes and extracellular matrix components did not alter the capacity of RPV to replicate in lymphocytes. This intercellular contact with endothelial cells increased the viability of certain lymphocyte subpopulations, but it alone could not explain the increased sensitivity to RPV. Intercellular signalling, which resulted in interleukin-2 receptor upregulation, probably played a role. In summary, monocytes are the main target for active, productive infection by RPV. Similar replication in lymphocytes depends on their activation state and on contact with accessory cells such as endothelial cells. These characteristics have important implications for virus traffic in vivo and the pathogenesis of this disease.

Rinderpest virus (RPV) is a member of the *Morbillivirus* genus of the *Paramyxoviridae* family. RPV causes disease in even-toed ungulates belonging to the order *Artiodactyla* (24) and most often infects cattle and buffalo. RPV has a particular affinity for lymphatic tissue, in which it causes severe necrotic changes. Lymphocytes within the B- and T-cell areas of the lymph nodes seem to be permissive to infection by RPV (4, 28, 33, 34, 36), and a profound leukopenia accompanies the acute phase of RPV. The latter is largely due to lymphopenia, but the precise mechanism of cell death is still unknown (24).

During experimental rinderpest in cattle, virus can be first isolated from the blood around the time when the animal develops fever. However, the duration and level of viremia varies with the RPV isolate used for infection. A low-grade viremia is detected after inoculation of vaccine strain RPV-RBOK, whereas a virulent strain like RGK/1 produces a viremia which starts earlier, lasts longer, and reaches higher titers than that with the avirulent vaccine strain (29). The infective character of blood from animals infected with RPV has been associated with leukocytes (17, 18). These observations have allowed speculations that lymphoid cells vary in their susceptibility to being infected by RPV or in their capacity to support the replication of RPV.

In vitro, bovine leukocytes have been infected with virulent and lapinized strains of RPV (16, 30). Studies by Rossiter and Wardley (22) suggested that RPV may grow better in populations of predominantly T lymphocytes after polyclonal mitogen activation, although udder macrophages could also be productively infected. More recent studies (21) have shown that bovine lymphoblastoid cell lines, transformed by the protozoan parasite *Theileria parva* and possessing B-cell or T-cell phenotypes, could be productively infected by the RPV-RBOK vaccine strain. However, the precise leukocyte target for productive infection in the blood has not been clearly defined. It is difficult to interpret the above in vitro results in the context of natural infection, during which it is unlikely that all blood lymphocytes are polyclonally activated or transformed by a parasite.

Recently, we have demonstrated that peripheral blood bovine monocytes and monocyte-derived macrophages serve as hosts for a relatively slow but productive infection by the vaccine strain of RPV (20). This study addressed the issue of leukocyte tropism for such active replication and productive infection by RPV. To this end, the capacity of RPV to undergo active replication in different subpopulations of bovine blood mononuclear cells, as well as the role played by lymphocyte activation, was examined. Bovine monocytes were found to be the main mononuclear target for active replication by RPV under nonstimulation conditions. In lymphocytes, active replication was noted only after the activation of cells, which included interaction with accessory endothelial cells.

MATERIALS AND METHODS

Cells and virus. African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection. Vero cells were grown in minimal essential medium (Gibco) supplemented with 10% (vol/vol) fetal calf serum and used to propagate the RPV-Saudi isolate. This isolate was recovered during an outbreak of rinderpest in the early 1980s that caused nearly 100% mortality in cattle and is considered to be a highly pathogenic isolate. It was obtained from the Virus Diagnostic Department at the Institute for Animal Health, Pirbright, United Kingdom, and used at low-level in vitro passage.

PBMC, monocytes, monocyte-derived macrophages, and endothelial cells. Blood samples were obtained from a blood donor calf by jugular venipuncture and collected into Alsever's solution. Peripheral blood mononuclear cells (PBMC) were separated from the buffy-coat fraction by centrifugation at room

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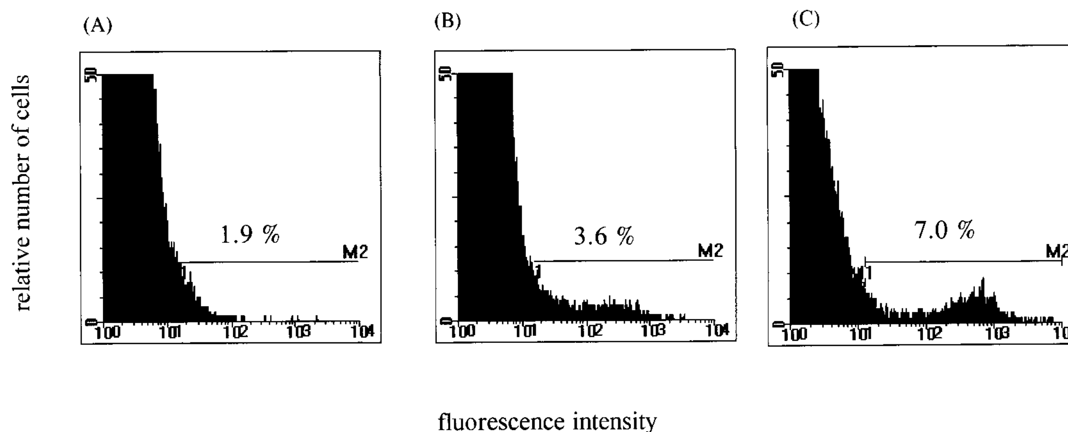

FIG. 1. Flow cytometry analysis of RPV H glycoprotein expression on bovine PBMC infected with RPV strain Saudi at an MOI of 0.1 $TCID_{50}$ per cell. Unfixed cells were stained with the anti-H glycoprotein MAb C1 on days 1 (A), 2 (B), and 3 (C) p.i. Data are plotted as relative fluorescence intensity (*x* axis) versus cell number (*y* axis).

temperature through endotoxin-free Ficoll-Hypaque (Pharmacia) at $1,000 \times g$ for 30 min. Cells were washed three times ($250 \times g$, 10 min) with PBS-A (phosphate-buffered saline [PBS] without Ca^{2+} and Mg^{2+}). Culturing was done in Teflon vials (Tuf-Tainer; Pierce) or on tissue culture 12-well plates (Falcon). The medium was phenol red-free Dulbecco's modified Eagle medium (Gibco) supplemented with 10% (vol/vol) nonmitogenic newborn calf serum (selected to support the growth of bovine PBMC without being mitogenic to cells), 1% (vol/vol) nonessential amino acids, 2 mM glutamine, and 1 mM sodium pyruvate (all from Gibco) (complete medium). Monocyte and macrophage cultures were obtained from PBMC which had been incubated for 24 h and 7 days, respectively, in plastic tissue culture flasks, at which time the nonadherent cells were removed by repeated washing with warm PBS.

