Dose-Dependent Changes in Influenza Virus-Infected Dendritic Cells Result in Increased Allogeneic T-Cell Proliferation at Low, but Not High, Doses of Virus

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During the acute phase of infection with influenza A virus, the degree of lymphopenia correlates with severity of disease. Factors that contribute to T-cell activation during influenza virus infection may contribute to this observation. Since the immune response is initiated when dendritic cells (DC) interact with T cells, we have established an in vitro system to examine the effects of influenza virus infection on DC function. Our results show that allogeneic T-cell proliferation was dependent on the dose of A/PR/8/34 used to infect DC, with enhanced responses at low, but not high, multiplicity of infection. The lack of enhancement at high virus doses was not primarily due to the increased rate of DC apoptosis, but required viral replication and neuraminidase (NA) activity. Clusters that formed between DC or between DC and T cells were also dependent on the viral dose. This change in cellular interaction may oppose T-cell proliferation in response to DC infected with high doses of PR8, since the increased contact between DC resulted in the exclusion of T cells. The enhanced alloreactive T-cell response was restored by neutralization of transforming growth factor β1 (TGF-β1). It is likely that NA present on viral particles released from DC infected with high doses of PR8 activates TGF-β1. Future studies will determine the mechanism by which TGF-β1 modifies the in vitro T-cell response and address the contribution of this cytokine to the lymphopenia observed in severe disease.

Influenza A virus infection results in a spectrum of clinical responses ranging from asymptomatic infection to a primary viral pneumonia that rapidly progresses to a fatal outcome. During acute illness (14) or induced infection (6), lymphopenia is evident as reduced numbers of B and T cells. This may reflect migration of lymphocytes to the site of infection, and it would therefore be reasonable to expect that lymphopenia would correlate with recovery from infection. However, in the recent influenza A virus H5N1 outbreak, low leukocyte counts correlated with severity of disease (29). In addition, the T cells present during acute infection are functionally impaired, with reduced lectin-induced stimulation (6, 14), suggesting that these quantitative and qualitative changes may not simply be due to migration of cells.

A number of factors probably contribute to these observations. For example, virus load, as well as viral components that confer pathogenicity, may influence the milieu of cytokines and the composition of responding cells. These factors may act on both naïve and effector B and T cells to result in lymphopenia. The cell type that may mediate this lack of response is the dendritic cell (DC), since it transports virus to the draining lymph node (9) and has direct contact with T cells. The interactions between DC and naïve and memory T cells determine both the magnitude and quality of the immune response. Our previous results showed that influenza virus alters this interaction in vitro (18). In this in vitro system, we examined the effects of influenza virus infection on DC function. DC were cultured from H-2d bone marrow and then used to stimulate H-2d allogeneic T cells. Since this response is not virus specific, the ramifications of influenza virus infection were determined by comparing T-cell proliferation stimulated by uninfected and virus-infected DC.

When DC were infected with a low dose of A/PR/8/34 (PR8), there was increased T-cell proliferation in response to influenza virus-infected DC (18). This altered response was dependent on viral neuraminidase (NA) and did not require infection of the DC with influenza virus. One or more mechanisms may mediate this effect when sialic acid is removed from glycoconjugates at the DC surface. This may include changes that facilitate interactions between the major histocompatibility complex (MHC) class I-peptide complex with the T-cell receptor, B7-1 with CD28, and adhesins with their ligands or changes in charge at the cell’s surface that result in a general increase in contact. However, our current results show that this enhanced proliferative response is not observed when DC are infected with high doses of PR8.

