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*J Immunol* 2001; 167:1060-1065; doi: 10.4049/jimmunol.167.2.1060
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Respiratory Syncytial Virus Predisposes Mice to Augmented Allergic Airway Responses Via IL-13-Mediated Mechanisms

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Received for publication March 29, 2001. Accepted for publication May 21, 2001.

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1 This work was supported, in part, by National Institutes of Health Grants HL31963 and AI6302.

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3 Abbreviations used in this paper: CRA, cockroach allergen; RSV, respiratory syncytial virus; AHR, airway hyperreactivity; m, mouse; h, human; MIP, macrophage-inflammatory protein; MCP, monocyte chemotactic protein; MDC, macrophage-derived chemokine.

The development of severe childhood asthma may be influenced by several factors including environmental and infectious stimuli. The causal relationship between infectious viral responses, such as respiratory syncytial virus (RSV), and severe asthma during early childhood is unclear. In these studies, the ability for an initial RSV infection to exacerbate and promote a more severe asthmatic-type response was investigated by combining established murine models of disease. We examined the ability of RSV to induce exacerbation of allergic disease over a relatively long period, leading to development of severe airway responses including airway inflammation and hyperreactivity. The preferential production of IL-13 during a primary RSV infection appears to play a critical role for the exacerbation of cockroach allergen-induced disease. The depletion of IL-13 during RSV infections inhibited the exacerbation and acceleration of severe allergen-induced airway hyperreactivity. This was indicated by decreases in airway hyperreactivity and changes in lung chemokine production. These data suggest that the airway responses during asthma can be greatly affected by a previous RSV infection, even when infection occurs before allergen sensitization. Overall, infection of the airways with RSV can induce an IL-13-dependent change in airway function and promotes an environment that contributes to the development of severe allergic asthmatic responses. The Journal of Immunology, 2001, 167: 1060–1065.

The incidence of severe childhood asthma, including emergency room visits and deaths, has increased significantly over the past several years (1–3). The increased incidence in asthma in the United States over the past 20 years has been accompanied by a >100% increase in mortality with health care costs ballooning to over 7.5 billion dollars annually. Disproportionate increases have been observed in inner-city children. Compared with the their rural counterparts with asthma, these children appear to have an increased exposure to environmental hazard and also a significant alteration of allergen sensitization. Recent studies have identified that many of the severe asthmatic cases in the inner city have links to cockroach allergen (CRA) sensitivity (4–8). Up to 70% of urban asthmatics have significant reactive responses to CRA compared with their rural counterpart, where response to this allergen is virtually nonexistent. This is only one of the several environmental allergens that has drastically increased the incidence of asthma. To better understand the specific responses that are associated with environmental cockroach Ag sensitivity, our laboratory has developed a murine model of CRA-induced hyper-reactive airway inflammation (9–11).

A second factor may be the influence of infectious diseases early in childhood. Respiratory syncytial virus (RSV) infections within the first 2 years of life can significantly impact the respiratory health of children (12–14). There are >90,000 documented hospital admissions due to RSV infections annually, with 1–2% of those hospitalized dying due to the infection. Epidemiological evidence also indicates that many of those children hospitalized with RSV later go on to develop severe childhood asthma (15–17). This correlation is likely more than circumstantial. Thus, understanding the mechanisms that are involved and the factors that influence the progression of disease will be crucial.

A number of cytokine mediators have been identified in allergic asthma. Most recently, it has been recognized that IL-13, a Th2-type cytokine, may play an important role in the progression of asthmatic inflammation, both in the activation of the lung environment and in the induction of mucus that accumulates and causes airway congestion (18–20). Our laboratory has recently identified that severe RSV infection in mice is related to the level of IL-13 (21). These latter studies identified that IL-13 promotes airway hyperreactivity (AHR), increased numbers of mucus-producing cells in the airway, as well as mucus production during severe infections. In the present set of studies, we were interested in whether IL-13 produced during RSV infections was responsible for the subsequent increase in asthmatic-type inflammation in allergic mice. By combining a RSV model with an allergic CRA model of lung inflammation, we have begun to identify key cytokines that are produced during the RSV infection that impact lung function during subsequent allergic airway responses. Overall, the data suggest that an initial RSV infection can initiate a proasthmatic environment that promotes a more severe asthmatic response, even when the allergic response is initiated at a time after clearance of the RSV-induced reactions.