For some experiments, PBMC were cultured on plates precoated with fibronectin or collagen I (Becton Dickinson) or in flasks that contained confluent monolayers of bovine endothelial cells. These cells were isolated from blood samples of the same calf used as the blood donor and cultured in complete medium of which 50% was conditioned (taken from endothelial cell cultures which were at least 7 days old). They were cloned by limiting dilution, and their endothelial origin was determined by factor VIII staining using a polyclonal rabbit anti-human factor VIII antiserum (Dako) (1). All cultures were maintained in a 37°C CO_2 incubator at 1×10^6 to 2.5×10^6 PBMC per ml. When PBMC were cocultured with endothelial cells, the conditioned medium required by the latter was employed.

In vitro infection of bovine PBMC, monocytes, and monocyte-derived macrophages. Bovine PBMC were infected on the same day of isolation (unstimulated cells) or stimulated with concanavalin A (ConA; 10 μg/ml) or pokeweed mitogen (PWM; 10 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) for 3 days before infection. PBMC, monocytes, and macrophages were infected with RPV-Saudi at a multiplicity of infection (MOI) of 0.1 50% tissue culture infective dose ($TCID_{50}$) per cell; for mock-infected controls, an inoculum of uninfected cell lysate prepared in the same manner as that for the virus was used. The adsorption period was 2 h at 37°C, after which the cells were washed and cultured as described above, with or without the addition of mitogens.

MAbs. The following monoclonal antibodies (MAbs) were used to identify the different bovine lymphocyte subpopulations: CC8 (immunoglobulin G2a [IgG2a]; anti-bovine CD4) (11), CC63 (IgG2a; anti-bovine CD8) (10), and CC51 (IgG2b; bovine B-cell marker) (15), all of which were included in the First International Workshop on Leukocyte Antigens in Cattle, Sheep and Goats (10). They were obtained as hybridomas from the European Collection of Animal Cell Cultures and used as culture supernatants produced in our laboratory. Monocytes were identified with MY4 (IgG2b; Coulter) specific for CD14 of several species, including human, porcine, and bovine CD14 (19a, 37). MAbs BIg73A (IgG1; anti-bovine IgM), GC65A (IgM; bovine B-cell marker), GB21A (IgG2b; anti-bovine γ/δ), and CACT116A (IgG1; anti-bovine interleukin-2 receptor [IL-2R]) were also used (all from VMRD, Pullman, Wash.). The identification of viral protein expression was done with MAb C1 (2) specific for the H glycoprotein of RPV, kindly provided by J. Anderson (Institute for Animal Health). The isotype controls were UPC-10 (IgG2a), MOPC141 (IgG2b), MOPC104E (IgM), and MOPC21 (IgG1) (all from Sigma).

Flow cytometry. Nonadherent PBMC were removed by the agitation of culture flasks followed by pipetting to dislodge loosely adherent cells. Adherent cells were removed from the surfaces of tissue culture flasks by incubation with 5 mM EDTA in PBS-A for 15 min at 4°C. After being harvested, cells were washed once in CellWASH (Becton Dickinson) before staining. Double immunofluorescence labelling was achieved in a two-step procedure: (i) incubation with MAb C1 (anti-RPV H glycoprotein; IgG1) and the MAb of choice specific for the bovine CD marker under study and (ii) staining with isotype-specific antisera (phycoerythrin-conjugated anti-IgG1 for MAb C1 and fluorescein isothiocyanate-conjugated anti-IgG2a, anti-IgM, or anti-IgG2b for anti-CD MAbs). The anti-isotype conjugates were obtained from Southern Biotechnology (Birmingham, Ala.). Forward-scatter and side-scatter profiles were used to place gates around regions in which live lymphocytes or live monocytes would be found. Fluorescence staining was determined by flow cytometry using a FACScan flow cytometer (Becton Dickinson) and the Lysis II software program. At least 5,000 cells were acquired.

Virus infectivity assay. Cell and supernatant fractions from RPV-Saudi-infected and mock-infected monocyte and monocyte-derived macrophage cultures were harvested at different times postinfection (p.i.) and stored at −70°C until titration. For extracellular virus (ECV), the culture supernatant was centrifuged at 4°C for 10 min at $1,000 \times g$ before storage. Cell-associated virus (CAV) was released from adherent cells by treatment with deionized water by the method of Drastini et al. (5). Briefly, the medium was replaced with sterile distilled water and cultured flasks were stored at −70°C. After being thawed, the suspension was homogenized by repeated pipetting and normal isotonicity was restored with PBS (10×). Samples were centrifuged as described above and stored at −70°C. The amounts of ECV and CAV were determined by titration of serial 10-fold dilutions on semiconfluent monolayers of Vero cells. The virus titer was calculated by the method of Reed and Muench (19).

RESULTS

Relative capacity of unstimulated bovine PBMC to support active replication by RPV in vitro. In order to assess the leukocyte tropism of RPV, freshly isolated bovine PBMC were infected with RPV-Saudi in Teflon vials and analyzed by flow cytometry for viral H glycoprotein expression at 1, 2, and 3 days p.i. As shown in Fig. 1, only a small percentage of cells in the cultures showed active replication of this virus. This percentage increased with time after infection, but the maximum was still $<10\%$ at day 3 p.i.

