Lack of CD200 Enhances Pathological T Cell Responses during Influenza Infection


*J Immunol* 2009; 183:1990-1996; Prepublished online 8 July 2009;
doi: 10.4049/jimmunol.0900252
http://www.jimmunol.org/content/183/3/1990
Lack of CD200 Enhances Pathological T Cell Responses during Influenza Infection


Influenza virus infection can be accompanied by life-threatening immune pathology most likely due to excessive antiviral responses. Inhibitory immune receptors may restrain such overactive immune responses. To study the role of the inhibitory immune receptor CD200R and its ligand CD200 during influenza infection, we challenged wild-type and CD200−/− mice with influenza virus. We found that CD200−/− mice in comparison to wild-type controls when inoculated with influenza virus developed more severe disease, associated with increased lung infiltration and lung endothelium damage. CD200−/− mice did develop adequate adaptive immune responses and were able to control viral load, suggesting that the severe disease was caused by a lack of control of the immune response. Interestingly, development of disease was completely prevented by depletion of T cells before infection, despite dramatically increased viral load, indicating that T cells are essential for the development of disease symptoms. Our data show that lack of CD200-CD200R signaling increases immune pathology during influenza infection, which can be reduced by T cell depletion. The Journal of Immunology, 2009, 183: 1990–1996.

Viruses and the host immune system co-evolve together. The virus must escape immune control and ensure replication, while the host needs to optimize its antiviral response. However, at the same time the host must reduce potential pathological effects of the immune response. Infection with influenza virus can result in severe pathology, which may be due to both direct effects of viral infection and the antiviral immune response. The most deadly influenza pandemic was the 1918–1919 “Spanish flu”, which caused an estimated 40 million deaths worldwide. Interestingly, during that pandemic influenza- and pneumonia-related mortality was more than 20 times higher among young adults, most likely individuals with a competent immune system that were able to mount a vigorous antiviral response (4).

During influenza virus infection the adaptive immune response is initiated by dendritic cells (DCs) migrating from the respiratory tract to the lung-draining mediastinal lymph nodes and spleen (5). There they stimulate the development of influenza-specific cellular immunity. Clearance of influenza virus from the lungs is dependent on B cells and on CD8+ T cells assisted by functional CD4+ T cells (6–9). A too vigorous immune response to the virus may cause negative side effects leading to excessive pathology (10).

Host factors that determine the magnitude and pathogenicity of antiviral responses may act at many levels in the innate and adaptive response. Polymorphisms in various activating but also inhibitory receptors may be involved. CD200R is an inhibitory immune receptor initially described to be expressed on myeloid cells (11). In later studies CD200R expression was also found on B and T lymphocytes in mice and humans (12, 13). CD200R ligand, CD200, is widely expressed, for example on endothelial cells, neurons, and lymphocytes (14). Previously, CD200−/− mice were found to have increased susceptibility to induction of autoimmune diseases (15). Infection of CD200−/− mice with the intracellular pathogen Toxoplasma gondii resulted in increased activation of microglia and decreased intracerebral parasite burden, corresponding with increased survival (16). In a recent study CD200−/− mice showed disruption of lung immune homeostasis and developed more severe pathology after high-dose influenza infection in contrast to wild-type (WT) mice (17). It was suggested that the excessive pathology in CD200−/− mice is a result of hyperactivation of alveolar macrophages. However, this may be only one of the immunoinflammatory pathways deregulated in CD200−/− mice; also, DCs and T cells may respond more strongly in the absence of CD200 inhibitory signaling (18).

Here, we investigated the role of T lymphocytes in the context of CD200 deficiency and low-dose influenza infection. We demonstrated that CD200−/− mice showed severe pathology at viral doses that resulted only in subclinical symptoms in WT mice, despite mounting adequate influenza-specific adaptive immune responses and control of the virus. Importantly, pathology in CD200−/− mice was associated with the presence of T cells. Depletion of T cells before viral challenge ameliorated the clinical
disease course, even though it resulted in increased viral burden. Our data support a role for CD200 as an important host factor in the down-modulation of immune responses during influenza infection and point to T cells as the crucial effector cells in mediating the observed immune pathology.

Materials and Methods

Mice, influenza infections, and T cell depletion

Wild type C57BL/6J mice were obtained from Charles River Laboratories. CD200−/− mice were backcrossed for more than four generations onto C57BL/6J background and were bred at the specific pathogen-free unit at the Utrecht University Central Animal Laboratory. All animal experiments were approved by the Utrecht University Ethical Committee for Animal Experimentation and in accordance with current Dutch laws on animal experiments.

