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Reduced activation and proliferation of human lymphocytes exposed to respiratory syncytial virus compared to cells exposed to influenza virus

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Abstract

Both respiratory syncytial virus (RSV) and influenza A virus (IAV) may infect human peripheral blood mononuclear leukocytes (PBMC) during the immune response to viral challenge as the cells are recruited to the respiratory tract. The current studies demonstrated differences in PBMC responses to the two viruses very early after exposure, including reduced fos protein and CD69 expression and IL-2 production by RSV-exposed T lymphocytes. Exposure to RSV resulted in reduced lymphocyte proliferation despite evidence of a virus-specific T lymphocyte frequency equivalent to that for influenza virus. Reduced RSV-induced proliferation was not due to apoptosis, which was itself reduced relative to that of influenza virus-exposed T lymphocytes. The data indicate that differential immune responses to RSV and influenza virus are determined early after exposure of human PBMC and support the concept that the anamnestic immune response that might prevent clinically evident reinfection is attenuated very soon after exposure to RSV. Thus, candidate RSV vaccines should be expected to reduce but not prevent clinical illness upon subsequent infection by RSV. Furthermore, effective therapeutic agents for RSV are likely to be needed, especially for high-risk populations, even after vaccine development.

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Author contributions

E.H.F., E.E.O., J.E.N., A.R.S., and N.J.R. conceived and designed the experiments; E.H.F., E.E.O., J.E.N., and A.R.S. performed the experiments; E.H.F., E.E.O., J.E.N., M.K.O., and N.J.R. analyzed the data; and E.H.F., E.E.O., M.K.O., and N.J.R. wrote the paper.

Disclosure

The authors report no conflicts of interest relating to this work.

Keywords

Human Lymphocytes; Lymphocyte activation; Lymphocyte proliferation; influenza virus; respiratory syncytial virus

Introduction

Respiratory syncytial virus (RSV) is the major cause of respiratory illness in infants and young children and, in the USA alone, 100,000 children may require hospitalization for RSV infection in a single year [Salkind and Roberts, Jr. 1992]. Most children with RSV infection are previously healthy and active community-based surveillance for influenza-like illness has demonstrated a considerable burden of RSV-associated illness, including in older infants and children, that would not be identified through hospital-based surveillance [Nolan et al. 2015]. In adults, RSV is a frequent cause of illness that can be clinically indistinguishable from and often mistaken for illness caused by influenza A virus (IAV) [Salkind and Roberts, Jr. 1992; Saxena et al. 2017]. RSV has been associated with over 11,000 deaths yearly in the USA, and older individuals are most likely to die as a result of the infection [Thompson et al. 2003]. Both healthy elderly patients and high-risk adults can be infected, but severe disease in the elderly is related most closely to underlying medical conditions [Walsh et al. 2013].

Symptomatic reinfection with RSV is common, and although repeat infections are associated with less morbidity and mortality than the initial infection [Hall et al. 1991], the pathogenesis of reinfection is incompletely described. Although RSV does exhibit some strain variation, it has not been found to account for reinfection [Sullender et al. 1998; Collins and Melero 2011] that can occur despite pre-existing serologic and cellular evidence of sensitization and immunity to the challenging strain of RSV [Kimman and Westenbrink 1990]. Longitudinal studies of RSV infections in children showed that children can be naturally re-infected with the same strain of virus [Agoti et al. 2012] although subsequent disease severity is usually reduced, especially as the child ages [Ohuma et al. 2012]. A single RSV isolate has been shown to cause repeated experimental symptomatic infections in adult volunteers [Hall et al. 1991], effectively eliminating strain variation as the singular determinant of reinfection. Such evidence suggests that RSV reduces human innate immune responses and/or anamnestic adaptive immune responses acutely but incompletely, allowing clinically evident re-infection [Munir et al. 2011]. In contrast to RSV, IAV induces adequate immune protection against clinical infection with homotypic strains [Editorial 1986].