The phenotype of the subpopulation of PBMC expressing RPV antigen after infection was analyzed by double-labelling flow cytometry. MAbs specific for monocytes and lymphocyte subpopulations were used in association with the anti-virus H glycoprotein MAb. After staining and acquisition, cells were separated by gating into a monocytic region and a lymphocytic region. Figure 2 shows the FACScan profiles of the monocytic region stained with anti-CD14 and anti-RPV glycoprotein at different time points after infection; mock-infected cultures (Fig. 2A) served as the control. The majority of the cells in this region were indeed monocytes ($CD14^+$), as shown in Fig. 2A; the number of cells double positive for CD14 and H glycoprotein (upper right quadrant of each plot) increased over time p.i. (Fig. 2B to D). By day 5 p.i., all cells in the monocytic

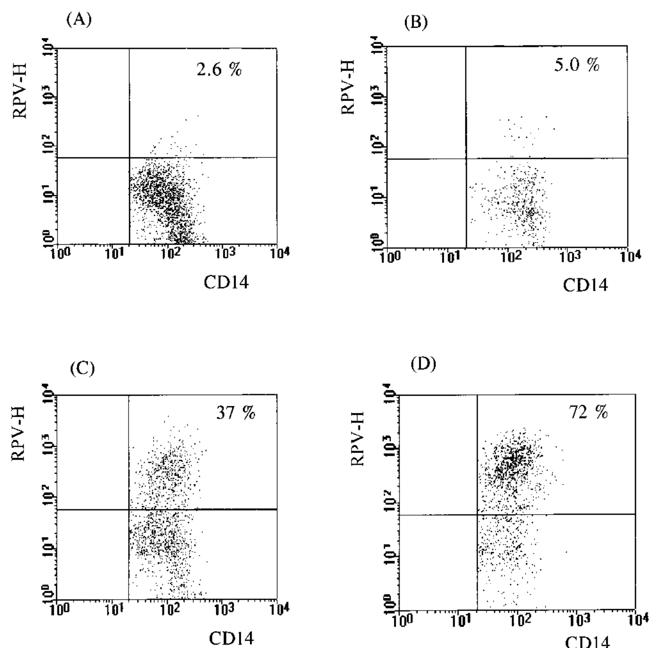

FIG. 2. Two-color flow cytometry analysis of bovine PBMC gated for monocytes and stained with MAbs MY4, against CD14 (*x* axis), and C1, against RPV-H glycoprotein (*y* axis). Cells were mock infected (A) or infected with RPV strain Saudi at an MOI of 0.1 $TCID_{50}$ per cell (B to D) and analyzed at 1 (B), 2 (C), and 3 (A and D) days p.i. The percentage of double-positive cells is shown in the upper right quadrant of each dot plot.

region were double positive (data not shown), showing that all $CD14^+$ cells supported active replication by RPV.

A different picture was obtained when the lymphocytic region was analyzed. Figure 3 shows the results obtained with mock-infected (A, C, E, G, and I) and RPV-infected (B, D, F, H, and J) cells tested at 3 days p.i. The subpopulation which showed the greatest evidence for signs of active replication was $CD8^+$ T lymphocytes, but here the number of H-glycoprotein-positive cells was never >2% of the total lymphocyte population, that is, <5% of the $CD8^+$ subpopulation.

It was noted, however, that the loss of B-cell viability was high in these unstimulated PBMC cultures. Bovine B lymphocytes, identified by MAb CC51, comprised between 20 and 25% of freshly isolated PBMC but decreased to about 10% of the viable population within 1 day of culture. Similar results were obtained when MAb BIg73A against bovine IgM was used to quantitate immunoglobulin-positive cells in the PBMC population (data not shown).

Influence of polyclonal mitogen activation of lymphocytes on their capacity to support active replication by RPV. It was of interest to determine if RPV underwent more efficient active replication in blasting lymphocytes than in resting lymphocytes. Freshly isolated bovine PBMC in plastic tissue culture plates were stimulated for 3 days with ConA before infection with RPV-Saudi was attempted. Plastic plates were used in place of Teflon vials in order that blasting colony formation could be visualized and nonadherent cells could easily be separated from adherent monocytic cells. At the time of infection, numerous colonies of large blast-like cells were detectable by microscopic examination and 95% of the cell population was viable. Only nonadherent populations of PBMC were analyzed because of previous observations that all monocytes supported active replication of this virus in the absence of mitogen stimulation. The main subpopulations of lymphocytes present in

