Influenza A induces dysfunctional immunity and death in MeCP2-overexpressing mice

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Introduction

Methyl-CpG-binding protein 2 (MeCP2) is an X-linked transcriptional regulator affecting a myriad of genes (1), and is capable of both genetic repression and activation (2). Transcriptional regulation by MeCP2 is critical for normal development and function, exemplified by the fact that both loss of function and overexpression of MeCP2 result in severe neurodevelopmental disorders Rett syndrome and MeCP2 duplication syndrome, respectively. MeCP2 plays a critical role in neuronal function and the function of cells throughout the body. It has been previously demonstrated that MeCP2 regulates T cell function and macrophage response to multiple stimuli, and that immune-mediated rescue imparts significant benefit in Mecp2-null mice. Unlike Rett syndrome, MeCP2 duplication syndrome results in chronic, severe respiratory infections, which represent a significant cause of patient morbidity and mortality. Here, we demonstrate that MeCP2Tg3 mice, which overexpress MeCP2 at levels 3- to 5-fold higher than normal, are hypersensitive to influenza A/PR/8/34 infection. Prior to death, MeCP2Tg3 mice experienced a host of complications during infection, including neutrophilia, increased cytokine production, excessive corticosterone levels, defective adaptive immunity, and vascular pathology characterized by impaired perfusion and pulmonary hemorrhage. Importantly, we found that radioresistant cells are essential to infection-related death after bone marrow transplantation. In all, these results demonstrate that influenza A infection in MeCP2Tg3 mice results in pathology affecting both immune and nonhematopoietic cells, suggesting that failure to effectively respond and clear viral respiratory infection has a complex, multicompartment etiology in the context of MeCP2 overexpression.
to 5-fold above normal, also demonstrate impaired IFN-γ levels during infection in vivo (7). This defect in IFN-γ production was hypothesized to account for the chronic infections seen in MeCP2 duplication syndrome (7). Importantly, Yang et al. demonstrated that transfer of wild-type CD4+ T cells into MeCP2 Tg3 mice decreased footpad swelling and increased IFN-γ levels during *Leishmania major* infection (7), suggesting that CD4+ T cells expressing normal levels of MeCP2 have the potential to ameliorate immune pathology in an MeCP2-overexpressing host.

More recently, defects in antibody production have been implicated in MeCP2 overexpression. In mice, it was shown that MeCP2 overexpression leads to development of anti-nuclear antibodies (18). In patients, deficiency of IgA and IgG2 and excessive levels of IgG1 and IgG3 were found, in addition to an elevated acute-phase response (19). Interestingly, this same study of MeCP2 duplication patients did not detect any obvious T cell abnormalities, including normal IFN-γ production (19).

Here, we demonstrate that MeCP2Tg3 mice are significantly more susceptible to influenza A infection compared with their wild-type counterparts. All MeCP2Tg3 mice succumbed between days 6 and 9 after infection at a viral dose where the majority of wild-type mice survive. We further demonstrate that although MeCP2Tg3 mice had an uneventful early response to infection, by day 5 a host of detrimental processes began to be manifested, including increased cytokine production, peripheral and pulmonary neutrophilia, and excessive corticosterone levels. In mice that survived to day 7 or 8, defective adaptive immune responses were observed with excessive virus accumulation in the lungs. Pharmacologic targeting of neither the increased cytokine production nor the elevated corticosterone was effective in preventing mortality, although some improvement in weight loss was observed. Despite the prevalence of immune defects, bone marrow transplant revealed that replacement of radiosensitive immune cells of the MeCP2Tg3 hematopoietic system to 5-fold above normal, also demonstrate impaired IFN-γ levels during infection in vivo (7). This defect in IFN-γ production was hypothesized to account for the chronic infections seen in MeCP2 duplication syndrome (7). Importantly, Yang et al. demonstrated that transfer of wild-type CD4+ T cells into MeCP2Tg3 mice decreased footpad swelling and increased IFN-γ levels during *Leishmania major* infection (7), suggesting that CD4+ T cells expressing normal levels of MeCP2 have the potential to ameliorate immune pathology in an MeCP2-overexpressing host.

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was insufficient to rescue mortality. Further investigation revealed that MeCP2
Tg3 mice experience significant vascular pathology during infection, including pul-
monary hemorrhage, reduced accumulation of fluid/proteins/cells into the air-
ways by day 7 or 8, and high pulmonary artery pressure with histologic evidence
of pulmonary arterial edema and luminal narrowing. Together, these results suggest
that susceptibility to influenza A infection in the context of MeCP2 overexpression
may involve pathologic responses of both immune cells and nonhematopoietic cells,
and that nonhematopoietic cells may play a key role in infection-related mortality.

Results
MeCP2Tg3 mice are highly susceptible to influenza A infection, with a normal initial immune response followed by adap-
tive immune failure. We chose to breed MeCP2Tg3 mice, which are commercially available on the FVB back-
ground, on a first generation C57BL6/J × FVB F1 cross to facilitate the use of genetic tools and reagents
available for C57BL6 mice. Importantly, the male MeCP2Tg3 mice used in this study only began to die of
natural causes after 200 days, with over 50% of mice surviving to 1 year (Supplemental Figure 1A; sup-
plemental material available online with this article; doi:10.1172/jci.insight.88257DS1). No defects were
observed in the baseline immune status of MeCP2Tg3 mice, as measured by flow cytometry of peripheral
blood (Supplemental Figure 1, B–D). Because MeCP2Tg3 mice did not begin to die of natural causes until
approximately 28 weeks of age and appeared healthy for the first several months of life, we performed our
experiments in adult MeCP2Tg3 and wild-type littermates between the ages of 10 and 15 weeks, with most
experiments performed at 12 weeks of age.