There may be multiple reasons for the lack of an enhanced response at high PR8 multiplicity of infection (MOI). For example, since influenza virus induces apoptosis of infected cells (10), greater numbers of virus particles may induce greater DC apoptosis, thereby reducing the number of effective stimulators in the culture. Alternatively, at high doses of virus, virions released from the DC may interact with T cells, resulting in their reduced proliferation. Viral NA could contribute to this reduced response by desialylation of T-cell surface glycoproteins. This would result in DC and T cells having equal charges, so that opposite attractive charges would no longer facilitate the interaction between them. Other reasons for the dose-dependent proliferative response may be that properties of DC that contribute to successful T-cell activation are altered at high virus doses, or that, under these conditions, cytokines that inhibit proliferation are secreted. We demonstrate in this report that at a high MOI, a number of changes occur in DC. The most notable physical change that provides a reasonable mechanism to explain the reduced response to DC infected...
with high doses of PR8 is the formation of DC clusters that exclude T cells. However, neutralization of transforming growth factor β1 (TGF-β1) restored the enhanced allogeneic T-cell response, suggesting that this cytokine plays a primary role in reducing proliferation.

**MATERIALS AND METHODS**

**Mice.** Five- to 6-week-old female C57BL/6 (H-2b) and BALB/c (H-2d) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and housed at Johns Hopkins University. They were used before age 12 weeks of age.

**Virus preparation, inactivation, and infection.** PR8 virus was cultured in 10-day-old embryonated chicken eggs. The infected allantoic fluid was harvested, and aliquots were stored at -80°C. An NA-deficient NWS-Mvi virus was a kind gift from Gillian Guerard (University of Oklahoma Health Sciences Center). A stock was cultured in MDCK cells in the presence of both trypsin (5 μg/ml; Quality Biologicals, Gaithersburg, Md.) and Vibrio cholerae (1 μg/ml; Boehringer-Mannheim, Mannheim, Germany) (15). Virus was inactivated by UV irradiation. The NA activity of live and UV-inactivated viruses was similar.

Virus titers were determined by infection of MDCK cells. Ten-fold dilutions of virus were made in serum-free Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Gaithersburg, Md.). MDCK cell monolayers in a 96-well plate were washed twice with serum-free DMEM, after which 100 μl of each virus dilution was added to the wells. After 1 h incubation at 37°C, the plates were rinsed with DMEM supplemented with 3% bovine serum albumin (BSA) and 0.5 μg of trypsin per ml was added to the culture plates. For NWS-Mvi, the final culture medium was also containing 1 mg of L-chloro-1,2-dihydroxybenzene (Boehringer-Mannheim) per ml. After 3 h of incubation at 37°C, 25 μl of the supernatant from each well was transferred to a round-bottom 96-well plate. Phosphate-buffered saline (PBS), 25 μl, and 0.5% chicken erythrocytes (50 μl) were added, and hemagglutination was observed after 30 min at room temperature (RT). The inverse of the dilution at which 50% of the wells showed hemagglutination was recorded as the 50% tissue culture infectious dose (TCID50). The titer of each of the virus stocks (PR8 and NWS-Mvi) was 10^8 TCID50/ml. Heat-inactivated and UV-inactivated PR8 did not contain any infectious virus particles.

To infect DC, different quantities of virus were added to tubes containing 10^6 cells in 2 ml of PBS to give MOI that ranged from 1.25 to 50 infectious virus particles/cell. After 1 h of incubation at 37°C, 10 ml of RPMI 1640 (Life Technologies, Gaithersburg, Md.) containing 10% fetal calf serum (FCS; Biosciences, Rockville, Md.), 2 mM glutamine, and antibiotics (Quality Biologicals, Gaithersburg, Md.) (complete medium) was added, and the cells were incubated for 3 h at 37°C. Uninfected DC were treated in the same way, except that virus was not added.

**Viral NA activity was measured by using a fluorescent substrate (8, 13). Briefly, serial dilutions of NA in 0.1 M sodium phosphate buffer (pH 5.9) were mixed with an equal volume (50 μl of 0.2 M 2-(4-methylumbelliferyl)-β-D-N-acetylneuraminic acid (MU-NA) and 50 μl of 0.2 M 2-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MU-NA) for hemagglutinin (HA) and neuraminidase [NA] activity, respectively). The reaction was stopped by addition of 150 μl of 0.1 M glycine buffer (pH 10.7) containing 25% ethanol. The fluorescence of released MU was determined on a Wallac fluorometer (excitation wavelength, 335 nm; emission wavelength, 460 nm). The amount of NA activity defined as the NA-associated loss of MU, that hydrolyzes 1 mM 2-acetyl-neuraminosyl-D-lactose within 1 min at 37°C, was used as a standard. All chemicals were purchased from Sigma (St. Louis, Mo.). Purified influenza virus NA (NS8) was a kind gift from Graeme Laver (John Curtin School of Medical Research). To NA treat cells, DC and T cells (10^6 cells/ml) were incubated with 2 μl of purified NA for 2 h at 37°C in PBS.