Coordinated recruitment, clonal expansion and activation of virus-specific effector T lymphocytes are critical to control, and recover from, a respiratory virus infection. Monocytes and monocyte-derived macrophages and lymphocytes migrate to the lungs in response to the respiratory virus challenge [Wyde and Cate 1978] on their mission to ensure defense and recovery, and thus are exposed to the virus. Even circulating leukocytes appear to be infected by RSV in vivo during initial but not subsequent natural infection [Domurat et al. 1985].

Our in vitro studies use human peripheral blood-derived mononuclear cells (PBMC) obtained from adults who would have been naturally and recurrently exposed to RSV and IAV in vivo [Wright Peter F. and Webster 2001;Ogra 2004]. Both RSV and IAV abortively infect both human monocytes/macrophages and lymphocytes [Domurat et al. 1985;Mock et al. 1987;McCarthy et al. 1989]. Past studies showed that exposure of the PBMC to RSV or to IAV results in substantial differences in both innate immune responses, such as interferon production [Chonmaitree et al. 1981;Bell et al. 1983], and adaptive immune responses, such as T lymphocyte expression of late activation markers and cell proliferation measured by classical assays seven days after exposure [Salkind et al. 1991;Salkind et al. 1991]. Notably, RSV-exposed monocytes-macrophages supported mitogen-stimulated lymphocyte responses as well as did control monocytes-macrophages, whereas RSV-exposed lymphocytes did not respond whether supported by control or RSV-exposed monocytes-macrophages [Roberts, Jr. 1982]. Thus, RSV appeared to affect lymphocyte function specifically in proliferative responses. The current studies were undertaken to examine early events associated with PBMC exposure to RSV, compared with cells that were sham-exposed or exposed to IAV, in order to identify alterations in lymphocyte function specifically that might result in attenuated or delayed defense against RSV [Salkind and Roberts, Jr. 1992], possibly contributing to the risk of reinfection.

Materials and methods

Viruses

Previously described standard techniques were used to produce and measure RSV and IAV [Chonmaitree et al. 1981;Salkind et al. 1991]. Influenza A/AA/Marton/43 (H1N1) virus was grown in embryonated hen's eggs and plaque assayed in MDCK cells; RSV A subtype (Long strain, VR-26, ATCC) was grown and plaque assayed in HEp-2 cells [Chonmaitree et al. 1981]. Virus was quickfrozen for storage at -70°C . Freshly isolated whole PBMC were exposed to RSV or IAV at a multiplicity of infection (MOI) of one for one hour in RPMI 1640, washed, and subsequently cultured in RPMI 1640 (Gibco, Invitrogen) with 10% defined FCS (Hyclone) at 37°C . Previous studies determined that similar results would be observed with exposures at other MOI.

Collection of PBMC and exposure to virus

Informed consent for withdrawal of blood was obtained from all donors, and these studies were approved by the Institutional Review Board of the University of Texas Medical Branch.. Donors were healthy and ranged in age from 18–45 years of age with no recent (2-month) illnesses or use of medication or receipt of vaccines. Each experiment for a study used a different donor. All comparisons were performed with autologous cell preparations. Equal numbers of male and female subjects were used as volunteer donors. It was expected that all donors experienced past in vivo exposure to RSV as well as IAV [Wright Peter F. and Webster 2001;Ogra 2004]. PBMC were separated by Ficoll-Hypaque sedimentation and maintained in RPMI 1640 supplemented with 10% FCS and 1% pen/strep [Salkind et al. 1991;Salkind et al. 1991;Chonmaitree et al. 1981]. Cells were counted and viability was determined (a) by the ability to exclude trypan blue under light microscopy or (b) by the use of propidium iodide with analysis by fluorescent microscopy and flow cytometry.

Freshly isolated PBMC were exposed or sham-exposed to RSV or IAV. In studies with analyses conducted later after exposure, the PBMC or purified monocytes-macrophages were washed in warm medium, centrifuged, and reincubated at 37°C in medium supplemented with 10% heatinactivated FCS after one hour.