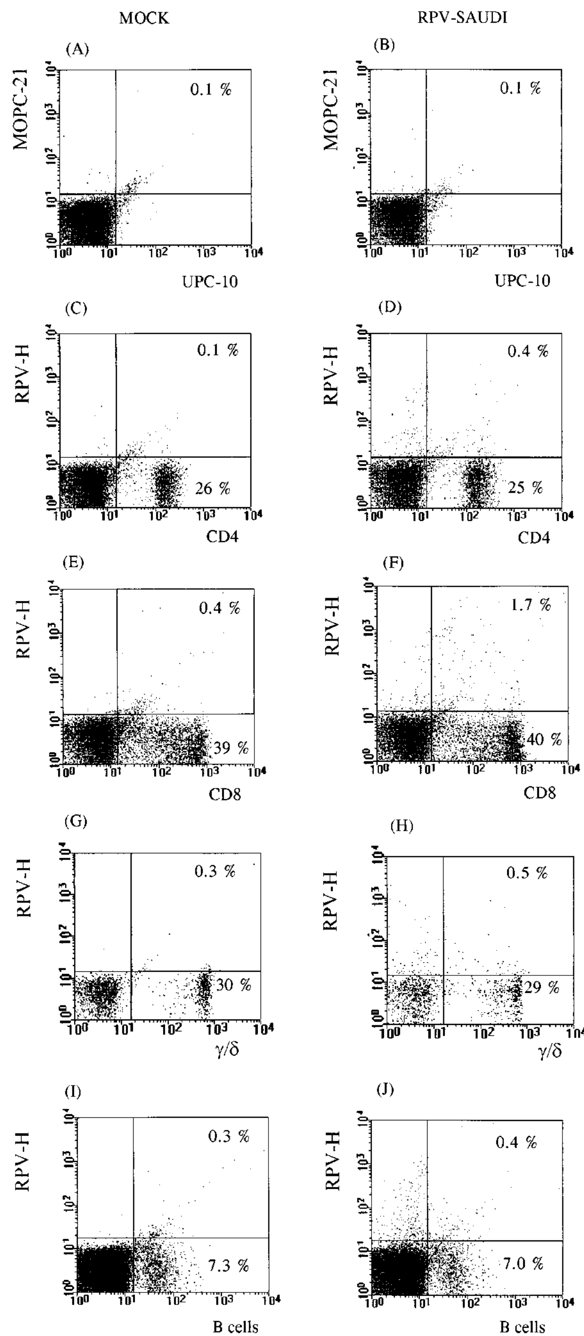

FIG. 3. Phenotypic characterizations of bovine PBMC gated for lymphocytes and double labelled with MAb C1 (anti-RPV) (C to J, *y* axis) and one of the following MAbs (*x* axis): CC8 (anti-CD4) (C and D), CC63 (anti-CD8) (E and F), GB21A (anti-γ/δ) (G and H), and CC51 (anti-B cell) (I and J). PBMC were mock infected (A, C, E, G, and I) or infected with RPV-Saudi at an MOI of 0.1 $TCID_{50}$ per cell (B, D, F, H, and J) and analyzed at day 3 p.i. The results of staining with isotype control MAbs UPC-10 (*x* axis) and MOPC21 (*y* axis) are shown in panels A and B. The percentage in the upper right quadrant of each dot plot is the number of cells double labelled, while the percentage in the lower right quadrant is the number of cells single labelled for the lymphocyte subpopulation marker.

ConA-stimulated PBMC cultures were of the $CD4^+$ and $CD8^+$ phenotype (Fig. 4C to F). Between 7 and 11% of the total population were γ/δ T cells (Fig. 4G to H), and <3% were B lymphocytes (data not shown). While the relative ratio

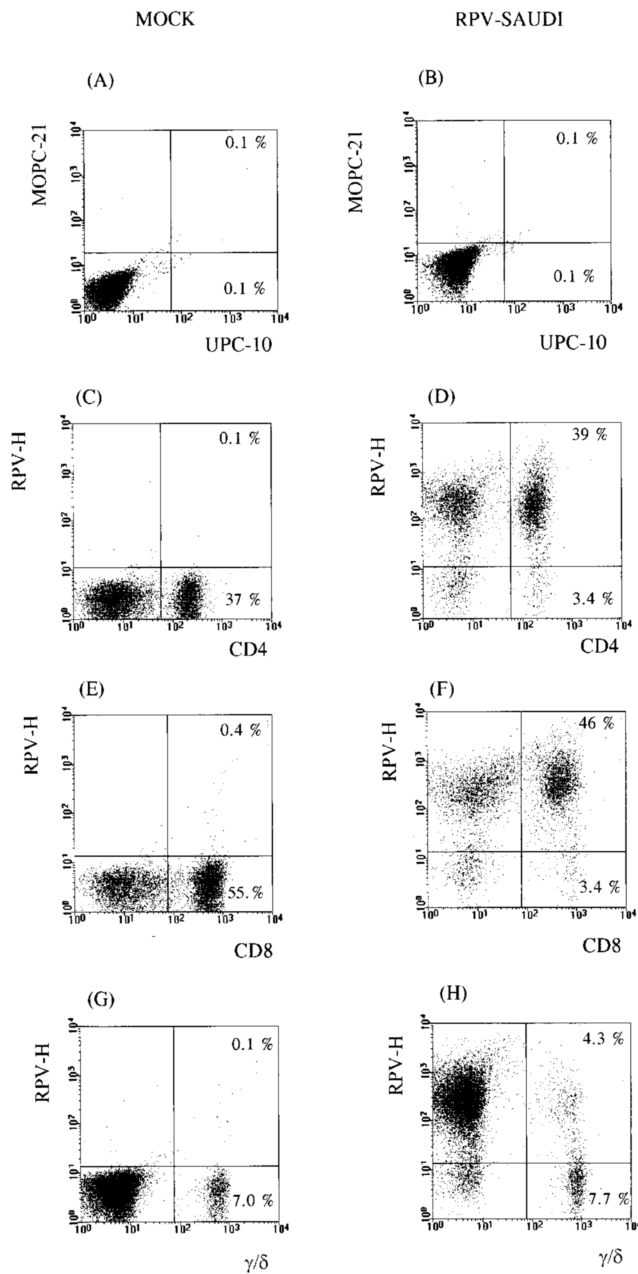

FIG. 4. Phenotypic characterizations of ConA-stimulated nonadherent bovine PBMC expressing RPV H glycoprotein at 3 days after mock infection (A, C, E, and G) or infection with RPV-Saudi at an MOI of 0.1 $TCID_{50}$ per cell (B, D, F, and H). Cells were stimulated with ConA for 3 days before infection. PBMC were stained with MAb C1 (anti-RPV) (C to H, *y* axis) and one of the following MAbs (*x* axis): CC8 (anti-CD4) (C and D), CC63 (anti-CD8) (E and F), and GB21A (anti-γ/δ) (G and H). The results of staining with isotype control MAbs UPC-10 (*x* axis) and MOPC21 (*y* axis) are shown in panels A and B. The percentage in the upper right quadrant of each dot plot is the number of cells double labelled, while the percentage in the lower right quadrant is the number of cells single labelled for the lymphocyte subpopulation marker.

of $CD4^+$ to $CD8^+$ cells remained similar to that in unstimulated cells (Fig. 4C and E; compare with Fig. 3C and E), the ratio of γ/δ to $CD4^+$ plus $CD8^+$ cells fell after ConA stimulation from 46 to 7.6% (Fig. 4G; compare with Fig. 3G). This was due to increases in the sizes of both $CD4^+$ and $CD8^+$ populations, as well as a decrease in the γ/δ population.