**DC.** Femurs and tibias from C57BL/6 mice were removed, washed with PBS, and transferred into a dish containing serum-free RPMI 1640 (Life Technologies). Both ends of each bone were removed, and the marrow was flushed out with 2 ml of serum-free RPMI 1640 in a syringe with a 25-gauge needle. Erythrocytes (RBC) were lysed with 0.5% NH4Cl, and the remaining cell suspension was washed with complete medium. Cells were finally resuspended at 5 × 10^6 to 10^7 cells/ml in complete medium containing 500 U of granulocyte-macrophage-colony-stimulating factor (GM-CSF; Pharmingen, San Diego, Calif.) per ml and cultured in six-well plates. On days 2 and 4, 75% of the medium was replaced with fresh medium containing 500 U of GM-CSF per ml. DC were infected with 2.5 or 25 MOI of PR8, or left untreated, for 1 h at 37°C. After incubation, or antibodies to neutralize IL-2 (clone JES6-1A12), IL-4 (clone 11B1), IL-10 (clone JES5-2A5), TGF-β1 (AT-72-1), and IFN-γ (clone R4-6A2) were added to wells at a final concentration to control for the specificity of these antibodies. All cytokines and antibodies were purchased from Pharmingen.

**Quantitation of IL-10 and TGF-β1 in DC cultures.** B and T cells were removed from bone marrow cultures with antibodies to B220, CD4, and CD8, followed by addition of anti-mouse Ig-coated magnetic beads (Dynal). The remaining DC were treated in the same way, except that virus was inactivated by UV irradiation. All cytokines were measured by ELISA plates as well as in acidified supernatants. Supernatants (10 μl) were added to wells that had been coated with specific antibody and then blocked. After overnight incubation at 4°C, cytokine-specific biotinylated antibodies were added to washed plates and incubated for 1 h. After being washed, the plates were incubated with 100 μl of 0.5 μg of phosphate-labeled streptavidin (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) for 30 min at RT. The plates were washed again, and 150 μl of Streptavidin-35 (Sigma) was added to washed plates, and the A_{405} was added after 1 h was measured on a Kinetic Microplate Reader (Molecular Devices, Palo Alto, Calif.). The amount of each cytokine was calculated from a standard curve generated from the titration of purified recombinant cytokines. To determine whether TGF-β1 is associated with DC, the amount of TGF-β1 in acidified medium was deducted from the amount of TGF-β1 in acidified DC culture supernatants.

**Immunostaining of infected cells.** To determine the degree of infection, cells were fixed for 15 min with 2% paraformaldehyde for NGAL mRNA expression. 3% paraformaldehyde (pH 7.4) was used as a standard. All chemicals were purchased from Sigma (St. Louis, Mo.). Purified influenza virus NA (NS8) was a kind gift from Graeme Laver (John Curtin School of Medical Research). To NA treat cells, DC and T cells (10^6 cells/ml) were incubated with 2 μl of purified NA for 2 h at 37°C in PBS. After fixation and permeabilization (BD Pharmingen, San Diego, Calif.), washed cells were stained with antibodies specific for hemagglutinin (HA) subtype H1 and neuraminidase [NA] subtype N1. After 4 h of incubation, single-cell suspensions were counted, and 10^6 to 10^7 cells were incubated with 10% normal mouse serum at 4°C for at least 20 min. After washing, 100 μl of a 1/100 dilution of goat polyclonal antimouse IgG (H+L) conjugated to FITC (Jackson ImmunoResearch) or 1/100 dilution of goat antimouse IgG (H+L) conjugated to PE (Jackson ImmunoResearch) was added to washes, and the A_{485} was added after 1 h was measured on a Kinetic Microplate Reader (Molecular Devices, Palo Alto, Calif.). The amount of each cytokine was calculated from a standard curve generated from the titration of purified recombinant cytokine. To determine whether TGF-β1 is associated with DC, the amount of TGF-β1 in acidified medium was deducted from the amount of TGF-β1 in acidified DC culture supernatants.