Flow cytometry analyses of phenotype and activation markers

Acquisition and analysis were performed using a FACSort flow cytometer and CellQuest software (Becton Dickinson). For analyses of lymphocytes, forward versus side light scatter was used for gating. Gating on CD3+ cells was used for specific analysis of T cells. T cell phenotyping was determined using fluorescein isothiocyanate (FITC) or peridinin chlorophyll protein (PerCP) conjugated antibodies specific for CD3 (Becton Dickinson) [Nichols et al. 2001]. Cell surface expression of CD69 was assayed using a monoclonal antibody conjugated to phycoerythrin (PE; Clone L78; Becton Dickinson).

Transcription factor analysis required an additional step for intracellular staining. Fixed cells were permeabilized with 0.1mL of 0.3% n-octyl β -D-glucopyranoside (Sigma Chemical Co.) for 2 minutes at room temperature, washed, and incubated with a 1:100 dilution of a rabbit polyclonal antibody specific for human Fos (Caltag). Secondary staining was done with a 1:500 dilution of a goat anti-rabbit F(ab')₂ antibody conjugated to PE (Tago).

Analyses of lymphocyte proliferation and IL-2 production

Cell division was determined by culturing PBMC with CFSE solution (Molecular Probes) at a concentration of 10 μ M in RPMI 1640 for 10 minutes at 37°C (5 $\times$ 10⁷ cells/mL) [Lyons and Parish 1994; Lyons 2000]. After incubation, cells were then washed with RPMI-1640 with 10% FCS at 4°C and then cultured in RPMI 1640 with 10% FCS at 37°C. Unlabeled cultures were analyzed to determine the contribution of autofluorescence and to set a baseline for CFSE-negative cells. PBMC supernatant fluids were frozen at -70°C until tested for soluble IL-2 levels using the human Th1/Th2 Cytokine Cytometric Bead Analysis (CBA) kit (BD Pharmingen), as described by the manufacturer.

Enumeration of virus specific T cells

PBMC were resuspended and washed twice in PBS to remove virus-specific antibodies present in the human serum. Subsequently, PBMC were resuspended in RPMI supplemented with streptomycin (100 U/ml), penicillin (100 μ g/ml), and glutamine (2 mM). They were then shamexposed or exposed to uv-inactivated RSV or IAV at 37°C for 1 hour at an MOI of 3. After 1h incubation at 37°C, the PBMC were spun down and re-suspended in RPMI supplemented with 10% fetal calf serum (FCS) at 37°C. Subsequently, Golgistop (Pharmingen) was added and left for a subsequent 5 h, at 37°C, as described previously [Boon et al. 2002]. The cells were then washed and stained with PerCP-conjugated anti-IgG, anti-CD3, anti-CD4 or anti-CD8 (BD Biosciences) in PBS with 2% FCS for 30 min at 4°C. Following two washing steps, the cells were fixed with 250 μ l of Cytotfix buffer (BD Biosciences) for 45 min at 4°C. Next, the cells were washed twice in permeabilization buffer and subsequently incubated for 30 min at 4°C in 100 μ l of permeabilization buffer containing anti-human IFN- γ -FITC or FITC-IgG (BD Biosciences). After two washing steps with permeabilization buffer, the cells were fixed with Cytotfix, and the number of cytokine-

positive cells was determined using flow cytometry (FACS scan, Becton–Dickinson). The percentage of cytokine-positive cells within the CD3+, CD4+ or CD8+ T cell fraction was calculated for each exposure to virus.

Analyses of cell apoptosis

Apoptosis was determined by quantitation of DNA strand breaks using the TUNEL Assay (In Situ Cell Death Kit, Boehringer Mannheim) [Nichols et al. 2001]. Cell phenotype was established by cell surface labeling with monoclonal antibodies prior to the TUNEL reaction. After staining, cells were fixed in 2% PAF and permeabilized for 10 minutes using 0.6% n-octyl glucopyranoside (Sigma Chemical Co.). The TUNEL reaction was carried out as described in the manufacturer's instructions.

Statistical analyses

Results are expressed as mean $\pm$ SD for the stated number of experiments. The statistical significance of differences in the means was calculated using the Student's t test performed by EXCEL software (Microsoft, Inc.), comparing RSV-exposed cells to IAV-exposed cells or RSV-exposed cells to sham-exposed cells. Mean differences in the values were considered significant when p was less than 0.05.