Materials and Methods

Animals

Specific pathogen-free BALB/c mice (H-2b) were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed in University of Michigan animal facilities under pathogen-free conditions.
**RSV infection**

BALB/c mice were infected intratracheally with 30 μl (~3 × 10^5 PFU) human RSV A2. The mice were anesthetized with sodium pentobarbital (50 mg/kg) and ketamine (40 mg/kg) given i.p. Tracheotomy was then performed, and RSV was injected directly into the trachea via a Hamilton syringe. The incision was closed with surgical staples, and mice were allowed to recover. No mice died from this level of viral infection or from anesthesia.

**Sensitization and induction of the allergic airway response**

Normal BALB/c mice were sensitized and challenged with cockroach Ag to induce a Th2-type response (9–11). Briefly, mice were immunized with 10 μg CRA (Bayer, Elkhart, IN) in IFA on day 0. On day 14, the mice were given an intranasal challenge of 10 μg CRA in 10 μl diluent to localize the response to the airway. This initial intranasal challenge with Ag induced little cellular infiltrate into the lungs of the mice upon histological examination. Mice were then rechallenged 6 days later by intratracheal administration of 10 μg CRA in 50 μl sterile PBS or with PBS alone (vehicle). Two days after the intranasal sensitization, some of the mice were given a RSV infection as described above and challenged on a schedule 4 days later with CRA. Control allergic mice were given a vehicle challenge instead of RSV or allergen, respectively.

**Combined RSV and CRA model**

To determine whether RSV alters allergen sensitization and predisposes animals to development of more severe allergen reactivity, we combined the models. The following depicts the procedure that was used to determine whether a primary RSV infection causes an increase in allergen-induced responses as has been indicated in clinical studies.

\[
\text{21 days RSV} \rightarrow \text{allergen sensitization} \rightarrow \text{21 days RSV} \rightarrow \text{allergen rechallenge} \rightarrow \text{24 h assessment of inflammation and hyperreactivity}
\]

The appropriate controls were allergen alone for the 21-day sensitization (see above) or RSV alone for 42 days of infection.

**Measurement of AHR**

AHR was measured using a Buxco mouse plethysmograph that was specifically designed for low tidal volumes (Buxco Electronics, Troy, NY) as previously described (9–11). Briefly, the mouse to be tested was anesthetized as previously described and intubated via cannulation of the trachea with an 18-gauge metal tube. The mouse was placed on a Harvard pump ventilator (Harvard Apparatus, Holliston, MA) (tidal volume, 0.4 ml; frequency, 120 breaths/min; positive-end expiratory pressure, 2.5–3.0 cm H₂O) and ventilated for 5 min before the methacholine challenge. The plethysmograph was sealed and readings monitored by computer. Because the box is a closed system, a change in lung volume was represented by a plethysmograph. The system was calibrated with a syringe that delivered a known measure of AHR. In each experiment in normal mice. The optimal dose was defined as the peak change in box pressure (i.e., pleural pressure), and to provide a measure of transpulmonary resistance as has been indicated in clinical studies.

\[
\left(\frac{P_{aw}}{P_{sw}}\right) = \frac{P_{aw} - P_{aw}}{P_{sw}}
\]

The tracheal transducer was calibrated at a constant pressure of 20 cm H₂O. Resistance is calculated by the Buxco software by dividing the change in pressure (P_{aw}) by the change in flow (F) (dp_{aw}/df; units, cm H₂O per milliliter per second) at two time points from the volume curve based upon a percentage of the inspiratory volume. Once baseline levels were stabilized and initial readings were taken, a methacholine challenge was given i.v. via cannulation of one of the tail veins with a 27-gauge needle. A dose-response curve (0.001–0.5 mg) was performed, and an optimal dose of 0.2 mg/kg of methacholine was determined in each experiment in normal mice. The optimal dose was defined as the highest concentration of methacholine that gave little or no increase in airway resistance but induced significant increases in airway resistance in allergen-challenged mice. After the methacholine challenge, the response was monitored, and the peak airway resistance was recorded as a measure of AHR.