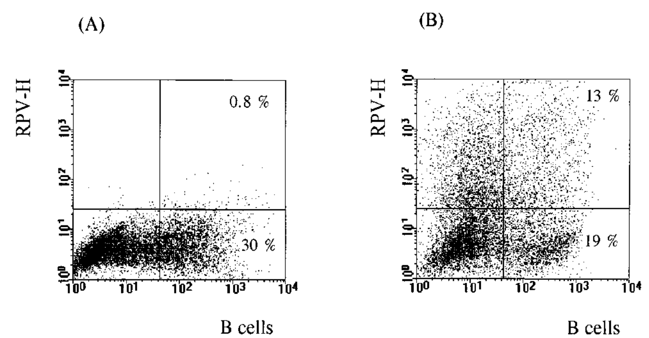

FIG. 5. Phenotypic characterizations of PWM-stimulated nonadherent bovine PBMC expressing RPV H glycoprotein at 3 days after mock infection (A) or infection with RPV-Saudi at an MOI of 0.1 $TCID_{50}$ per cell (B). Cells were stimulated with PWM for 3 days before infection. PBMC were stained with MAb C1 (anti-H glycoprotein) (*y* axis) and MAb GC65A (bovine B-cell marker), as indicated. The percentage in the upper right quadrant of each dot plot is the number of cells double labelled, while the percentage in the lower right quadrant is the number of cells single labelled for the B-lymphocyte subpopulation marker.

Mitogen-stimulated cultures were analyzed at 3 days p.i., as was the case with unstimulated cultures. At this time point, >80% of stimulated nonadherent cells expressed viral H glycoprotein. Double-labelling experiments showed that >90% of both $CD4^+$ (Fig. 4D) and $CD8^+$ (Fig. 4F) T-lymphocyte subpopulations expressed the H glycoprotein. In contrast, less than 40% of γ/δ T cells (Fig. 4H) were seen to support active replication of RPV.

To study the influence of activation on B-cell susceptibility to RPV, PWM was used for mitogenic stimulation. While ConA is a T-cell mitogen, PWM can activate directly both B lymphocytes and at least a subpopulation of T cells. As with ConA-stimulated cultures, the capacity of T cells to support active replication by RPV increased after activation (data not shown). B cells represented around 30% of the nonadherent population in these cultures (Fig. 5A). Almost 50% of these were found to be expressing viral H glycoprotein at 3 days p.i. (Fig. 5B). It should be noted that the identification of B cells was carried out with MAb GC65A. A second MAb, CC51, failed to recognize B cells in these cultures, suggesting that the epitope recognized by this MAb might be lost or altered during activation. The presence of B cells in PWM-stimulated cultures was confirmed by single-labelling staining with MAb BIg73A to identify immunoglobulin-positive cells (data not shown).

IL-2R expression on mitogen-activated lymphocytes. IL-2R expression was used to monitor the activation state of mitogen-stimulated cultures by flow cytometry. When ConA was used, stimulated cells were located as a single population that stained brightly with MAb CACT116A directed against IL-2R (Fig. 6A). This contrasted with nonstimulated cells, which gave a major population of negative cells and a minor population of cells (23% of the total) that were heterogeneous in the quantity of IL-2R expressed; this $IL\text{-}2R^+$ population in nonstimulated cultures was identified as γ/δ T cells and large $CD8^+$ T lymphocytes (data not shown).

Activation by PWM also upregulated IL-2R expression (Fig. 6B). In this instance, two populations of $IL\text{-}2R^+$ cells were noted. Not only did both of these have a higher intensity of IL-2R expression than that on the minor population of $IL\text{-}2R^+$ cells in unstimulated cultures (23% of cells therein), but the expression was higher than that obtained after ConA activation.

Increased capacity of bovine lymphocyte subpopulations to support active replication by RPV after coculture with extra-

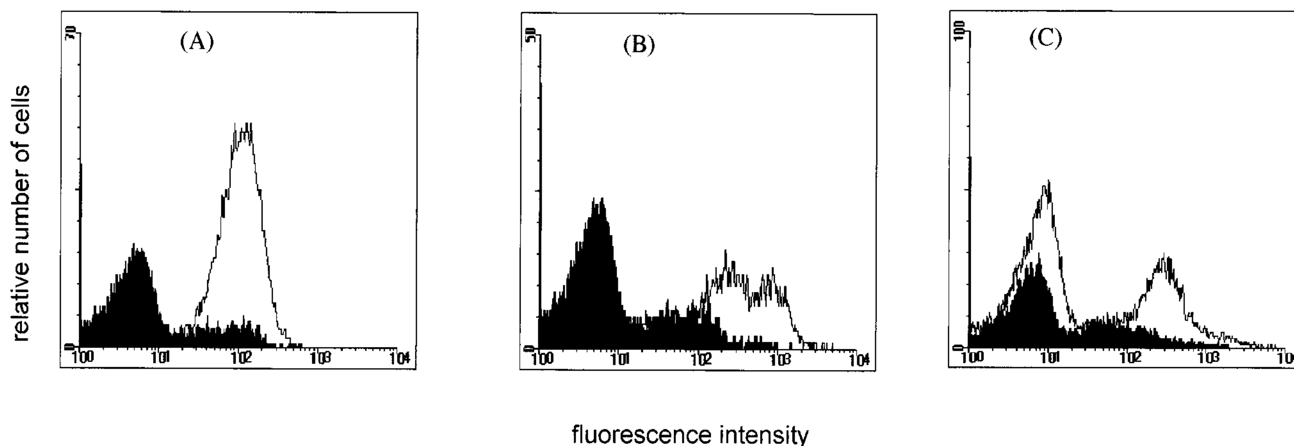

FIG. 6. Flow cytometry analysis of IL-2R expression on nonadherent bovine PBMC. Cultures were unstimulated (solid histograms), stimulated (open histograms) with ConA (A) or PWM (B) for 3 days, or cocultured (open histogram) for 3 days on endothelial cells (C) before being stained with MAb CACT116A (anti-IL-2R).