Results

Virus-induced T lymphocyte activation

In lymphocytes, Fos is an important immediate-early transcription factor involved in the regulation of expression of many effector molecules involved in lymphocyte proliferation and effector function. Expression of Fos was examined using flow cytometry analysis (Fig. 1). IAV-exposed CD3+ T cells demonstrated an increase in Fos expression as soon as 30 minutes after exposure. In contrast, RSV-exposed CD3+ T cells demonstrated no increase in expression of Fos at any time of measurement.

At 4 hours after exposure, both IAV and RSV led to a slight but not significant increase in CD3+ T cells expressing CD69 compared to sham-exposed cells (Fig 2), possibly indicating early activation of the very small percent of lymphocytes that were specific for each virus in addition to the cells that were nonspecifically activated by the experimental procedures. However, by 24 hours there was a dramatic increase in the percentage of cells expressing CD69 in the IAV-exposed T cells compared with sham- ($P = 0.003$) and RSV-exposed ($P = 0.002$) cells. In contrast, at 24 hours the percentage of sham-exposed and RSV-exposed T cells expressing CD69 were reduced and equivalent ($P = 0.341$).

Virus-exposed T lymphocyte proliferation and IL-2 production

To analyze proliferation in T cells exposed to IAV and RSV, we used CFSE which is serially diluted with cell division, allowing determination of both the extent and the kinetics of cellular proliferation [Lyons 2000]. PBMC were sham-exposed or exposed to the viruses and CD3+ T cells were subsequently analyzed for CFSE content on days 1, 3, and 7 after exposure (Fig 3). On day 1 and 3 after exposure, there were no major differences in the number of proliferating cells between the different PBMC. By day 7, there was a higher

percent of T cells proliferating in response to IAV than observed in the RSV-exposed cells (38.24 percent versus 15.87, respectfully). Furthermore, the T cells proliferating in response to RSV were dividing at a slower rate than the IAV-exposed cells as illustrated by the lower percentage of cells that exhibited sequentially reduced CFSE content (Table 1).

The amount of IL-2 produced by T cells can influence their proliferative responses. Soluble IL-2 was detectable by cytometric bead analysis of supernatant fluids that were collected from RSV-exposed PBMC after 24 h (Figure 4) but the amount was reduced in comparison to IAV-exposed PBMC supernatant fluids.

Virus-induced T lymphocyte apoptosis

Analyses of apoptosis showed that, whereas exposure to infectious influenza virus induced apoptosis as expected [Nichols et al. 2001], exposure of the PBMC to RSV induced far less CD3+ T cell apoptosis than did IAV (Fig. 5). Thus, reduced lymphocyte proliferation in response to RSV was not due to increased apoptosis relative to IAV. Exposure to inactivated IAV or RSV in the same experiments did not induce apoptosis relative to sham-exposed PBMC in the 48 hours after exposure (1.75 ± 0.54 , 1.09 ± 0.37 , and 1.29 ± 0.31 , mean percent $\pm$ SD, respectively).

Enumeration of RSV- and IAV-specific T lymphocytes

In order to assess virus-specific T cells present at the time of exposure to RSV and IAV, we measured the relative percentages of virus-specific T lymphocytes in PBMC of five healthy donors sham-exposed or exposed to uv-inactivated RSV or IAV, and compared the production of IFN- γ by the lymphocytes as early as 6 hours after exposure.

There were similar percentages of lymphocytes producing IFN- γ early (up to 6 h) after exposure to uv-inactivated IAV and RSV. This was true for IAV- and RSV-exposed total CD3+ cells (0.24 ± 0.11 and 0.26 ± 0.09 , mean percent $\pm$ SD, respectively) as well as CD4+ (0.28 ± 0.11 and 0.27 ± 0.09 , respectively) and CD8+ (0.30 ± 0.12 and 0.32 ± 0.17 , respectively) subpopulations. These results suggest that there are similar percentages of lymphocytes specific for RSV and IAV in the PBMC.