**ELISAs**

Assessment of cytokines and chemokines was quantitated from homogenized (PBS) lung aqueous extracts using a double-ligand ELISA system. The murine ELISAs had been developed in our laboratories using a previously described method (9–11). ELISAs were conducted as follows: Flat-bottom 96-well microtiter plates (Nunc ImmunoPlate 1 96-F; Nunc, Naperville, IL) were coated with capture Ab diluted to 3.2 μg/ml in coating buffer (borate-buffered saline, pH 8.6) and incubated overnight at 4°C. Non-specific binding sites were blocked with 2% BSA in PBS and incubated for 1 h at 37°C. Plates were washed and specimens added in triplicate followed by incubation at 37°C and washing. Biotinylated detection Ab was added and the plates incubated at 37°C for 1 h. Plates were washed and conjugated streptavidin peroxidase was added, followed by washing and the addition of chromogen substrate (o-phenylenediamine). Finally, plates were incubated at room temperature, and the reaction was terminated with 3 M H₂SO₄ and read at 490 nm in an ELISA reader. Our ELISAs routinely detect protein at concentrations above 50 pg/ml. These ELISAs are specific and do not cross-react to any other chemokine or cytokine. The cytokine levels were standardized to total nanograms of cytokine per lung.

**Real-time RT-PCR analysis**

Five micrograms of total RNA from specific samples was reverse transcribed into cDNA using a prescribed reverse transcriptase kit from PerkinElmer Applied Biosystems (Foster City, CA). Primers and probe sets for IL-13 and GADPH have been developed by PerkinElmer Applied Biosystems using a patented technique for optimal and specific amplification. Briefly, during PCR, a fluorogenic probe consisting of an oligonucleotide with both a reporter and a quencher dye attached, anneals specifically between the forward and reverse primers. When the probe is cleaved by the 5’ nuclease activity of the DNA polymerase, the reporter dye is separated from the quencher dye and a sequence-specific signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored during the PCR. This real-time detection generates quantitative data based on the PCR at early cycles when PCR fidelity is the highest. Just as important, the real-time PCR system has a linear dynamic range of at least five orders of magnitude, reducing the need for serial dilutions. These computer-linked operations, using the specialized software, make the quantitation of PCR products achievable and, because each well has its own internal standard (GAPDH) with a different fluorescent dye marker, the product can be instantly quantitated and compared with other samples.

**Production of anti-IL-13 Abs**

Rabbit anti-murine IL-13 Abs were prepared by multiple-site immunization of New Zealand White rabbits with recombinant murine IL-13 (R&D Systems, Rochester, MN) in CFA. Polyclonal Ab were titrated by direct ELISA and specifically verified by the failure to cross-react to mouse (m)IL-1α, mIL-1β, mIL-4, human (h)IL-13, mIL-10, mIL-12, mouse macrophage-inflammatory protein (MIP)-1α, hIL-6, mouse JE, hRANTES, human monocyte chemoattractant protein (MCP)-1, hIL-8, hRANTES, hMIP-1α, hSTNF, and hMIP-1β. The IgG portion of the serum was purified over a protein A column and used in a sandwich ELISA. Whole serum (0.5 ml) was used in vivo to block IL-13 during the RSV infection.

**In vivo neutralization of IL-13**

Neutralization of IL-13 was conducted using a polyclonal rabbit anti-murine IL-13 Ab developed in our laboratory as described above. The anti-IL-13 or control Ab was administered i.p. 1 h before RSV infection and every other day until day 12. The in vivo half-life of the Ab was ~30 h, and no detectable circulating anti-IL-13 could be found at the time of allergen sensitization.

**Statistics**

Statistical significance was determined by ANOVA, and significance was determined as values of p < 0.05.