cellular matrix components or endothelial cells. It was not clear if activation was a prerequisite for lymphocytes to support active replication by RPV or merely reflected the increased viability of cells and thus availability for infection. Consequently, PBMC were cultured on different supports in order to determine how this influenced their susceptibility to RPV. Plates precoated with the extracellular matrix components fibronectin and collagen were used first. Upon culture of mock-infected and RPV-Saudi-infected PBMC under these conditions, no effect on viability or the capacity to support active replication of RPV was found, in comparison with cultures in Teflon vials (data not shown). It was noted, however, that primarily monocytes and macrophages had bound to the matrix components. A second approach was then employed. Freshly isolated PBMC were infected with RPV-Saudi and, after an adsorption period of 2 h at 37°C, washed to remove nonadsorbed virus. These cells were divided into two lots: one was plated in Teflon vials, as shown in Fig. 3, and the other was cocultured on confluent monolayers of blood-derived endothelial cells. Analyses were made at 3 days after infection. Such conditions reflected more accurately the natural environment, in which endothelial cells play an important accessory role in intercellular communications and signalling with lymphocytes. Indeed, both lymphocytes and monocytic cells were seen microscopically to bind to endothelial cells. This coculture also increased the viabilities of both the B-lymphocyte and γ/δ T-cell populations compared with those in Teflon vials (Fig. 7). No significant influence on the viability of $CD4^+$ or $CD8^+$ cells was noted. An increased capacity of lymphocytes to support active replication of RPV, which was observed in all subpopulations, was detected (Fig. 8; compare with Fig. 3). The most significant increases in this capacity were noted in the $CD4^+$ and B-cell populations, of which >25 and >45%, respectively, were positive for viral H glycoprotein. Among the other subpopulations, 15% of $CD8^+$ cells and almost 10% of γ/δ lymphocytes were seen to support active replication of this virus. There was no change in the high capacity of monocytic cells to support active replication of RPV; all monocytes and macrophages in these cocultures of PBMC on endothelial cells were seen to express viral H glycoprotein.

IL-2R expression was also measured in these cultures, as shown in Fig. 6C. Two populations of cells were found among PBMC cocultured on endothelial cells. The IL-$2R^+$ population constituted >40% of cells. This compared with only 23% IL-$2R^+$ cells among PBMC cultured in Teflon vials in the absence of endothelial cells. Furthermore, IL-2R expression was higher on IL-$2R^+$ cells from cocultures, in much the same manner as that seen after mitogen stimulation.

Production of infectious virus in bovine monocytes and monocyte-derived macrophages after RPV-Saudi infection. Monocytes and monocyte-derived macrophages, the most sensitive mononuclear cells to active replication by RPV, were compared in terms of the amount of infectious progeny virus produced therein (Fig. 9). Infectious virus was detected first on day 1 p.i. in the ECV fraction of infected monocytes, but this probably reflected residual input virus. By day 3 p.i., virus was detected in the ECV and CAV fractions of both infected monocytes and macrophages. The amount of virus produced increased over time, and peak titers were obtained by day 10 p.i.

DISCUSSION

A number of viruses have been shown capable of causing immunosuppression by infecting cells of the immune system.

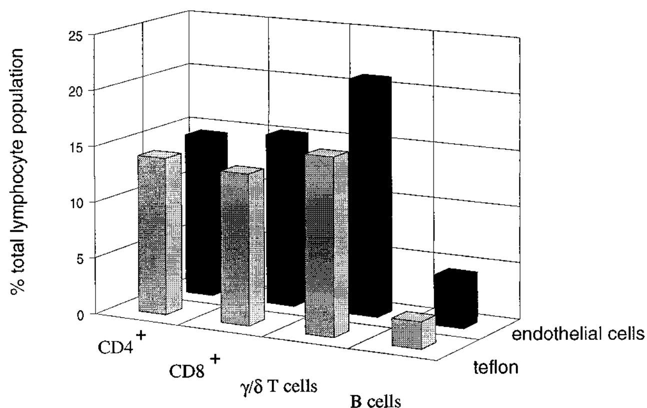

FIG. 7. Lymphocyte subpopulations present under different culturing conditions. PBMC were cultured for 3 days in Teflon vials or in tissue culture flasks containing confluent monolayers of bovine endothelial cells and then stained with MAbs CC8 (anti-CD4), CC63 (anti-CD8), GB21A (anti-γ/δ), and CC51 (B-cell marker). Data are the percentages of different lymphocyte subpopulations present in cultures.