Discussion

The data from the current studies support the concept that recurrent RSV infection may be related at least in part to early RSV effects on lymphocyte function [Salkind and Roberts, Jr. 1992] rather than due only to inadequate generation or persistence of immunity after previous infections as suggested by some animal models [Chang and Braciale 2002]. Virus-induced early responses of human lymphocytes measured after exposure to RSV were consistently reduced or delayed compared with determinations of cell function in response to exposure to IAV. Early effects on an anamnestic immune response such as observed in these studies, especially the likely reduced clonal expansion of RSV-specific lymphocytes, might allow RSV reinfection to progress to clinically evident illness.

Alterations in the magnitude and kinetics of immune responsiveness to RSV could contribute to the limited adaptive immunity to the virus that has been reported in both human

and experimental mouse models of RSV infection [Bont, V 2002] and explain at least in part clinical RSV reinfection [Chang and Braciale 2002]. Both antibody and CD8+ T cell responses are established during a primary response to RSV but ex vivo analyses of PBMC from infected children suggest that responses to the virus may not be boosted by episodes of reinfection [Bont, V 2002]. RSV-specific memory T lymphocytes present in mice as a result of primary virus infection or peptide vaccination were present in the lung at the time of RSV reinfection but responding at low frequency [Chang and Braciale 2002]. Thus, although RSV induces fairly strong chemokine production responses by respiratory epithelial cells [Zhang et al. 2001], the recruited leukocytes may not be expected to abruptly interrupt the progression to clinically evident illness.

Two proto-oncogenes that are particularly influential in the regulation of T cell responses are *c-fos* and *c-jun*. Shortly after activation, increased levels of their respective proteins, Fos and Jun, are detected and combine to form activator protein-1 (AP-1), which regulates the transcription of genes that control cytokine production, proliferation, and other T cell responses [Crabtree 1989]. Reduced IL-2 synthesis was in fact noted in our studies after exposure of PBMC to RSV, and our earlier studies showed that lymphocyte expression of the IL-2 receptor was induced by exposure of PBMC to IAV but not by exposure to RSV [Salkind et al. 1991]. Fos and Jun transcription factors also regulate the expression of CD69 [Castellanos et al. 1997], an indicator of T lymphocytes that have been activated through the T cell receptor [Testi et al. 1989]. Few T lymphocytes exposed to RSV expressed CD69 on their cell surface in comparison with those exposed to IAV.

Earlier studies using tritiated thymidine incorporation assays or cell cycle analyses suggested that there was minimal proliferation of virus-specific lymphocytes after exposure of PBMC to RSV [Salkind et al. 1991]. The current studies used a CFSE proliferation assay that allowed both cumulative and kinetic measurements, and suggested that RSV-exposed human PBMC do respond to the virus with a decreased, and possibly delayed, but not absent T lymphocyte proliferative response. There was a degree of asymmetry in CFSE distribution with lymphocyte division, with recruitment of cells to the proliferative response, and the asymmetry and time lags of lymphocyte division as expected [Bocharov et al. 2013]. More T lymphocytes had completed more cell divisions by day seven in the IAV-exposed PBMC than in the PBMC exposed to RSV. Rather than being completely unresponsive to RSV, the T lymphocytes were proliferating, but at a decreased pace. It is quite clear, however, that a small percentage change in cell cycle rate in a population of cells that should be expanding exponentially can produce outcome differences of more than two orders of magnitude within a week's time, given the rate at which lymphocytes multiply [Gett and Hodgkin 2000].

The current studies did not establish the antigen-specific nature of the cells, and the responding activated T lymphocytes could include both virus-specific and bystander cells [Tripp et al. 1995] although we have shown that the small percentage of lymphocytes responding early after exposure are virus-specific [Domurat et al. 1989]. The lymphoproliferative response to viral challenge involves a rapid expansion of cells. Murine studies have shown a dramatically rapid and large increase in the number of restimulatable (memory) precursor CTL by 2–4 days post-immunization with influenza virus [Owen et al. 1988]. While the virus-specific response proceeds, bystander T cell activation in response to