It was previously demonstrated that MeCP2-overexpressing mice (MeCP2Tg) and humans exhibit
impaired IFN-γ production by Th1 CD4+ T cells, and this was suggested to be the primary etiology of
chronic infection in MeCP2 duplication syndrome patients (7). However, it has also been shown that IFN-γ-null mice are able to effectively respond to, and clear, primary influenza A infection (20). Thus, we hypothesized that if IFN-γ accounts for the major immune deficit in MeCP2Tg3 mice, they should effectively clear primary influenza A infection. Surprisingly, MeCP2 Tg3 mice infected at 12 weeks of age universally succumbed to infection approximately 6–9 days post infection (p.i.), demonstrating significantly increased mortality as compared with wild-type mice infected with the same dose of virus (Figure 1A). The percentage of body weight loss was similar between MeCP2Tg3 and wild-type mice (Figure 1B).

Analysis of the host response in the lungs on day 3 p.i. revealed that immune cell counts were overall comparable between MeCP2Tg3 and wild-type mice (Figure 1C). This is a time when the innate immune cells have entered the lungs in response to infection but prior to the onset of the adaptive immune response in the lungs, i.e., when the adaptive response is being initiated in the lung-draining lymph nodes. In order to assess the ability of MeCP2Tg3 T cells to mount an antigen-specific CD8+ T cell response, we crossed MeCP2 Tg3 mice with T cell receptor (TCR) transgenic OTI mice to produce MeCP2 Tg3::OTI+/– and wild-type::OTI+/– littermates. OTI mice express, on CD8+ T cells, a transgenic TCR specific for chicken ovalbumin (OVA) (21), and thus these T cells can be used to assess antigen-specific CD8+ T cell responses to the OVA antigen. In order to assess the antigen-specific CD8+ T cell response in the context of influenza infection, 2 million wild-type or MeCP2Tg3::OTI T cells were transferred into wild-type or MeCP2Tg3 hosts. Twenty-four hours following T cell transfer, mice were infected with influenza A/PR/8/34 virus engineered to express the OVA epitope recognized by OTI T cells, and the antigen-specific T cell response in the draining mediastinal lymph node...
Table 1. Luminex analysis of bronchoalveolar lavage (BAL) fluid in wild-type and MeCP2Tg3 mice

| Molecule | Wild type | MeCP2Tg3 | P Value |
|----------|-----------|----------|---------|
| IL-17    | 1.606 ± 0.2453 | 2.630 ± 0.4033 | 0.0619  |
| IL-15    | 21.55 ± 1.742 | 43.26 ± 3.210 | 0.0003  |
| IL-13    | Not detected  | Not detected | N/A     |
| IL-12p70 | 5.366 ± 0.5995 | 11.12 ± 1.127 | 0.002   |
| IL-12p40 | 3.412 ± 0.5398 | 7.082 ± 1.236 | 0.0262  |
| IL-10    | 39.68 ± 19.36 | 14.14 ± 2.959 | 0.2285  |
| IL-9     | 72.20 ± 7.842 | 157.2 ± 11.89 | 0.0003  |
| IL-7     | 0.4960 ± 0.1437 | 1.682 ± 0.2160 | 0.0018  |
| IL-6     | 2058 ± 186.2 | 8623 ± 800.1 | < 0.0001 |
| IL-5     | 28.78 ± 5.473 | 130.7 ± 22.27 | 0.0022  |
| IL-4     | 0.9500 ± 0.2524 | 1.764 ± 0.2816 | 0.0635  |
| IL-3     | 1.778 ± 0.2475 | 3.634 ± 0.3583 | 0.0028  |
| IL-2     | 3.292 ± 0.2591 | 4.634 ± 0.2328 | 0.0049  |
| IL-1β    | 21.70 ± 2.000 | 44.59 ± 4.538 | 0.0017  |
| IL-1α    | 10.82 ± 2.318 | 26.18 ± 2.795 | 0.0029  |
| IFN-γ    | 996.6 ± 477.5 | 538.4 ± 141.8 | 0.3845  |
| TNF-α    | 37.81 ± 2.808 | 73.43 ± 7.207 | 0.0017  |
| CXCL10   | 2770 ± 405.6 | 5412 ± 587.7 | 0.006   |
| CXCL9    | 2610 ± 628.5 | 3485 ± 250.8 | 0.2318  |
| CXCL5    | 4.808 ± 1.805 | 55.90 ± 12.97 | 0.0045  |
| CXCL2    | 131.1 ± 3.970 | 174.1 ± 15.35 | 0.0267  |
| CXCL1    | 804.9 ± 60.80 | 1372 ± 155.4 | 0.0094  |
| CCL5     | 48.91 ± 3.036 | 100.9 ± 14.23 | 0.0073  |
| CCL4     | 643.9 ± 87.99 | 1552 ± 327.5 | 0.028   |
| CCL3     | 225.2 ± 12.51 | 406.9 ± 30.69 | 0.0006  |
| CCL2     | 1451 ± 199.0 | 4007 ± 419.2 | 0.0037  |
| LIF      | 182.2 ± 9.589 | 1179 ± 211.6 | 0.0015  |
| VEGF     | 21.99 ± 5.641 | 56.67 ± 6.091 | 0.0031  |
| Eotaxin  | 125.4 ± 16.57 | 488.8 ± 73.97 | 0.0014  |
| MCSF     | 19.48 ± 1.389 | 40.43 ± 3.524 | 0.0006  |
| GMCSF    | 50.18 ± 5.760 | 109.4 ± 12.78 | 0.0029  |
| GCSF     | 1606 ± 140.2 | 5473 ± 752.1 | 0.001   |