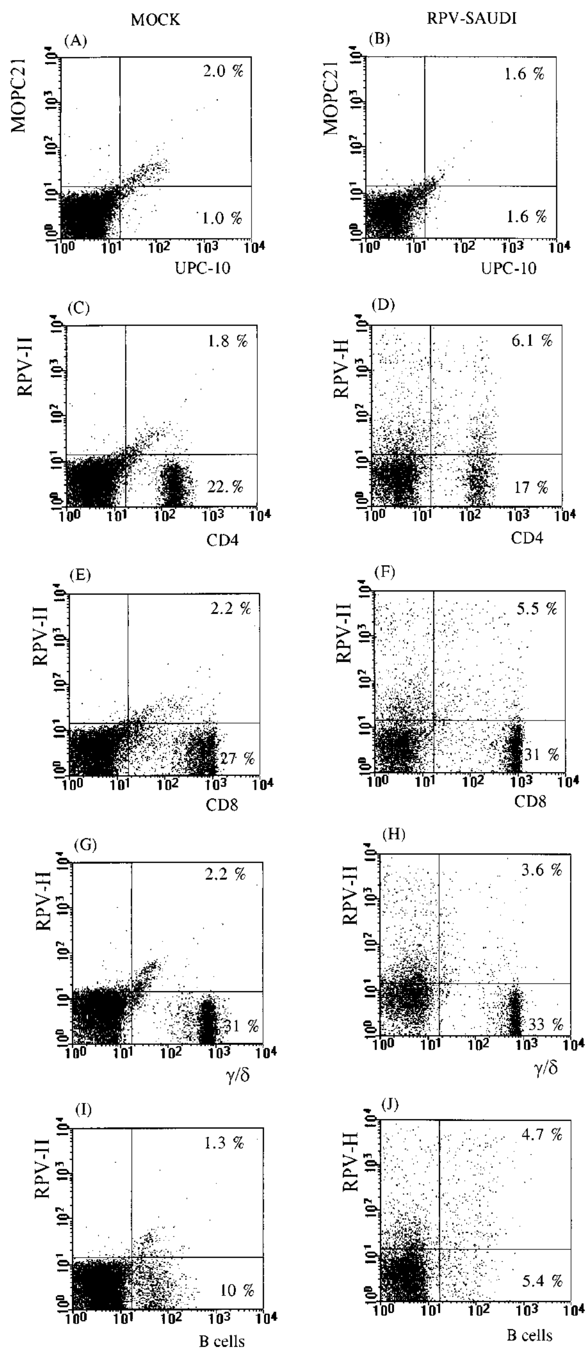

FIG. 8. Phenotypic characterizations of bovine PBMC gated for lymphocytes and double labelled with MAb C1 (anti-RPV) (C to J, *y* axis) and one of the following MAbs (*x* axis): CC8 (anti-CD4) (C and D), CC63 (anti-CD8) (E and F), GB21A (anti-γ/δ) (G and H), and CC51 (B-cell marker) (I and J). PBMC were mock infected (A, C, E, G, and I) or infected with RPV-Saudi at an MOI of 0.1 $TCID_{50}$ per cell (B, D, F, H, and J). After infection, PBMC were cocultured on confluent monolayers of bovine endothelial cells and analyzed on day 3 p.i. The results of staining with isotype control MAbs UPC-10 (*x* axis) and MOPC21 (*y* axis) are shown in panels A and B. The percentage in the upper right quadrant of each dot plot is the number of cells double labelled, while the percentage in the lower right quadrant is the number of cells single labelled for the lymphocyte subpopulation marker.

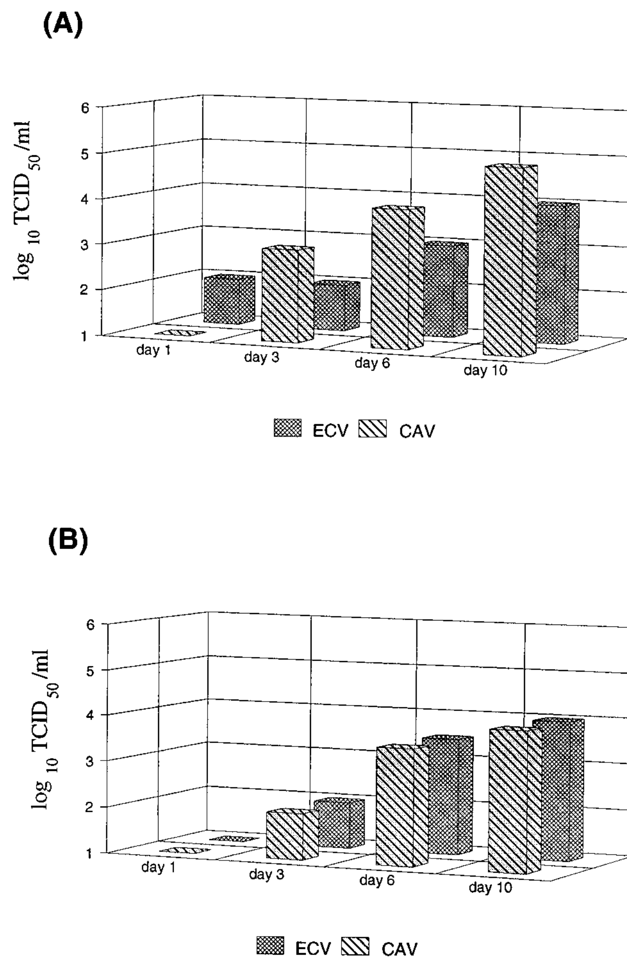

FIG. 9. Titers of RPV in the supernatant (ECV) and cell (CAV) fractions of infected bovine monocytes (A) and monocyte-derived macrophages (B). Cells were infected with RPV-Saudi at an MOI of 0.1 $TCID_{50}$ per cell. Both ECV and CAV were harvested at the indicated times (up to 10 days p.i.) and titrated on monolayers of Vero cells.

Measles virus (MV), a morbillivirus closely related to RPV (25), was the first virus reported to cause immunosuppression (32). Both this and the secondary infections associated with MV infections probably result from the ability of this virus to replicate in leukocyte subpopulations and lymphoid organs. In vitro, MV can replicate in B and T lymphocytes and monocytes (12, 13). Recent evidence, however, has identified monocytes as the primary leukocyte target during natural MV infection (6).

Experiments with cattle (14) and rabbits (33) have shown that RPV can also act as a potent immunosuppressive agent, destroying lymphocytes in the T- and B-cell-dependent areas of lymph nodes. Little is known, however, about the mechanism of this destruction and its contribution to the pathogenesis of this disease. Rossiter and Wardley (22) reported that RPV could grow in cultures of T-cell-enriched bovine PBMC and udder macrophages collected after infusion of the mammary gland with a solution of lipopolysaccharide. Although RPV could actively replicate in the majority of udder leukocytes isolated by adherence, less than 20% of lymphocytes showed the same phenomenon and then only after mitogenic stimulation. Furthermore, this virus grew better in T-cell-enriched cultures, although it was not clear exactly in which

lymphocyte subpopulations this virus actively replicated, compared with T-cell-depleted cultures. Bovine lymphoblastoid cell lines, transformed with the parasite *T. parva*, of either B- or T-cell phenotype could also support replication, in this case, that of vaccine strain RPV-RBOK (21). By using this same vaccine strain, we (20) demonstrated that RPV productively infected blood-adherent cells. These were shown clearly to be monocytes and monocyte-derived macrophages by double-labelling experiments using MAbs against the H glycoprotein of RPV and the CD14 marker characteristically found on monocytic cells.

Consequently, the present work was designed to identify the relative capacities of different leukocyte subpopulations to support active replication by RPV, as well as the influence of the activation state of the target cell on this interaction. It was not infection per se but the ability of this virus to undergo active replication which was investigated because of the influences this could have on the functioning of the immune system. Inapparent infection of quiescent lymphocytes may be possible with MV (23, 31), but the objective of the current investigation was to determine active replication of RPV in bovine leukocyte populations. In order to identify active replication of RPV, flow cytometric analyses were employed. These analyses used double labelling with a MAb against viral H glycoprotein plus a MAb specific for a particular leukocyte subpopulation; this permitted the simultaneous detection of viral antigen expression and the phenotype of the cell in which replication occurred. After infection, <10% (maximum) of the viable PBMC population were seen to express virus antigen. Phenotypic analysis demonstrated that these were mainly monocytes; as previously reported (20), the majority of these $CD14^+$ cells supported active replication of RPV. Among lymphocytes, <2% expressed viral antigen after infection, primarily within the $CD8^+$ T-cell population.