viral challenge, such as exhibited by expression of CD69, has been well documented. Such activation is TCR-independent and also occurs via cytokine stimulation in infections such as LCMV, HSV, HIV and SIV, dengue, and others [Freeman et al. 2012] [Lin et al. 2000], potentially leading to apoptosis of the cells. Human monocytes-macrophages produce an extensive pro-inflammatory and anti-inflammatory repertoire of cytokines as well as cytokine inhibitors [Arango and Descoteaux 2014;Nichols et al. 1993] upon virus challenge. For example, they secrete both IL-1 and IL-1-inhibitors in response to both influenza virus and RSV, with the net biological response to influenza being pro-inflammatory and the net response to RSV being anti-inflammatory [Roberts, Jr. et al. 1986]. Our earlier studies [Domurat et al. 1989] combined with the current data and the investigations by others that are cited above together suggest that the earliest activation/proliferation response to influenza virus is exhibited by the small percent of virus-specific T cells, followed by the recruitment of bystander cells in response to the cytokines produced by monocytes-macrophages, evolving into a primarily virus-specific proliferative and effector response. Such a complex response overall is reduced or delayed after exposure to RSV.

Fewer proliferating T lymphocytes were observed in the RSV-exposed PBMC than in the IAV population in spite of similar percentages of virus-specific precursor lymphocytes. Our earlier studies also showed RSV-specific lymphocytes in PBMC from adults, with robust proliferation in response to inactivated RSV but markedly reduced proliferation after exposure to infectious RSV [Salkind et al. 1991]. In the current studies, the reduced proliferation of the RSV-exposed PBMC was shown not to be due to virus-induced apoptosis, which was itself reduced compared to that of the IAV-exposed cells. Prior studies showed that the percentage of cells that are IAV-infected is less than the percentage of apoptotic cells, suggesting that direct effects of cell infection by the virus cannot account fully for a high level of cell death [Nichols et al. 2001]. Furthermore, for the IAV-exposed cells, apoptosis was unlikely to contribute to diminished cellular CFSE since apoptotic lymphocytes would be engulfed by monocytes-macrophages [Fujimoto et al. 2000], and not analyzed by the flow cytometric gates set for lymphocytes.

IAV and RSV infect similar percentages of human monocytes-macrophages and lymphocytes [Domurat et al. 1985]. RSV infection of adult PBMC appears to be recognized as vigorously as IAV as measured by early biophysical changes in the small populations of lymphocytes that were in turn shown to be virus-specific by deletion and reconstitution experiments [Domurat et al. 1989], although the IAV-exposed cells proliferated and the RSV-exposed cells showed cell cycle arrest [Salkind et al. 1991]. Our experiments that evaluated the frequency of virus-specific T cells used exposure to uvinactivated IAV and RSV for enumeration of virus-specific T cells. This method preserved antigenic sites while eliminating effects due to viral replication. The data showed that there were similar percentages of lymphocytes producing IFN- γ early after exposure to RSV and IAV. Our results regarding percentages of IAV- and RSV-specific lymphocytes in adult peripheral blood are similar to those reported by others [He et al. 2003;Lee et al. 2005]. In spite of a similar number of precursor cells, there was a decreased response in the RSV-exposed population compared to IAV-exposed cells over time that correlated with the findings of a decreased IL2 production after infection, and decreased IL-2 production has been associated with a decreased proliferation of lymphocytes [Sia and Chou 1988] as seen in our studies.

A limitation of our studies is that they were not designed to elucidate the molecular mechanism responsible for attenuated or delayed immune responses to RSV. They were designed to establish the existence of such effects of the virus and provide a rationale and incentive for such future studies. Although the current and earlier cited studies provide evidence supporting the potential for RSV infection to acutely and adversely affect human innate immune responses and/or anamnestic adaptive immune responses, such a concept does not in any way diminish the need for a vaccine for RSV. It is clear that the host does have anamnestic immune responses that render the second and subsequent infections with RSV less severe than the initial infection. The existence and participation of a protective recall response is supported by the current data, showing clear although diminished proliferative responses, and induced but low IL-2 production compared to IAV, as well as our earlier studies showing low-level but clearly induced IFN- γ production [Salkind et al. 1991]. Thus, an RSV vaccine candidate should only be expected to render the otherwise severe first natural infection similar in severity to reinfection. The vaccine should not be expected to prevent clinical illness from infection or reinfection, a common criterion for vaccine efficacy. The cellular and humoral defenses that are induced by the vaccine are likely to be offset at least partially by the ability of the naturally encountered infectious virus to counteract innate and adaptive immune responses.