BAL fluid from wild-type and MeCP2Tg3 mice on day 5 after infection with influenza A/PR/8/34 was assessed for cytokine, chemokine, and growth factor levels. Values are displayed as pg/ml ± SEM. Two-tailed Student’s t test was performed for statistical analyses, n = 5 mice per group.

was assessed on day 3.5 p.i. We found that there was no difference in T cell proliferation based on genotype of the T cells; however, MeCP2Tg3 hosts actually had an increased number of OTI T cells and increased proliferation of OTI T cells on day 3.5 (Supplemental Figure 2, A–C), suggesting that host genotype, rather than T cell genotype, may be driving any differences in influenza A response.

Although we found increased antigen-specific OTI T cell proliferation in MeCP2Tg3 hosts on day 3.5, we were surprised to find that the entire adaptive immune cell and antibody complement in the lungs appeared to be blunted by day 8 p.i. T and B cell numbers in the MeCP2Tg3 lung parenchyma were reduced, while innate immune cell numbers were normal or elevated at day 8 (Figure 1D and Supplemental Figure 2D). Influenza A–specific antibody titers were greatly reduced in bronchoalveolar lavage (BAL) fluid (Figure 1, E and F), and influenza A viral titers were significantly elevated above wild type, in which no virus was detectable on day 8 (Figure 1G). In addition to the reduced overall number of T cells, there was also a decreased percentage of influenza A–specific CD8+ T cells among total CD8+ T cells as measured by nucleoprotein (NP) and polymeric acid (PA) tetramer staining (Supplemental Figure 2E). In agreement with this finding and previously published data (7), we found that both CD4+ and CD8+ MeCP2Tg3 T cells exhibited significantly reduced IFN-γ production upon restimulation by influenza A–virus–loaded bone marrow–derived dendritic cells (BMDCs), with minimal differences based on the genotype of BMDC used as antigen-presenting cells (APCs), i.e., wild-type or MeCP2Tg3 bone marrow origin (Supplemental Figure 2, F and G). Therefore, there was both a reduced percentage of IFN-γ–producing T cells and reduced overall T cell numbers, which likely combine to explain IFN-γ deficiency observed in the context of MeCP2 overexpression. Although this finding is consistent with previously published data (7), we find that the entire adaptive cell and antibody complement in the lungs appeared to be blunted by day 8 p.i.

Influenza A infection of MeCP2Tg3 mice results in severe lung pathology by 5 days p.i. Because the innate immune response appeared normal in these mice at day 3 p.i., but the adaptive response in the lungs was defective at day 8, we decided to perform serial complete blood count (CBC) measurements on peripheral blood to detect any obvious global changes occurring during the course of infection. These data revealed a robust and specific increase in granulocytes on day 5 p.i. (Figure 2A). Interestingly, those mice that succumbed prior to blood collection on day 7 had a significantly elevated granulocyte count at day 5 p.i., while those that survived to day 7 did not have significantly elevated granulocyte counts on day 5 (Figure 2B). Flow cytometric analysis of peripheral blood and BAL fluid on day 5 demonstrated that neutrophils were significantly elevated in both compartments (Figure 2, C and D). This widespread neutrophilia suggested global exacerbation of innate inflammation in MeCP2Tg3 mice on day 5 p.i., that is, prior to the onset of the adaptive response in the infected lungs. In agreement with this, Luminex analysis of day 5 BAL fluid revealed a widespread increase in inflammatory cytokines and chemokines (Figure 3A and Table 1), consistent with infection-induced excess cytokine production and enhanced pulmonary injury (22). Accordingly, we attempted to reduce inflammation via the use of the COX-2–specific inhibitor, celecoxib. Daily celecoxib treatment normalized neutrophil levels (Figure 3B), and weight loss was attenuated (Figure 3C) without any effect
on survival (Figure 3D). Importantly, there was no difference in weight loss (Figure 3C) or neutrophil levels (Figure 3B) after celecoxib treatment in wild-type mice, demonstrating a specific role for excessive inflammation in MeCP2Tg3 mice.