**Results**

**RSV induces increased allergen-induced responses**

We have previously used a CRA model of asthmatic-type reactions to examine cytokine and chemokine involvement in airway responses (9–11). To determine how RSV impacts these allergen-induced responses, we examined the responses by first infecting the mice with RSV (3 × 10^5 PFU). RSV induces increased AHR that returns to background levels by day 21 (Fig. 1A). The induction of AHR correlates well with and is dependent upon the expression of IL-13 (21), which peaks at day 8 of RSV infection (Fig. 1B). At day 21 after RSV infection, the animals were immunized with CRA for another 21 days, as indicated in Methods. The allergen-rechallenged mice were then examined for
changes in AHR. The data in Fig. 2 illustrate that there was a significant increase in AHR in the immunized mice that had previously been infected with RSV compared with either the RSV or allergen alone. Histologically, the lungs appeared to have significantly more peribronchial inflammation (Fig. 3). Thus, the previous RSV infection model exacerbated the allergic response.

Neutralization of IL-13 blocks RSV-induced AHR during allergen challenge

The increased expression of IL-13 induced by RSV was suggestive of the exacerbation of AHR during allergen rechallenge. We have recently identified IL-13 as an important cytokine involved in primary RSV-induced AHR (21). To examine whether an IL-13-mediated mechanism was functioning within this response, allergic mice were treated with anti-IL-13 or control Ab at the time of RSV infection every other day for 14 days. A total of 7 days after the final Ab treatment, the mice were sensitized with allergen (See Materials and Methods). After 21 days of allergen sensitization, the animals were intratracheally rechallenged with allergen, and AHR and inflammation were examined. The RSV plus allergen-challenged mice treated with anti-IL-13 demonstrated significantly reduced AHR responses compared with control Ab-treated mice (Fig. 4). Thus, the neutralization of IL-13 during the initial RSV infection appeared to affect the allergen response even though it was far removed from the initial RSV infection.

RSV infection predisposes allergic animals to IL-13-dependent increases in pulmonary cytokine and chemokine production

We were next interested in determining whether IL-13 produced during the RSV infection had an impact on increased cytokine expression during the allergen response. The data in Fig. 5 indicate that animals that had previously been infected with RSV had higher levels of pulmonary IL-13 (Fig. 5). In addition, when IL-13 was neutralized during the primary RSV infection (see Materials and Methods), an abrogated increase in IL-13 during the allergen response.
responses was observed (Fig. 5). Interestingly, there was no effect on IL-4 levels. We also examined the regulation of the chemokines and found that there was also increased expression of CC chemokines observed in the RSV plus allergen-challenge group compared with allergen alone, including C10, MCP-3, and macrophage-derived chemokine (MDC) (Fig. 6). The levels of C10, MCP-3, and MDC were significantly reduced in the anti-IL-13-treated animals. Interestingly, RANTES production was not further elevated in the RSV plus allergen group compared with allergen alone, but was significantly reduced in the anti-IL-13-treated group. These studies demonstrate that IL-13 functions as an activating factor inducing the production of additional chemotactic factors known to be involved in asthmatic-type responses (9, 10, 22–24). Thus, the initial RSV infection appears to set up a detrimental environment via an IL-13-dependent mechanism that promotes a more severe allergen-induced response.

The histological examination of the animals with a previous RSV infection indicated that there was an increase in peribronchial inflammation. To assess whether the number of eosinophils was affected, we enumerated the peribronchial eosinophils. The data in Fig. 7 show a modest but significant increase in eosinophils in the animals that had previously been infected with RSV. However, the neutralization of IL-13 during the first 14 days of RSV infection had no significant effect on the increased peribronchial eosinophil accumulation.