The majority of lymphocytes acquired the capacity to support active replication by RPV only after their activation, as effected through mitogen stimulation. In contrast to a previous report (22) in which 20 to 30% of cells in ConA-stimulated cultures expressed viral antigen after infection, the present results showed that >90% were positive. Phenotypic analysis of these cells showed that activated $CD4^+$ and $CD8^+$ cells were the main lymphocytic targets for this active replication of RPV. This is in contrast to the infection of *T. parva*-transformed lymphoblastoid cell lines; all lines, $CD4^+$, $CD8^+$, and γ/δ T lymphocyte-like and B lymphocyte-like, supported active replication by RPV (21). The present results demonstrate that only a proportion of γ/δ T cells (<40%) supported active viral replication. It is known that sheep γ/δ T cells show poor proliferative responses when stimulated in vitro with T-cell mitogens (8). Although studies of bovine γ/δ T cells are limited, similar results would be expected. This could explain the lower capacity to support active replication, since it appears that this would occur only in lymphocytes after their activation. The infected γ/δ T cells may have been activated indirectly through cytokines produced by other cells in the cultures. Evidence that mouse γ/δ T cells can be activated by IL-1, IL-7, and IL-12 has been reported (27).

B lymphocytes constitute a minor population in unstimulated PBMC cultures. As with T cells, very few resting B lymphocytes showed signs of active replication by RPV after infection. The stimulation of PBMC with ConA results in very low levels of B cells in cultures, so the B-cell mitogen PWM was used to activate B lymphocytes. As with T cells, the activation of B lymphocytes increased their capacity to support active viral replication, but not all activated B cells appeared to be sensitive to this virus. Once again, this is in contrast to the results obtained with lymphoblastoid cell lines (21).

When bovine PBMC are placed in culture without activation or otherwise supplying a costimulatory signal, a proportion of cells die, particularly within the B-lymphocyte population. It is possible that the lower capacity of resting lymphocytes to support active replication of RPV is related to this problem of viability. When signals were provided by the extracellular matrix components fibronectin and collagen, no differences in the capacity of cells to support active viral replication were observed (data not shown), nor was any improvement noted in the viability of nonadherent cells. This is not surprising, since monocytes rather than lymphocytes would more likely be the cell type which could efficiently interact with matrix components and thus receive the appropriate signalling. Microscopic examination revealed that the majority of cells bound to the matrix component were morphologically monocyte or macrophage in nature and were difficult to remove. Since monocytic cells are already capable of supporting active replication by RPV and the objective was to provide signalling to lymphocytes, further analysis employed cocultures of PBMC with blood-derived endothelial cells.

There is increasing evidence that endothelial cells, and the microenvironment to which endothelial cells contribute, play a decisive role in immune responses (26). An important component for the traffic of PBMC is adherence to and subsequent passage through the endothelium. Transendothelial transit occurs under noninflammatory conditions, although it increases during periods of inflammation. Thus, endothelial cells frequently come in contact with circulating leukocytes and may modulate activation events that could influence the course of a virus infection. Recent evidence has shown that the replication of macrophage-tropic and T-cell-tropic strains of human immunodeficiency virus is augmented by macrophage-endothelial cell contact and that this enhancement can be blocked with a MAb directed against the β2-integrin molecule LFA-1 (7). Human immunodeficiency virus itself can modulate LFA-1 expression (9). Evidence that morbilliviruses can also modulate LFA-1 expression has been demonstrated with MV (3). The infection of purified peripheral blood leukocytes or the monocytic cell line U-937 leads to altered expression of LFA-1 and increased cell-cell interaction. This results in efficient dissemination of virus among leukocytes (3, 35).

Coculture of bovine PBMC with endothelial cells did not give the same results for active replication by RPV as those of PBMC on matrix components. Both lymphocytic and monocytic cells were seen microscopically to adhere to endothelial cells; the removal of leukocytes required only gentle pipetting. As for the viability of lymphocytes, there was an increase in the number of viable B cells and γ/δ T cells after contact with endothelial cells, but there was no influence on other T-lymphocyte populations. When the capacity to support active replication of RPV was analyzed, this also was increased in all lymphocyte populations. The increase was most apparent in B-cell and, particularly, $CD4^+$ T-lymphocyte susceptibility to RPV. Consequently, in contrast to contact with matrix components, intercellular contact between lymphocytes and endothelial cells increased the capacity of the former to support active replication by RPV. Whether the mechanism involved is that reported for human immunodeficiency virus and MV, through modulation of LFA-1, remains to be determined. What is clear is that increased lymphocyte viability alone does not explain the susceptibility to RPV; otherwise, the greatest increase in this susceptibility would have been seen with γ/δ T cells and B lymphocytes after adherence to endothelial cells. Active signalling between lymphocytes and endothelial cells is probably

essential. It was noted that contact with endothelial cells upregulated IL-2R expression on 40% of lymphocytes. This might be related to the in vivo observations of Wohlsein et al. (33) that lymphocytes within the B- and T-cell areas of lymphoid organs seem to be particularly permissive to both mild and highly pathogenic isolates of RPV.

In summary, monocytes and macrophages are clearly the main targets for what is seen to be productive infection by RPV. Lymphocytes in the quiescent nonactivated state do not support active viral replication. It is possible that inapparent infection of quiescent lymphocytes may have occurred, as appears to be possible with MV (23, 31), but the objective of the current investigation was to determine active replication of RPV in bovine leukocyte populations. Activation of these cells renders them susceptible to this virus, as does intercellular communication with endothelial cells but not extracellular matrix components. The role of endothelial cells is not simply to maintain lymphocytes in a viable state but also to deliver appropriate intercellular signals which can be manifest by upregulation in IL-2R expression on certain lymphocytes. This mechanism is not identical to mitogen-dependent activation; otherwise, all lymphocytes would have upregulated IL-2R expression and become sensitive to this virus. The demonstration that monocytic cells are capable of supporting active replication by RPV, as are certain lymphocytes which have interacted with endothelial cells, has important implications for virus traffic in vivo and the pathogenesis of this disease. Increased viral replication could enhance the vascular endothelial adhesiveness of infected cells, with the recruitment of increased numbers of monocytes as well as lymphocytes, thus promoting inflammatory responses.

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