Previous reports have implicated MeCP2 in the control of glucocorticoid response (13, 23, 24). In addition, MeCP2-overexpressing mice have been shown to have an enhanced response to stress (25). Therefore, we measured serum corticosterone levels during infection, and found a significant increase starting on day 5 and persisting through day 8 (Supplemental Figure 3A). Importantly, glucocorticoids are potent inhibitors of both T and B cells (26), and could explain adaptive immune suppression observed between day 3 and 8. However, similar to celecoxib treatment, treatment with either the glucocorticoid inhibitor mifepristone or combination treatment with mifepristone and celecoxib resulted in attenuated weight loss (Supplemental Figure 3B), but with no effect on MeCP2Tg3 survival after influenza A infection (Supplemental Figure 3C). These results suggest that glucocorticoids play a role in the pathology observed in MeCP2Tg3 mice exposed to influenza A virus, but are insufficient to explain the full extent of disease.

Radioresistant cells are essential for influenza A–induced mortality in MeCP2Tg3 mice. Based on these cumulative results, we questioned the overall role of hematopoietic cells in explaining influenza A–induced mortality in MeCP2Tg3 mice. Towards this end, we transplanted wild-type or MeCP2Tg3 mice with either wild-type or MeCP2Tg3 bone marrow and infected with influenza A/PR/8/34. Survival was not improved by wild-type bone marrow, nor was survival impaired by MeCP2Tg3 bone marrow, regardless of host genotype (Figure 4A). This strongly suggests that radioresistant cells of the immune system alone do not account for mortality in MeCP2Tg3 mice, because the MeCP2Tg3 immune system was insufficient to cause death in wild-type hosts, and a wild-type immune system was unable to rescue survival in MeCP2Tg3 hosts. Thus, we concluded that radioresistant cells must play an essential role in mortality upon influenza A infection in MeCP2Tg3 mice.

We had previously noted that BAL fluid from infected MeCP2Tg3 mice was bloody, suggesting increased vascular leakage. When we measured BAL red blood cell (RBC) numbers via flow cytometry on day 5 p.i., we found significantly more RBCs in BAL fluid of infected MeCP2Tg3 mice (Figure 4B). In agreement with these findings, we observed that MeCP2Tg3 lungs appeared grossly erythematous on day 7 (Figure 4C). Analysis of BAL fluid from uninfected, day 5, and day 7 p.i. MeCP2Tg3 mice revealed a progressive increase in airway hemorrhage. By contrast, there was no blood evident in BAL fluid from infected wild-type mice (Figure 4D). As an additional measure of vascular leakage, Evans Blue dye accumulation was measured in the BAL fluid of infected mice 1 hour after i.p. dye injection. Interestingly, while wild-type mice demonstrated Evans Blue in their airways only on day 7 (as would be expected with increasing pulmonary inflammation), some MeCP2Tg3 mice had Evans Blue in their airway starting on day 5, but had significantly less than wild type by day 7 (Figure 4D). This suggested that although airway hemorrhage was occurring on day 5, transport of protein/fluid from the vasculature to the airways was impaired by day 7 p.i. In agreement with this finding, on day 7 MeCP2Tg3 lungs displayed marked areas of vascular congestion even after pulmonary vascular perfusion with PBS (Figure 4E). We also found that, in contrast with results on day 3 p.i., the number of infiltrating airway cells was significantly reduced in MeCP2Tg3 BAL fluid on day 8 (Figure 4F), again supporting the possibility that access to the pulmonary vascular compartment is compromised in MeCP2Tg3 mice after day 5 of infection.

Histological analysis of lungs from mice day 7 p.i. revealed capillary congestion and alveolar hemorrhage, likely reflecting damage occurring on and after day 5 p.i. (Supplemental Figure 4A). Importantly, overall tissue integrity as detected by histology was comparable between wild-type and MeCP2Tg3 mice, suggesting that the observed vascular defects represented the major tissue damage leading to death upon infection in MeCP2Tg3 mice. Because we had observed defects in pulmonary perfusion, we decided to measure pulmonary function, which includes pulmonary artery pressure. While resistance and compliance were similar to that of wild type (as expected, since no increased tissue destruction was evident in H&E staining), pulmonary artery pressure was significantly elevated in MeCP2Tg3 mice on day 8 (Figure 4G). H&E analysis revealed both perivascular edema and pulmonary arterial narrowing in MeCP2Tg3 mice (Figure 4H and Supplemental Figure 4B), consistent with pulmonary arterial hypertension (Figure 4G) and inability to effectively perfuse the infected MeCP2Tg3 lungs (Figure 4E). In sum, these data suggest that MeCP2Tg3 mice experience pulmonary hemorrhage starting on day 5, with subsequent high pulmonary artery pressure and arterial narrowing, which may ultimately contribute to infection-related mortality.
Here we demonstrated that influenza A infection in MeCP2-overexpressing mice results initially (up to day 3 p.i.) in a normal immune response, but abnormally progresses into excessive innate inflammation, high corticosterone levels, defective adaptive immunity, uncontrolled virus replication, vascular defects, and death approximately 6–9 days p.i. Most pathologic findings began on day 5 p.i., indicating this as a critical turning point. Much of our data regarding adaptive immune system function agrees with previous

**Discussion**

Here we demonstrated that influenza A infection in MeCP2-overexpressing mice results initially (up to day 3 p.i.) in a normal immune response, but abnormally progresses into excessive innate inflammation, high corticosterone levels, defective adaptive immunity, uncontrolled virus replication, vascular defects, and death approximately 6–9 days p.i. Most pathologic findings began on day 5 p.i., indicating this as a critical turning point. Much of our data regarding adaptive immune system function agrees with previous
reports (7, 18, 19), and suggests that the adaptive immune response is markedly impaired in the context of MeCP2 overexpression. We have also identified multiple other pathologic processes during infection in MeCP2Tg3 mice, suggesting that the impairment of the adaptive immune response is just one of a complex set of abnormalities occurring during infection in the context of MeCP2 overexpression.