Discussion

The most common cause for exacerbation of asthmatic responses is the activation of the airways by viral infections (25). Interestingly, some viruses, especially RSV, seem to have a strong propensity for inducing airway responses that exacerbate or may even promote asthma-like inflammation (16, 17, 26–31). In most cases, it appears that the severe asthmatic syndrome may surface only after the RSV infection has cleared and evidence of the disease has dissipated. The findings in these studies indicate that one of the main activating factors that mediates the exacerbation of the allergen responses is IL-13 produced during the RSV infection. Recent studies have clearly demonstrated that IL-13 plays a prominent role in asthmatic-type responses (18–21). Additional data have also indicated that RSV preferentially induces IL-13 production in the airway and promotes AHR and airway damage (21). Although this has not yet been established in humans with severe RSV-induced responses, our data predict that it may be observed.
and contributes to the progression of disease. The primary characteristic of patients with RSV and severe asthma is the overproduction of mucus within the airway, which may be the most detrimental aspect of IL-13 functions. Interestingly, IL-4 levels were not increased over allergen alone during RSV-induced enhancement of the allergic airway responses in these studies, nor has increased IL-4 production been observed during primary RSV infections (21, 32). In contrast to the present study, a recent publication has demonstrated that IL-13 was not up-regulated by RSV during an allergic response (32). However, this latter study gave RSV to allergen-sensitized mice and waited only 5 days after RSV infection before giving a final allergen challenge. Our data indicate that IL-13 production from an initial RSV infection peaks later in the response and may subsequently set up an altered immune environment in the lung, leading to heightened allergen responses. The impact of IL-13 may also be indirect, through the activation of chemokines that augment recruitment of specific cell populations such as eosinophils or Th2-type lymphocytes, which can worsen and prolong the allergen-induced responses. As shown in these studies, the specific upregulation of C10, MCP-3, and MDC appears to correlate with increased inflammation and AHR observed when the allergen-induced responses were overlaid on a previous RSV infection. Thus, the overproduction of IL-13 may alter multiple mechanisms that detrimentally impact disease progression.

Previous studies have clearly demonstrated that IL-5 plays a crucial role in the exacerbation of allergic airway responses induced by RSV infection (33–35). This aspect appeared to be a function of eosinophil-related mechanisms for which IL-5 is absolutely required. This would correlate very well with the present studies because IL-13 appears to be the cytokine that induces increased chemokine production locally within the lung and subsequently causes increased accumulation of leukocytes. Multiple studies have now shown that although IL-5 is required for eosinophil maturation and differentiation in the bone marrow, it also is important for increasing the responsiveness of the eosinophils to chemotactic stimuli (36–38). Thus, it is logically a coordinated response that relies on IL-5 for eosinophil maturation and movement into the circulation from the bone marrow, followed by increased tissue chemokine production for localized migration of eosinophils into the airways. Surprisingly, although the neutralization of IL-13 during RSV infection altered the chemokine production, it did not significantly reduce the peribronchial eosinophilia. However, the levels of eosin, a potent eosinophil chemoattractant, were neither altered by the previous RSV infection nor by the neutralization of IL-13 during RSV.

The cytokine cascades that are induced by a primary RSV infection appear to determine the phenotype of the subsequent responses. The activation of specific chemokines by IL-13 has previously been examined in vitro (22, 39–42). However, when RSV-induced IL-13 overproduction is overlaid on a Th2-type allergic response, the synergistic relationship appears to be detrimental. The cytokine environment set up by these initial infectious responses may ultimately lead to increased recruitment of Th2-type cells, mucus production, and eosinophil accumulation and activation. Together, these detrimental responses contribute to lung dysfunction and AHR. Thus, a key to controlling these responses chronically may be to alter pivotal cytokines that will impact the detrimental inflammatory reactions without affecting the host’s immune responses to properly clear infectious agents.

These results raise important clinical questions about the role of RSV in exacerbation of asthmatic responses and the specific cytokines that are involved. It appears that IL-13 produced during the RSV response can significantly increase the AHR that occurs after an allergen rechallenge. Accompanying these responses is the increased production of chemokines that have previously been associated with AHR and inflammation in asthma-type responses. There is no doubt that there are numerous pathways that are activated during asthmatic responses that can cause exacerbation of disease. However, IL-13-induced responses may be one of the more critical pathways that are activated. Additional studies in our laboratory have suggested that the IL-12/IL-13 axis may be important for determining whether RSV induces a severe or mild airway response. This hypothesis correlates very well with recent observations in human RSV infections that indicate that those children with the most severe disease have a decreased ability to produce IL-12, and levels of IL-12 are inversely related to disease severity (43, 44). In addition, recent work in our laboratory has indicated that IL-12 production during RSV infection may control pulmonary IL-13 levels (45). Taken together, these studies suggest that RSV may induce an altered cytokine environment within the lung that ultimately provides for an exacerbated asthmatic-type response. Although these studies underscore the role of IL-13, it will be interesting to investigate the role of IL-13-induced chemokines in the development of the exacerbated allergic response.