Influenza strains A/HK/2/68 and A/HKx31 were grown in fertilized hen’s eggs. Tissue culture-infective dose (TCID)50 dose was calculated according to Reed and Muench (31) after titration on MDCK cells. Unless stated otherwise, mice were infected with A/HK/2/68 strain. Infection was performed under light ether anesthesia with 50 μl of PBS-diluted influenza virus. Mice were monitored once every 24 h for symptoms of illness (weight loss, ruffled fur, and hunched posture).
CD4⁺ and CD8⁺ T cells were depleted by injection of 20 μg of anti-CD4 Ab (clone YTA 3.1.2) and 50 μg of anti-CD8 Ab (clone YTS 169.4) on days −2 and −1 relative to influenza virus inoculation. Flow cytometry and cell isolation Abs against mouse CD3, CD4, CD8, CD44, and CD62L were obtained from BD Biosciences. CD200R Ab was supplied by AbD Serotec. Allophycocyanin-labeled tetramers of the mouse MHC class I H2-Db H chain, β2 microglobulin, and the influenza virus nucleoprotein (NP)366–374 peptide ASNENMDAM were prepared as described (19) and used for immunofluorescence in combination with the indicated Abs. Blood cells were stained with Abs followed by erythrocyte lysis and cell fixation. Spleen, mediastinal lymph node, and lung cells were isolated by straining the organs through a nylon cell strainer and lysing erythrocytes. Cells were stained with Abs and analyzed by flow cytometry.

Flow cytometry and cell isolation

Abs against mouse CD3, CD4, CD8, CD44, and CD62L were obtained from BD Biosciences. CD200R Ab was supplied by AbD Serotec. Allophycocyanin-labeled tetramers of the mouse MHC class I H2-Db H chain, β2 microglobulin, and the influenza virus nucleoprotein (NP)366–374 peptide ASNENMDAM were prepared as described (19) and used for immunofluorescence in combination with the indicated Abs.

Blood cells were stained with Abs followed by erythrocyte lysis and cell fixation. Spleen, mediastinal lymph node, and lung cells were isolated by straining the organs through a nylon cell strainer and lysing erythrocytes. Cells were stained with Abs and analyzed by flow cytometry.

Microscopic evaluation and protein content quantification

Lungs were inflated and fixed in EAF (40% ethanol, 5% acetic acid, 10% formalin in PBS). Paraffin-embedded sections of 5 μm were stained with...
For calculation of statistical significance, the Mann-Whitney U test was used. Analysis was done only when $n \geq 3$ (*, $p < 0.05$ and **, $p < 0.02$).

**Results**

**Lack of CD200 causes severe pathology at subclinical doses of influenza**

To investigate the role of CD200-CD200R interaction in the regulation of the immune response during viral infection, we inoculated CD200$^{-/-}$ and WT C57BL/6J mice intranasally with increasing doses of A/HK/268 virus. We monitored the disease by daily body weight measurement. Mice were sacrificed once they experienced experimental-animal pathologist.

For protein quantification, BAL fluid was precleared from cells by centrifugation, and an aliquot was used to determine protein concentration with a BCA kit (Pierce) according to the manufacturer’s instructions. Dilutions of BSA standard were used to obtain a standard curve.

**Quantitative influenza PCR and influenza-specific Ab ELISA**

Viral load measurement was performed by RT-PCR. Cycle threshold ($C_t$) value for influenza RNA in lungs was determined by quantitative PCR as described previously (20). The relative fold difference was calculated using the formula $2^{(C_{t1} - C_{t0})}$, where 45 is the total number of PCR cycles and $C_t$ is the number of cycles required to reach the threshold signal.

Relative amounts of influenza-specific Abs were measured with ELISA. The procedure was performed as described before (12) with the modification that $4 \times 10^4$ TCID$_{50}$ influenza virus was coated before assay in 96-well plates.

**Statistical analyses**

For calculation of statistical significance, the Mann-Whitney $U$ test was used. Analysis was done only when $n \geq 3$ (*, $p < 0.05$ and **, $p < 0.02$).
after influenza infection, suggesting sustained inflammation in CD200−/− mice (Fig. 1D). To assess the extent of lung epithelium damage we measured the total protein content in BAL fluid (21) and found increased protein concentrations in the BAL fluid of CD200−/− mice (Fig. 1E). All of these data support more severe inflammation in CD200−/− mice during influenza infection than in WT mice.