In addition to defects in immune cells, radioresistant cells were also essential to infection-associated lethality. Bone marrow transplant revealed no effect of immune system genotype on mortality, and substantial vascular impairment (including perivascular edema, pulmonary arterial hypertension, impaired perfusion, and alveolar hemorrhage) was observed in MeCP2Tg3 mice. These results suggest that either direct response of vasculature, or upstream response by other nonhematopoietic cells, may play an important role in disease pathogenesis. In order to identify which cell type(s) are responsible for susceptibility to respiratory infection in MeCP2-overexpressing mice, it would be necessary to either overexpress or correct MeCP2 expression levels in specific nonhematopoietic cell types. Unfortunately, such genetic tools to target specific cell types do not currently exist.

Alternatively, radioresistant immune cells, such as tissue-resident macrophages and innate lymphoid cells, may still be playing a key role in pathology. In particular, alveolar macrophages may contribute to the abnormal responses observed here, as these cells are relatively radioresistant compared with circulating immune cells (27). Alveolar macrophages are essential for effective response to and protection from influenza infection (28–30), and we have previously demonstrated that Mecp2 regulates macrophage response to multiple stimuli, including inflammation and glucocorticoids (13), 2 stimuli that are critical in the context of influenza infection. In addition, several groups have demonstrated abnormal inflammatory and glucocorticoid responses with either MeCP2 loss or gain of function in multiple cell types and contexts (7, 10, 12, 19, 23–25, 31, 32). In this study, both abnormal inflammation and excessive glucocorticoid levels were observed, lending support to the possibility that these pathways are disrupted in the context of MeCP2 overexpression. Future work should endeavor to both create and utilize cell-specific tools to better understand which pulmonary cell types are critical to mortality associated with respiratory infection in the context of MeCP2 overexpression.

The excessive innate inflammation seen in this study may correlate to excessive acute-phase responses observed in MeCP2 duplication syndrome patients (19), and could represent a common defect in the immune response when MeCP2 is overexpressed. Interestingly, significant levels of CXCL5 were observed in MeCP2Tg3 BAL fluid (Table 1), whereas wild-type mice had little to no CXCL5, potentially implicating immune cells, may still be playing a key role in pathology. In particular, alveolar macrophages may contribute to the abnormal responses observed here, as these cells are relatively radioresistant compared with circulating immune cells (27). Alveolar macrophages are essential for effective response to and protection from influenza infection (28–30), and we have previously demonstrated that Mecp2 regulates macrophage response to multiple stimuli, including inflammation and glucocorticoids (13), 2 stimuli that are critical in the context of influenza infection. In addition, several groups have demonstrated abnormal inflammatory and glucocorticoid responses with either MeCP2 loss or gain of function in multiple cell types and contexts (7, 10, 12, 19, 23–25, 31, 32). In this study, both abnormal inflammation and excessive glucocorticoid levels were observed, lending support to the possibility that these pathways are disrupted in the context of MeCP2 overexpression. Future work should endeavor to both create and utilize cell-specific tools to better understand which pulmonary cell types are critical to mortality associated with respiratory infection in the context of MeCP2 overexpression.

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hemorrhage, impaired perfusion, and arterial narrowing, this hypothesis may deserve additional investigation in the future. In essence, perhaps the immune system cannot fight infection owing to breakdown of vascular access and subsequent uncontrolled viral replication. Although T cell–extrinsic mechanisms may explain these defects, as outlined above, it is still important to note that we did in fact measure significantly decreased IFN-γ production upon influenza-loaded-BMDC stimulation in both CD4+ and CD8+ T cells taken from infected MeCP22/3 lungs. While this could be explained by differences in T cell activation and trafficking, it does not on its own contradict the previous finding that MeCP22/3 CD4+ T cells are intrinsically impaired in IFN-γ production (7). However, our findings do suggest that IFN-γ deficiency is unlikely to explain the entirety of respiratory pathology in MeCP2 duplication syndrome.

Another potential point of future investigation is the role of type I and III interferons, which have been well established as playing a role in controlling influenza virus infection, especially in epithelial cells (37). It is therefore possible that a failure to either produce or signal through type I and/or III interferon pathways in MeCP22/3 mice may contribute to failure to control viral infection, which might explain the role of radioresistant cells in mortality.

Our findings could inform future work on respiratory infections in MeCP2 duplication syndrome by suggesting that some potential therapies focusing only on the hematopoietic system, such as bone marrow transplant, may not be viable. Treatment of respiratory infection in MeCP2 duplication syndrome may require therapies either targeting or genetically correcting nonhematopoietic cells. It was recently shown that antisense oligonucleotides can effectively knock down MeCP2 expression in MeCP2-overexpressing mice, and ameliorates neurologic symptoms (38). A similar strategy could be employed in the context of infection in order to correct nonhematopoietic defects. Such an approach may represent the most promising therapy for MeCP2 duplication patients, given our results demonstrating the potential importance of nonhematopoietic cells in the context of infection.