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Abbreviations used

IAV	influenza A virus
MOI	multiplicity of infection
RSV	respiratory syncytial virus

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Figure 1.

Fos expression by sham-, IAV-, and RSV-exposed CD3⁺ T lymphocytes. MNL were sham-exposed or exposed to IAV or RSV and collected up to 24 h after exposure.

Immunofluorescent staining and flow cytometric analysis were then used to detect T cells producing the transcriptional regulating protein Fos. Mean results $\pm$ SD are shown for eight experiments using cells from different donors. Data from 10,000 gated cells were collected for each assay. Fos product expression by IAV-exposed cells was increased 0.5 ($P = 0.04$) and 24 h ($P = 0.03$) after exposure, whereas the RSV- and sham-exposed cells did not differ ($P = 0.42$ to 0.99) in response at any time point.

Figure 2.

CD69 expression by sham-, IAV-, and RSV-exposed CD3+ T lymphocytes. Cells were collected 4 or 24 h after sham exposure or exposure to virus. Mean percents of cells expressing CD69 $\pm$ SD are shown for four experiments using cells from different donors. Data from 10,000 gated cells were collected for each cell preparation. The response of IAV-exposed cells 24 h after exposure was significantly greater than that of RSV-exposed ($P = 0.002$) or sham-exposed ($P = 0.003$) cells, and the RSV- and sham-exposed cells did not differ ($P = 0.341$) in response.

Figure 3.

CFSE proliferation assay of Sham-, IAV-, and RSV-exposed CD3⁺ T lymphocytes. Human PBMC were loaded with a maximum of 10mM of CFSE and then exposed to virus at an MOI of one for one hour. (A) CFSE fluorescence was analyzed by flow cytometry at days 1, 3 and 7 after virus-exposure (5, 4, and 7 experiments, respectively, using cells from different donors) and is shown as percent CD3⁺ cells that have divided (i.e., that demonstrate reduction in CFSE fluorescence). For each sample, data from 10,000 cells were collected. Mean results $\pm$ SD are shown for each graph. The IAV-exposed CD3⁺T cells demonstrated significant increased proliferation by day 7 compared to sham- ($p = 0.004$) and RSV- ($p = 0.0025$) exposed cells. (B) Representative histograms of a day-7 assay demonstrating an analysis of divided (“D”) and undivided (“U”) CD3⁺ T lymphocytes.

Figure 4.

IL-2 concentrations in supernatant fluids of IAV- and RSV-exposed PBMC. PBMC were sham-exposed, exposed to IAV or RSV at an MOI of one for one hour. Supernatants were harvested at 24 hours and IL-2 levels were determined for each group by cytometric bead array analysis. For each assay, 2×10^6 cells were used. Mean results $\pm$ SD are shown for four experiments using cells from different donors.

Figure 5.

Apoptosis-related cell death of sham-exposed and virus-exposed T lymphocytes. Results from nine experiments using cells from different donors are presented as mean percentage of apoptotic (TUNEL+) cells $\pm$ SD. Data from 10,000 gated cells were collected for each sample. Differences between IAV-exposed and RSV-exposed cells were significant ($P < 0.0002$) at 4, 8, 12, 24 and 48 hours.

TABLE 1
Extent of CD3+ lymphocyte division after sham exposure or exposure to IAV or RSV*

Exposure	Quadrants of Divided Cells				Undivided Cells
Sham	0.44	1.07	2.39	4.55	91.58
IAV	3.57	6.96	13.84	10.91	61.76
RSV	0.57	2.16	6.52	6.18	84.13

* Distribution of CD3⁺ cells among the undivided population and four equal quadrants of the divided regions of CFSE content, from most extensively divided to least divided (left to right) as illustrated in Fig. 3B. Results are shown as mean percent of total CD3⁺ cells based on seven experiments using cells from different donors with measurements at 7 days after exposure. For each sample, data from 10,000 cells were collected.

IAV, influenza A virus; RSV, respiratory syncytial virus.