**Quantitation of sialic acid.** Sialic acid in the cell supernatant was determined by a modified method of Mrukoci et al. (17). DC were resuspended at 10^6 cells/ml in RPMI, and incubated with different doses of PR8 or purified viral NA for 2 h at 37°C. Cells were pelleted, and 400 μl of each supernatant was mixed with 150 μl of sodium m-periodate (25 mM in 125 mM H_2SO_4). After 30 min of incubation at 37°C, 100 μl of sodium m-arsenite (6% in 0.5 M HCl) and 100 μl of thiobarbituric acid (6% [wt/vol], adjusted to pH 8 to 9 with NaOH) were added. After the mixture was incubated for 30 min at 95°C, 500 μl of dimethyl sulfoxide (DMSO) was added to each reaction tube, and 200 μl was aliquoted into a 96-well plate for reading at A_{492} on a microplate reader (Molecular Devices, Palo Alto, Calif.). NANA was used as a standard. All chemicals were purchased from Sigma.

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were then en bloc stained overnight in Kellenberger’s uranyl acetate, dehydrated through a graded series of ethanol, and subsequently embedded in Epon. Sections were cut (80 μm) on a Leica UCT ultramicrotome and then observed and photographed on a Philips 420 transmission electron microscope at 80 kV.

Cell cluster formation. Cultured DC were infected with PR8 for 4 h at 37°C. These, as well as uninfected DC, were washed, and $5 \times 10^5$ cells were resuspended in 2 ml of complete medium containing 500 U of GM-CSF per ml. Each group of DC was mixed with an equal volume of allogeneic T cells ($5 \times 10^6$ cells) and incubated at 37°C for either 30 min or 12 h. The cell suspension was then gently transferred onto 5 ml of RPMI containing 50% FCS in a 15-ml conical tube. After 30 min of incubation at room temperature, 6 ml was removed from the upper layer of suspension. Cells in the bottom were redistributed into 96-well plates to examine cluster formation under a light microscope.

Analysis of data. Data are expressed as the average of quadruplicate cultures or tests followed by the standard deviation (SD). Some results show the average of several different assays followed by the standard error (SE). In the latter case, the number of assays performed (n) is also shown. The significance of the difference between mean values was compared by using the nonparametric Wilcoxon rank test.

RESULTS

Alloreactive T-cell proliferation to influenza virus-infected DC is dependent on virus dose. We tested the consequence of influenza virus infection on the ability of DC to stimulate an allogeneic immune response in a standard mixed-lymphocyte reaction. Cultured H-2b DC were infected with PR8 at different MOI and then incubated with H-2d T cells. Each assay used serial dilutions of DC to stimulate $3 \times 10^5$ T cells/well. The alloreactive T-cell response to DC was enhanced when these cells were infected with a low dose of PR8 (Fig. 1A). However, when DC were infected with increasing doses of PR8, the enhanced proliferative response was no longer observed (Fig. 1A). When up to $10^7$ DC were added to each well, the alloreactive proliferation to virus-infected cells was equivalent to the response to uninfected DC (Fig. 1B). This was dependent on the number of DC in each well, since greater numbers of PR8-infected DC in a well resulted in a response that was even less than that to uninfected DC (Fig. 1B). There may be multiple reasons for this apparent disparity in T-cell responses when DC are infected with different doses of PR8. Assays in which $[^{3}H]$ thymidine was added at either 48, 72, or 96 h after culture showed consistent differences, indicating that T cells stimulated by DC infected with a high dose of PR8 did not simply respond with different kinetics (results not shown). In subsequent assays, $[^{3}H]$ thymidine was added to the cultures at 72 h, the time point that resulted in greatest incorporation.