In sum, this work provides evidence that influenza A infection–related morbidity and mortality in the context of MeCP2 overexpression is a complex process involving defects in both immune and nonhematopoietic cells. Future studies should endeavor to further understand which cell types drive mortality upon respiratory infection in the context of MeCP2 overexpression, which may lead to targeted therapies for these patients.

**Methods**

**Mice and infections.** FVB-Tg(MECP2)3Hzo/J (Jackson Labs stock 008680), C57BL/6J (Jackson Labs stock 000664), C57BL/6-Tg(UBC-GFP)30Scha/J (Jackson Labs stock 004353), and B6.129S7-Rag1tm1Mom Tg(TcraTcrb)1100Mjb (Taconic) were maintained by in-house breeding. The background strain for all mice used in experiments was first-generation F1 C57BL6 × FVB (MeCP22/3 female on FVB background bred with C57BL6 background male) to allow for use of transgenes only available on the C57BL6 background. The MeCP22/3 mice contain a human MeCP2 transgene on the X chromosome. Female mice are hemizygous for the transgene, and therefore 50% of male offspring will receive a copy of the transgene. Mice used for infection experiments were all male to most closely mimic the human disease, and were typically 12 weeks of age, with a range of 10 to 15 weeks, and were age matched within each experiment. Mice were anesthetized via i.p. injection of ketamine/xylose prior to intranasal infection with influenza A/PR/8/34 in 50 μl serum-free Iscove’s modified Dulbecco’s medium (IMDM; Gibco). Unless otherwise specified, the standard dose used was LD100 for MeCP22/3 mice, 3,500 egg-infectious doses (EID50). Mice were euthanized based on a scoring system of 0 to 3 for three criteria: body weight changes (0 = normal up to 3 = greater than 15% weight loss), activity level changes (0 = normal up to 3 = immobile), and posture changes (0 = normal up to 3 = lying prone). Euthanasia was performed when either a total score of 8 or a maximal score in 2 categories was reached.

**Flow cytometry.** After tissue preparation, cells were stained for 30 minutes at 4°C in flow cytometry buffer (1% BSA, 2 mM EDTA, and 0.1% sodium azide in PBS). Flow cytometry antibodies against CD45 (clone 30-F11), CD11b (clone M1/70), CD11c (clone HL3), CD103 (clone M290), F4/80 (clone T45-2342), Ly6C (clone AL-21), Ly6G (clone 1A8), MHCIi (clone M5/114.15.2), TCRb (clone H57-597), CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8 (clone 53-6.7), B220 (clone RA3-6B2), NK1.1 (clone PK136), IgD (clone 11-26c.2a), CD19 (clone 1D3), Ter119 (clone TER-119), Siglec-F (clone E50-2440 or IRN44N), and IFN-γ (clone XMG1.2) were purchased from BD Pharmingen or BCell. Flow cytometry antibodies against CD115 (clone AFS98) were purchased from Biolegend, and CCR2 (clone...
475301) from R&D Systems. Tetramers were from MBL (OTI tetramer) or the Trudeau institute (NP and PA tetramers). For tetramer staining, samples were incubated for 30 minutes at room temperature. Samples were washed with 5 ml flow cytometry buffer, centrifuged for 10 minutes at 300 g, 4°C, and decanted. For intracellular staining, samples that had already been stained with extracellular antibodies were fixed and permeabilized using IC fixation buffer (eBioscience) and stained/washed using permeabilization buffer (eBioscience), according to the manufacturers’ instructions. Samples were either fixed in 1% paraformaldehyde (PFA) for later analysis or immediately run on a flow cytometer. Cytometers used were an LSR Fortessa (BD) in the University of Virginia Flow Cytometry Core, and a Gallios (Beckman Coulter) in the Center for Brain Immunology and Glia, University of Virginia.

Preparation of blood for flow cytometry. Blood samples for flow cytometry were collected after animal sacrifice via blood collection from the eye. Briefly, the eye was removed, and several drops of blood were collected into a heparinized tube to be used for whole blood cell count, and then the majority of blood was collected into flow cytometry buffer containing heparin (1% BSA, 2 mM EDTA, 0.1% sodium azide, 10 U/ml heparin in PBS), centrifuged at 300 g and 4°C for 10 minutes, supernatant was aspirated, and 5 ml ACK lysis buffer was used to lyse RBCs by incubation for 5 minutes at room temperature. ACK buffer was washed by addition of 45 ml 4°C PBS, and centrifugation at 300 g and 4°C for 10 minutes. Samples were then decanted and used for flow cytometry staining. Whole blood cell counts were obtained by 1:20 dilution of heparinized whole blood into acridine orange/propidium iodide staining solution in PBS and analysis on a Nexcelom Cellometer Auto 2000. Absolute blood cell counts were obtained by multiplication of the percentage of each population among total live cells measured via flow cytometry by the total live cell count.