**CD200−/− mice mount an adequate and sustained antiviral adaptive immune response**

We next determined if the development of severe disease by CD200−/− mice after influenza virus infection was associated with increased lung viral load. The viral mRNA load from day 2 to 10 after infection was similar in both mouse strains with slightly delayed virus clearance by CD200−/− mice at day 10 (Fig. 2A). Interestingly, adaptive influenza-virus-specific responses in WT and CD200−/− mice were also similar. We measured influenza-virus-specific T cells using an H2-D9 MHC class I tetramer loaded with NP366–374 (ASNENMDAM) peptide. Starting at day 6 after infection, influenza-specific CD8+ T cells appeared in the lungs, with the percentage being comparable between WT and CD200−/− mice (Fig. 2B), indicating that CD200−/− mice generate a normal influenza-virus-specific CD8+ T cell response. Next we examined the percentage of naïve, memory, and effector CD4+ and CD8+ T cells during the course of influenza infection in lung draining (mediastinal) lymph nodes. Again, we found that levels of these cell populations are overall comparable in the WT and CD200−/− mice during the first days of infection (Fig. 2C). Furthermore, we did not observe significant differences in T cell populations up to day 10 after the infection (data not shown). To assess the magnitude of B cell responses, we measured the levels of influenza-specific Abs in serum. During the course of infection the levels of anti-influenza IgG2a, IgG2b, and IgG3 were comparable; however, only at day 6 the level of IgM Ab was significantly higher in CD200−/− mice (Fig. 2D). Overall, the data presented herein indicate that CD200−/− mice have adequate antiviral responses and that loss of control of virus replication is not the cause of the enhanced disease.

Next, we studied the cells infiltrating the airways of infected mice in more detail. There were no significant differences in the numbers of either monocytes or neutrophils (Fig. 3, A and B). Interestingly, at day 6 after infection, relatively large numbers of lymphocytes were present in the airways of CD200−/− mice when compared with WT mice (Fig. 3C). Lymphocyte infiltration coincided with the body weight loss in CD200−/− mice and the rise of the influenza-specific NP366–374 CD8+ T cells as seen by us (Fig. 2B) and others (22). We determined the expression of CD200R on CD4+ and CD8+ T cells in the BAL fluid of infected mice (Fig. 3, D and E). The percentage of T cells with CD200R expression was increased in the CD200−/− mice in both T cell populations at all time points. This suggests that the presence of the ligand normally results in down-regulation of receptor expression, which does not occur during an absence of ligand.

Since T cells are one of the main cell populations involved in the antiviral response, we decided to investigate their role in disease development. The differences in lung infiltration by lymphocytes suggest T cell involvement in disease progression in CD200−/− mice.

**T lymphocytes are essential for the development of pathology during influenza infection**

To assess the role of T cells in the hyperactive response of CD200−/− mice upon influenza infection, we depleted T cells by simultaneous injection of anti-CD4 and anti-CD8 Abs at days −2 and +1 relative to infection. This procedure resulted in depletion of CD3+ cells to 2–5% of peripheral blood lymphocytes, with a slow recovery in time (Fig. 4A).
To determine which T cell population was responsible for immune pathology, we depleted either CD4⁺ or CD8⁺ cells before infection. This treatment had no significant effect on WT mice (Fig. 5A). However, in CD200⁻/⁻ mice depletion of either population had an ameliorating effect on influenza-induced weight loss. Depletion of CD4⁺ cells almost completely prevented weight loss, while the effect of CD8⁺ cell depletion was not as pronounced (Fig. 5B). This might be due to a slightly better efficiency of depletion of CD4⁺ cells, although both populations were depleted almost completely (Fig. 5, C and D). Depletion of each of the T cell populations delayed virus clearance, although viral mRNA levels detected after CD4⁺ or CD8⁺ depletion were not as high as after total T cell depletion (data not shown). These data suggest that both CD4⁺ and CD8⁺ T cells contribute to influenza clearance as well as to immune pathology.