References

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CORRECTIONS


In Figure 1, panel C was omitted. The corrected figure is shown below. The error has been corrected in the online version, which now differs from the print version as originally published.

In the author line, the sequence of the first two authors is reversed. The corrected author line is shown below.

Krishnamurthy Malathi, Xiaogui Li, Olga Krizanova, Karol Ondrias, Kirk Sperber, Vitaly Ablamunits, and Thottala Jayaraman


The fourth author’s name, Cindy Banh, was omitted. The correct list of authors and affiliations is shown below.

Valerie Pasquetto,* Huynh-Hoa Bui,* Rielie Giannino,* Cindy Banh,* Fareed Mirza,† John Sidney,* Carla Oseroff,* David C. Tscharke,§§ Kari Irvine,§ Jack R. Bennink,§ Bjoern Peters,* Scott Southwood,* Vincenzo Cerundolo,§ Howard Grey,§ Jonathan W. Yewdell,* and Alessandro Sette*†

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In Materials and Methods, in the first sentence under the heading Intranasal administration of recombinant adenovirus-containing HO-1 cDNA, the source for adenoviral HO-1 cDNA was incorrectly attributed. The source is stated in the corrected sentence below.

Mice were anesthetized with methoxyflurane, and then 5 × 10⁸ PFU of adenoviral HO-1 (Ad-HO-1) (a gift from K. Kolls, University of Pittsburgh Medical Center, Pittsburgh, PA, and J. Alam, Alton Ochsner Medical Foundation, New Orleans, LA) (29) or adenoviral β-galactosidase (Ad-LacZ) (BD Biosciences) were administered intranasally to each mouse in a volume of 50 μl as described previously (12).

The authors also wish to add the reference shown below.

In Figure 1, a sentence regarding the solid and broken lines was omitted from the legend. The corrected legend is shown below.

**FIGURE 1.** Specificity of the CM4 mAb. A, YB2 or RNK cells transfected with Ly49 constructs were stained with medium or first layer Abs followed by AF488 goat anti-mouse Ig. Solid lines: staining by CM4. Left broken line: medium control. Right broken line: staining by positive control Abs Ly49A = A1, Ly49B = 1A1, Ly49C = 4D12, Ly49D = 4E5, Ly49E = 4D12, Ly49F = HBF, Ly49G = 4G11, Ly49H = 3D10, Ly49I = YBI. B, Cross-competition between Abs. YB2 cells transfected with Ly49E (YB2-E) and RNK cells transfected with Ly49F (RNK-F) were incubated with medium or saturating quantities of the unlabeled Ly49 Abs shown on the y-axis. After 20 min, AF488-labeled CM4, 4D12, or HBF Ab was added, and incubation was continued for an additional 20 min. Median fluorescence values were determined by flow cytometry, and the percentage inhibition caused by pretreatment with each unlabeled Ab is plotted on the y-axis. The likelihood that the inhibition observed was due to chance variation was determined by Student’s t test (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). The experiments shown are representative of three similar experiments of each type that were performed.

In Figure 9A, the gel image labeled Ly49A is inverted. The corrected figure is shown below.

Figure 10, demonstrating intracellular trafficking of HLA-DR after the introduction of HIV proteins, is incorrect. The corrected figure is shown below.

![Corrected Figure](image)


In *Materials and Methods*, in the first sentence under the heading *RSV infection*, the designation of the virus type should be human RSV A strain, not A2 strain.


In *Materials and Methods*, in the first sentence under the heading *Virus and infection*, the designation of the virus type should be human RSV A strain, not A2 strain.

Figure 3B, demonstrating the apoptotic effect of gp120 on CD4 and CD8 cells; Figure 4B, depicting the apoptotic effect of Fas-FasL interactions in CD4 and CD8 T cells cocultured with 43\textsubscript{HIV} cells; and Figure 6B, showing the apoptotic activity of fractionated supernatant from the 43\textsubscript{HIV} cell line, are inaccurate. The corrected figures are shown below.

In Figure 5, demonstrating the inability of HIV-1-infected 43 cells to present antigen to HLA-DR2 and DR4 T cells, panels A and B are the same. The corrected figure is shown below.