Preparation of lung tissue for flow cytometry. After animals were euthanized, mice were transcardially perfused with 4°C PBS to remove blood, both systemically (via left ventricle) and within the pulmonary vasculature (right ventricle). Lungs were placed into 4°C IMDM (Gibco) until tissue collection was completed. Lungs were then miniced and incubated at 37°C for 40 minutes, on a shaker, in petri dishes filled with 5 ml IMDM containing penicillin, streptomycin, and 183 U/ml collagenase type II (Worthington). Digested lungs were gently pressed and filtered through a 70-μm filter, washed in 50 ml flow cytometry buffer, centrifuged at 300 g and 4°C for 10 minutes, decanted, RBC lysis was performed with ACK buffer (same method as described above in Preparation of blood for flow cytometry), and cells were used for flow cytometry staining. Lung cell counts were obtained by acridine orange/propidium iodide staining solution in PBS and analyzed on a Nexcelom Cellometer Auto 2000. Total lung cell counts were obtained by multiplication of the percentage of each population among total live cells measured via flow cytometry by the total live cell count.

Anti-PR8 antibody ELISA. Ninety-six-well ELISA plates were coated with influenza A/PR/8/34 virus stock diluted 1:100 in PBS (7 × 10^7 EID₅₀ per ml) overnight at 4°C. Plates were then washed 3 times with PBS containing 0.05% Tween-20 (PBST). Plates were then blocked with 2% BSA in PBST for 1 hour at room temperature, and washed 3 times with PBST. BAL fluid was then added to each well (50 μl volume) starting at 1:10 BAL diluted in PBS and then serially diluted 1:5. Samples were incubated at room temperature for 2 hours, and then wells were washed 3 times with PBST. Anti–mouse IgG or IgM conjugated to horseradish peroxidase (HRP) (SouthernBiotech) was diluted 1:10,000 in PBS and then serially diluted 1:5. Samples that had already been stained with extracellular antibodies were fixed and permeabilized using serum-free media, twice. Positive-control virus, negative control (PBS), or BAL fluid was prepared by mixing an initial 20 μl of sample with 180 μl of serum-free media containing trypsin, and serially diluting 1:10, seven times. Serial dilutions were added to MDCK cells in 96-well plates, and allowed to adhere overnight at 37°C. The following day, serum was washed off the cells using serum-free media, twice. Positive-control virus, negative control (PBS), or BAL fluid was prepared by mixing an initial 20 μl of sample with 180 μl of serum-free media containing trypsin, and serially diluting 1:10, seven times. Serial dilutions were added to MDCK cells in 96-well plates, in triplicate, and incubated for 3 days. The plates were spun down, and 50 μl supernatant was pipetted into new 96-well round-bottom plates. Chicken RBCs (50 μl of 1%) in PBS were added and mixed. Plates were incubated at room temperature for 30 minutes and hemagglutination was assessed. The tissue culture infectious dose (TCID₅₀) is reported as the average number of wells with hemagglutination between technical replicates for each sample.
Transfer of OTI T cells and assessment of proliferation during infection. OTI+/– CD8+ T cells were sorted by positive selection from either wild-type/OTI+/– or MeCP2TG3/OTI+/– donors using CD8+ selection magnetic beads (Miltenyi Biotec) per the manufacturer’s instructions. Sorted OTI+/– CD8+ T cells were CFSE labeled and 2 million cells were injected via tail vein into each host. One day later, mice were infected with OVA-expressing influenza A/PR/8. On day 3.5, mice were sacrificed and mediastinal lymph nodes were collected to assess for OTI+/– CD8+ T cell proliferation by CFSE dilution.

Culture of BMDCs and restimulation of T cells with virus-loaded BMDCs. Bone marrow was collected from femurs and resuspended in DC media (RPMI without L-glutamine, 10% FBS, penicillin/streptomycin, 0.05 mM β-mercaptoethanol, and 2 mM L-glutamine) at a concentration of 10⁶ cells per ml. DC media (10 ml) containing 20 ng/ml GM-CSF (eBioscience) was placed in a non-TC-treated 100-mm petri dish, and 200 µl of the concentrated bone marrow (2 x 10⁶ bone marrow cells) were carefully pipetted as a colony in the middle of the petri dish. After resting for 10 minutes to allow cells to settle, petri dishes were moved to a tissue culture incubator at 37°C, with care taken not to disperse the concentrated bone marrow cells in the center of the dish. On day 3, an additional 10 ml of DC media containing 20 ng/ml GM-CSF was slowly added to each petri dish so as not to disturb the cells. On days 6–9, BMDCs were harvested by collecting floating and semi-adherent cells in each petri dish. Cells were washed with serum-free RPMI twice. BMDC pellets were resuspended in 200 µl serum-free RPMI, and mixed with 500 µl stock influenza A/PR/8/34 virus for 5 minutes on ice, followed by incubation at 37°C for 30 minutes. Then, 20 µl of prewarmed 37°C DC media containing 20 ng/ml GM-CSF was added, and cells were incubated at 37°C for 5 hours. BMDCs were then washed with DC media, counted, and mixed with effector cells (prepared via lung digestion, as described above) at a ratio of 1:1, in a 6-well non-TC-treated plate. Cells were incubated at 37°C for 1 hour, and then brefeldin A (eBioscience) was added to each well, followed by an additional 4-hour incubation at 37°C. Cells were then collected and stained for cytokine production by flow cytometry.