Since MOI greater than 1 were used, we did not expect there to be differences in the number of cells infected with different doses of infection. This was confirmed by immunostaining infected cells with polyclonal anti-N1 and anti-H1 antibodies that showed approximately the same proportion of cells infected by 2.5 and 25 influenza virus particles/cell (Fig. 2A). As expected, the levels at which HA and NA were expressed were greatest when cells were infected with larger numbers of virus particles (Fig. 2B).
Apoptosis of DC does not play a significant role in the dose-dependent response. The lack of an enhanced response at a high influenza virus MOI may be due to apoptosis induced in DC by the infection, thereby reducing the number of stimulators in the culture. We therefore determined the degree of apoptosis induced by low and high doses of influenza virus 4, 12, and 24 h postinfection (p.i.). By 24 h p.i. with either low or high doses of virus, most infected cells were apoptotic (Fig. 3A). However, at low MOI, the percentage of apoptotic cells at 4 h.p.i. was less than that observed at high MOI (approximately 14 and 30%, respectively). This difference was smaller, but still evident, at 12 h.p.i. This may contribute to the reduced ability of DC at high MOI to enhance the allogeneic T-cell response. If this were the case, we would expect that stimulation of T cells with greater numbers of high-dose-infected DC would result in increased proliferation. This was not the case; T-cell proliferation stimulated by greater numbers of viable DC infected at high MOI was even weaker than that observed in infected DC (Fig. 1B). These results therefore suggest that induction of apoptosis in DC by influenza virus does not contribute significantly to the reduced incorporation of [3H]thymidine.

Virions or soluble products released from infected DC may inhibit T-cell proliferation or even induce apoptosis in T cells. When the number of viable cells was counted, it was clear that the proportion of viable cells was least when T cells were stimulated by DC infected at high MOI (Fig. 3B). Ten-fold greater amounts of purified NA did not inhibit proliferation, suggesting that the decreased proliferation observed at high MOI was not due to the increased activity of NA on the DC surface.

**TABLE 1. Quantity of sialic acid released from DC after PR8 infection or viral NA treatment**

<table>
<thead>
<tr>
<th>DC treatment</th>
<th>Sialic acid concn (μg) in cell supernatants</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.087 ± 0.016</td>
</tr>
<tr>
<td>Infection with PR8 (MOI)</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0.312 ± 0.019</td>
</tr>
<tr>
<td>5</td>
<td>1.212 ± 0.088</td>
</tr>
<tr>
<td>25</td>
<td>1.624 ± 0.113</td>
</tr>
<tr>
<td>NA (mU/10⁶ cells)</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.202 ± 0.024</td>
</tr>
<tr>
<td>0.5</td>
<td>0.886 ± 0.076</td>
</tr>
<tr>
<td>1</td>
<td>1.221 ± 0.098</td>
</tr>
<tr>
<td>5</td>
<td>1.998 ± 0.337</td>
</tr>
</tbody>
</table>

*a* DC (10⁶) were resuspended in 1 ml of PBS and incubated with different doses of PR8 or NA for 2 h at 37°C. The amount of sialic acid in the supernatant was determined as described in Materials and Methods. NANA was serially diluted in PBS and used as a standard.

*b* Mean ± SD in three supernatants.
The alloreactive proliferation in response to DC treated with UV-inactivated PR8 supported this result, since only the enhanced, NA-dependent response was observed (Fig. 4B). This result also showed that viral replication was required to obtain the diminished response at high MOI.

**NA contributes to the diminished response at high virus dose.** To examine the contribution of NA more closely, DC were infected with PR8 at low and high MOI in the presence or absence of a virus NA-specific inhibitor, zanamivir. Inhibition of NA activity during the infection phase (first 4 h) or during the entire culture resulted in an inhibition of the enhanced response when 2.5 virus particles were used to treat each DC (Fig. 5A). When cells infected at high MOI (25 virus particles/cell) were used as stimulators, the decreased response was still evident when treatment was discontinued after 4 h. When DC were infected with Mvi, a replication-competent, NA-deficient virus, alloreactive proliferation did not decrease at high doses (Fig. 5). In fact, the response was slightly enhanced with increasing numbers of virus particles per cell, suggesting that NA may contribute to the decreased proliferation in response to DC infected at high MOI.