**Discussion**

In this paper we demonstrate that CD200⁻/⁻ mice develop severe pathology after influenza infection at viral doses that resulted only in subclinical symptoms in WT mice. Severe disease of CD200⁻/⁻ mice developed despite adequate influenza-specific adaptive immune responses that control the virus. The pathology observed in CD200⁻/⁻ mice was dependent on the presence of T cells. Depletion of T cells before infection ameliorated the clinical disease symptoms, although it substantially increased viral load. Interestingly, although to a lesser extent than in CD200⁻/⁻ mice, disease caused by influenza virus infection in WT mice was also prevented by depletion of T lymphocytes. This leaves room for additional mechanisms besides the CD200/CD200R axis to control pathological T cells. Most immune cells indeed express multiple inhibitory immune receptors, and some of them have already been implied in the control of viral pathology. Recently, the inhibitory receptor programmed death-1 (PD-1) has attracted attention in the context of enhanced antiviral immunity (23, 24). On the contrary, a critical role for PD-1 in preventing hyperactivation of nonpathogenic SIV infection was demonstrated in sooty mangabeys (25). PD-1⁻/⁻ mice, when infected with adenovirus, indeed have enhanced antiviral immunity and improved viral clearance, but they also have a more severe hepatitis after infection, pointing to a role for PD-1 in the control of immune pathology (26). Furthermore, a recent study in mice deficient in Qa-1b (a murine MHC class Ib molecule), the ligand for the inhibitory CD94/NKG2A complex (CD159a), demonstrated enhanced CD8⁺ T cell-mediated lung pathology upon influenza infection (27). Thus, there is an increasing body of evidence that immune inhibitory receptors regulate and diminish immune pathology.

It is not clear what might be the mechanism by which the CD200-CD200R axis may regulate the immune viral response. A recent report suggested involvement of macrophages and showed increased levels of proinflammatory cytokines found in lungs of CD200⁻/⁻ mice after influenza infection (17). However, we were not able to repetitively measure increased levels of these cytokines in CD200⁻/⁻ mice. This apparent discrepancy may be explained by the use of 800- to 2000-fold higher infection doses of influenza in the studies by Snelders et al. compared with our study. Possibly, massive initial contact with virus may cause more fierce response.

Based on our experiments in CD200⁻/⁻ and WT mice we postulate that T cells may be responsible for the pathological outcome of influenza infection in CD200⁻/⁻ mice. The earlier proposed role of hyperactivated alveolar macrophages might be proximal to the pathological T cell activation (17). Of note, macrophages were...
found to be dispensable for the adaptive immune response to respiratory syncytial virus, where alveolar macrophages seem to have an important role only in the early antiviral response and depletion of macrophages does not affect weight loss after respiratory syncytial virus infection (28). Alternatively, the enhanced T cell responses in CD200−/− mice may be caused by aberrant activation of APCs. CD200−/− macrophages have increased intrinsic activation and they may be the underlying cause of the pathology executed by the T cells. Furthermore, DCs directly sample lung airway surfaces and express high levels of CD200R, which is upregulated during influenza infection (17). Differences in activation of DCs could also result in different antiviral T cell responses and pathology.

Interestingly, although Snegurova et al. focused their study on macrophages, they did notice a massive influx of CD8+ and CD4+ cells into the airways of CD200−/− mice upon high-dose influenza infection (17), suggesting a role for lymphocytes in the severe disease outcome in these mice. Earlier, the importance of T lymphocytes in host-dependent pathology was already reported in the context of influenza virus infection in mice (29), where depletion of CD4+ and CD8+ T cells before influenza inoculation prevented morbidity, in agreement with our findings. However, the interplay of these populations is still unclear: depletion of CD4+ cells might result in lack of help to pathological CD8+ cells. Interestingly, in respiratory syncytial virus infection CD8+ T cells seem to be more important than CD4+ T cells for the development of pathology (30), but complete prevention of immune pathology after respiratory syncytial virus infection was achieved only by total T cell depletion.

Of note, the increased viral load in T cell-depleted mice did not result in severe disease. Thus, the antiviral T cell response resembles a double-edged sword. Inadequate T cell responses will allow the virus to escape the immune control, whereas excessive T cell responsiveness will result in pathological side effects. Our current study suggests that CD200−/− mice may be caused by aberrant activation of APCs. CD200−/− macrophages have increased intrinsic activation and they may be the underlying cause of the pathology executed by the T cells. Furthermore, DCs directly sample lung airway surfaces and express high levels of CD200R, which is upregulated during influenza infection (17). Differences in activation of DCs could also result in different antiviral T cell responses and pathology.

Acknowledgments
We thank Utrecht University Central (UMC) Animal Facility for animal care and Frans Hofniss and Vivienne Verweij for animal experiments as assistance. We thank Peter van Kooten and Dr. Martin Glennie for the kind care and Frans Hofhuis and Vivienne Verweij for animal experiments as assistance. We thank Dr. Marianne Boes, Dr. Christine Jansen, and Anneke Reinierse for the kind assistance. We thank Peter van Kooten and Dr. Martin Glennie for the kind care and Frans Hofhuis and Vivienne Verweij for animal experiments as assistance.

Disclosures
The authors have no financial conflicts of interest.

References