An explanation for these results may be that at high doses of virus, NA removes sialic acid from both the DC and the T-cell surface, diminishing the charge differences, and hence attractive force, between cells. Our previous results showed that treatment of both DC and T cells with bacterial NA resulted in alloreactive proliferation that was diminished compared to proliferation when one cell type only was desialylated (18). Treatment of either uninfected DC or T cells with purified influenza virus NA gave similar results (results not shown), suggesting that the reduced proliferation to high-dose-infected DC may result from desialylation of glycoconjugates on the T-cell surface. This can only happen if virus particles are released into the supernatant from infected DC. Unlike monocytes, DC do not support the formation of virions in PR8-infected cells—virus release from DC infected with a low MOI of PR8 was not observed by electron microscopy (2). To determine whether the DC in our system were indeed infected, the supernatants of cells infected at increasing PR8 MOI were harvested after 24 h of infection, and the presence of virus particles was determined after amplification on MDCK cells. The amount of virus as well as the quantity of NA in the supernatant increased as the MOI was raised (Table 2). However, as reported previously, the number of virus particles released from DC was small and could not be observed in electron micrographs of cells infected at low MOI. Virus par-
This was particularly clear after 12 h of incubation (Fig. 8). However, at high MOI, light microscopy showed that the cluster formation between DC predominated, with very little inclusion of T cells. As for uninfected DC after 12 h of incubation, small lymphocytes were observed independent of cell clusters (Fig. 8).

The dose-dependent response is blocked in the presence of antibodies that neutralize TGF-β1. It is feasible that different doses of influenza virus could influence the type or quantity of cytokines secreted by either DC or T cells. Since some cytokines inhibit proliferation, this may be a mechanism by which the T-cell response is inhibited when DC are infected at high MOI. Cytokines that inhibit T-cell proliferation include IL-10 (22) and TGF-β1 (1, 27). To determine the effect of IL-10 and TGF-β1 on allogeneic T-cell proliferation stimulated with DC infected with low doses of PR8, these cytokines as well as IL-2, IL-4, and IFN-γ were added to T-cell cultures stimulated with allogeneic DC. Proliferation to DC infected with a low dose of PR8 was decreased in the presence of TGF-β1 (Fig. 9A). Addition of IL-10 to these cultures also reduced proliferation, but to a lesser degree. Proliferation was enhanced by the addition of IL-2 and IFN-γ.

To determine the role of these cytokines in our system, antibodies that neutralize IL-10 and TGF-β1, as well as IL-2, IL-4, and IFN-γ, were added to cultures containing H-2b DC infected with various doses of PR8 and H-2b T cells. The enhanced alloreactive proliferation to infected DC with high-MOI PR8 was restored in the presence of antibodies that inhibited TGF-β1 (Fig. 9B). Antibodies that neutralized IL-10 only partially increased the response to high-dose-infected DC, while antibodies to IL-2, IL-4, and IFN-γ inhibited proliferation (Fig. 9B). An isotype-matched control antibody had no effect on the dose-dependent response.

When cells were counted in the presence of trypan blue, the viability of T cells stimulated by high-dose-infected DC was 60%, compared to 85% in the presence of anti-TGF-β1. In contrast, T cells stimulated by low-dose-infected DC in the presence of antibodies that neutralized TGF-β1 had the same viability. This suggests that TGF-β1 contributes to T-cell death, and therefore decreased proliferation, in this system.

To determine whether the source of either of these cytokines was the infected DC themselves, the supernatants from a large number of DC (10⁶ cells in 200 μl) were harvested at several time points after infection with either low or high doses of PR8. The quantity of IL-10 and TGF-β1 in these supernatants was dependent on the infectious dose of PR8 (Fig. 10). Maximum amounts of TGF-β1 and IL-10 were measured in supernatants collected 24 h and 48 h after infection, respectively. When medium alone was acidified to activate TGF-β1, the amount of TGF-β1 was equivalent to the amount measured in the supernatants of DC infected with low MOI of PR8, but larger amounts of TGF-β1 were present in supernatants from DC infected with high doses of PR8. Although the majority of TGF-β1 was activated from latent molecules present in the medium, a small amount was associated with DC infected with high doses of PR8.

### DISCUSSION

Alloreactive T-cell proliferation is enhanced when DC are infected with low doses of influenza virus (18). This enhanced response is, however, dependent on the dose of virus and is no longer observed when DC are infected with high doses of PR8 (Fig. 1A). There may be multiple reasons for the reduced alloreactive T-cell proliferation in response to DC infected with high PR8 MOI. In this report, we assessed the contribu-
tion of apoptosis, viral NA activity, cluster formation, and cytokines to the reduced response.

Although the rate of apoptosis in DC was proportional to the amount of infectious virus (Fig. 3A), our results show that the addition of greater numbers of infected viable DC did not increase the response. This suggests that the difference in apoptosis does not contribute much to the lack of T-cell proliferation at high virus doses. Also, decreased proliferation was not observed when DC were infected with an NA-deficient Mvi virus that is infectious and replication competent. Alternate explanations to account for reduced T-cell proliferation at a high MOI of PR8 include changes that result in reduced activation of T cells, or T-cell death. When T cells from these mixed cultures were counted with trypan blue to exclude dead cells, it was clear that there were greater numbers of viable T cells in the cultures stimulated by DC infected with low doses than with high doses of PR8 (Fig. 3B). At high doses of virus, virions released from the DC, cytokines in the milieu, or physical changes to the DC, may induce apoptosis in the responding T cells. Since influenza virus induces apoptosis, it could be

FIG. 7. Morphology of uninfected and PR8-infected DC. DC were prepared by in vitro culture of H-2b bone marrow and infected with a PR8 MOI of 2.5 or 25 for 4 h. After three washes with RPMI containing 10% FCS, 5 × 10^6 cells were fixed and prepared for electron microscopy. (A) Uninfected DC. (B) DC infected with 2.5 infectious particles/cell. (C, D, and E) DC infected with 25 virus particles/cell. Arrows point to the extensive uropod network that forms a close association between cells. Intercellular and intracellular uropod contacts are demonstrated in panels D and E respectively. Each bar in panels A, B, and C represents 0.2 μm, and each bar in panels D and E is 1 μm. The nucleus of each cell is marked (n).
proposed that at high MOI, T cells become infected. This, however, is unlikely, since trypsin (or a trypsin-like enzyme), which is required to cleave HA and is required for infection, was not present in the mixed cultures. Also, as demonstrated in Fig. 9, T-cell proliferation was restored when cells were cultured in the presence of antibodies to TGF-β1, suggesting that this cytokine may contribute to T-cell death.

TGF-β1 has various seemingly opposite effects on immune responses, acting on various cell types to influence both the initiation and resolution of the immune response (27). It may facilitate the initiation of responses by recruiting inflammatory cells (28), supporting the differentiation of naïve T cells (20) and enhancing DC activity by protecting DC progenitors from apoptosis (19). TGF-β1 can also facilitate DC function by potentiating DC differentiation and cluster formation in collaboration with engagement of flt3 ligand (23).

In contrast, TGF-β1 is best known for its immunosuppressive properties and uses this property to resolve the inflammatory response (27). Whether TGF-β1 results in immune enhancement or suppression usually depends on the activation status of the responding cell, or the mixture of cytokines in the environment (27). Addition of anti-TGF-β1 to T cells stimulated by high-dose-infected DC restored the proliferative response to levels observed when T cells were stimulated with low-dose-infected DC (Fig. 9B). When 200 pg of TGF-β1 per ml was added to the latter cultures, T-cell proliferation was reduced (Fig. 9A), supporting the results of others that demonstrate that TGF-β1 inhibits proliferation of activated CD4+ T cells (27).

Each of the TGF-β isoforms is expressed as a latent preprotein that requires extracellular processing to release the active homodimer. One way in which TGF-β is activated is by removal of carbohydrate moieties from the latent molecules: bacterial as well as influenza virus NA (4, 21) can activate TGF-β1. A significant amount of TGF-β1 was activated in the presence of DC infected with high doses of PR8. Most of this was due to the activation of latent molecules in the tissue culture medium, but some latent TGF-β1 was clearly associated with the infected DC. The TGF-β1 associated with the DC was probably secreted by the infected cells and was not sequestered in the cell matrix, since the amount present in acidified supernatants from high-dose-infected DC was greater than the amount present in supernatants from low-dose or uninfected DC. These results are supported by others that demonstrate secretion of TGF-β1 by DC (3). We therefore propose that when DC are infected with high doses of PR8, TGF-β1 secreted by the DC or in the milieu is activated by the...
activity is proportional to the inoculum dose (Table 2) and correlates with the amount of TGF-β1 in the supernatant. Since influenza virus NA is not secreted from cells, it can be assumed that this enzyme is in association with virus particles. Newly budded virus particles were observed by electron microscopy of DC infected with high doses of PR8 (Fig. 6). These results show that viral NA is present at a location that can facilitate activation of TGF-β1.

There was a little less proliferation in the presence of anti-TGF-β1 than when T cells were stimulated by DC infected with low doses of PR8. This suggests that other mechanisms, for example, other cytokines or apoptosis of DC, may also contribute to the dose-dependent response. In addition to TGF-β1, IL-10 was present in the supernatant of PR8-infected DC in a dose-dependent manner. Although anti-IL-10 did not restore proliferation to maximum levels (Fig. 9B) and addition of 50 ng of IL-10 per ml did not completely inhibit the alloreactive response to DC infected with a low dose of PR8 (Fig. 9A), this cytokine plays a pivotal role in regulating the type of the T helper response and therefore could influence the quality of the T-cell response. Our results show that IL-10 was produced by cells infected with high doses of PR8 (Fig. 10A). Others have demonstrated high levels of IL-10 mRNA in freshly isolated DC from the lungs of rats (24). As these authors suggest, IL-10 production by DC may contribute to the induction of a type 2 response. This idea is supported by results obtained in our in vitro system that show production of IL-4, a typical type 2 cytokine, by allosreactive T cells that are stimulated by DC infected with high, but not low, doses of PR8 (manuscript in preparation).

Electron microscopy also identified changes in DC morphology that were dependent on PR8 infection dose. DC infected at a high MOI had large numbers of dendritic extensions (uropods) that in many instances were “stuck” to adjacent uropods or to those on neighboring cells (Fig. 7). Formation of clusters with T cells is a hallmark of activated DC (5, 12) and was enhanced when DC were infected with low doses of PR8 (Fig. 8) or when DC were treated with NA (results not shown). However, when DC were infected with high doses of PR8, they formed a close network with one another and excluded T cells (Fig. 7 and 8). Although it is reported that TGF-β1 facilitates cluster formation (23), our results do not address whether this is the mechanism that results in enhanced DC clustering. Other factors that may contribute to the enhanced contact between DC when infected with influenza virus include direct desialylation of DC (7) and enhanced interaction between CD2 and LFA-3. The latter interaction results in increased formation of clusters between CD8+ T cells and influenza virus-infected target cells (26).

Our results show that allosreactive T-cell proliferation to influenza virus-infected DC is dependent on the dose of virus. The enhanced proliferation observed in response to low-dose-infected DC is due to the activity of viral NA on the DC surface (18) and therefore reflects the activity of the input virus. Although multiple factors may contribute to the reduced response at high doses of PR8, TGF-β1 plays a prominent role. This cytokine is best known for suppressing both B- and T-cell proliferation and may therefore contribute to the lymphopenia observed during acute influenza virus infection. TGF-β1 is activated by NA that is present on virions released from DC infected with high doses of PR8. The reduced T-cell response is therefore dependent on the output virus. TGF-β is increased in the serum of mice infected with influenza virus (21). Further studies will determine whether the production of TGF-β1 in vivo is dependent on viral NA and whether this is a mechanism that reduces the number of lymphocytes in circulation. If this
hypothesis is true, NA inhibitors that facilitate viral clearance by restricting spread of virus particles may also protect the host from the consequences of pneumonia.

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