CBC. For serial CBCs, lateral tail veins were lanced every other day and a minimal amount of blood was taken for analysis (~50 µl). Samples were collected into EDTA tubes for anticoagulation and analyzed on a VetScan Abaxis HM5 CBC analyzer.

BAL. After mice were euthanized, skin and muscle over the trachea was removed, a small incision made in the trachea, a 25-G needle sheathed with soft plastic tubing was inserted into the trachea, secured with sutures, and used to administer 0.5 ml PBS to lavage the lungs. Supernatants from the first lavage were used for cytokine, viral titer, and antibody titer analysis. For flow cytometric analysis, an additional 1 ml of PBS was used to lavage the lungs a second time, and cells from both lavages were used for flow cytometry staining.

Luminex. BAL fluid samples were collected as described and stored at –80°C until analysis. BAL fluid samples were submitted to and analyzed by the University of Virginia Flow Cytometry Core using Millex reagents per manufacturer’s instructions (EMD Millipore).

Celecoxib and mifepristone treatment. Celecoxib (LKT Laboratories) and/or mifepristone (Sigma-Aldrich) was suspended in 0.4% carboxymethylcellulose, low-viscosity (Sigma-Aldrich) in PBS. Suspensions were prepared by vortexing followed by brief sonications in a water bath sonicator. Doses used were 25 or 100 mg/kg for celecoxib, and 25 mg/kg mifepristone, given by daily i.p. injection starting 1 hour prior to infection.

Corticosterone measurement. Serum samples were collected after animal sacrifice via blood collection from the eye. Briefly, the eye was removed, and blood was collected in a BD microtainer serum gel tube. Blood was allowed to fully clot for 45 minutes at room temperature and serum was collected by centrifugation at 15,000 g for 90 seconds and collection of supernatant (serum). Serum samples were frozen at –80°C until analysis by the University of Virginia Ligand Assay and Analysis Core using a mouse and rat corticosterone ELISA (IBL).

Bone marrow transplant. Four- to 10-week-old mice were lethally irradiated with 1,000 rad, and injected with 5 million bone marrow cells via tail vein 5 hours after irradiation. Hosts were C57BL6 × FVB F1 hybrids (either MeCP2TG3 or wild type), and donor bone marrow was of the identical background strain. Donor mice were either MeCP2TG3 or wild type, and some donors were additionally UBC1GFP+ in order to allow for assessment of engraftment efficiency prior to use of mice in downstream experiments. Mice were given water containing sulfamethoxazole and trimethoprim for 2 weeks after transplant. Infection experiments were performed 10 or more weeks after transplant.

Evans Blue assay for assessment of vascular permeability. Mice were injected i.p. with 400 µl 2% Evans Blue solution in PBS 1 hour prior to collection. Mice were euthanized by Euthasol, and transcardially perfused with ice-cold PBS both systemically (left ventricle) and throughout the pulmonary vasculature
(right ventricle) to remove vascular blood and Evans Blue dye. After weighing and photographing organs and BAL fluid, samples were mixed with 1 volume of 50% trichloroacetic acid and incubated overnight at 4°C to precipitate out proteins and other particulates. Samples were centrifuged at 15,000 g and 4°C for 30 minutes. Supernatants were analyzed by 96-well plate reader at 620 nm (Multiskan FC, Fisher Scientific) and a standard curve was generated using known concentrations of Evans Blue dye in PBS mixed 1:1 with 50% trichloroacetic acid.

**Pulmonary function measurement.** Lung function was measured using a buffer-perfused mouse lung system (Hugo Sachs Elektronik). Briefly, mice were anesthetized with ketamine and xylazine and ventilated with room air at 100 strokes/min with a tidal volume of 7 μl/g body weight with a positive end expiratory pressure of 2 cmH2O using the MINIVENT mouse ventilator (Hugo Sachs Elektronik). The animals were exsanguinated by transecting the inferior vena cava. The pulmonary artery was cannulated via the right ventricle, and the left ventricle was immediately tube-vented through a small incision at the apex of the heart. The lungs were then perfused at a constant flow of 60 μl/g body weight/min with Krebs-Henseleit buffer containing 2% albumin, 0.1% glucose, and 0.3% HEPES. The perfusate buffer and isolated lungs were maintained at 37°C throughout the experiment. Isolated lungs were allowed to equilibrate on the apparatus during a 5-minute stabilization period. After equilibration, data were recorded for an additional 10 minutes. Hemodynamic and pulmonary parameters were continuously recorded during this period by the PULMODYN data acquisition system (Hugo Sachs Elektronik).

**Histology.** Tissues were collected either with or without vascular perfusion and inflation of lungs with 4% PFA as indicated in each figure. Tissues were placed in 4% PFA for 2 days, followed by transfer to 70% ethanol. Tissues were submitted to the University of Virginia Research Histology Core, where they were paraffin embedded, sectioned, mounted, and stained with H&E.

**Statistics.** Flow cytometry data were analyzed using FlowJo (TreeStar). All statistical analyses were performed in Prism 6 (GraphPad) using the statistical tests described in each figure legend. P-value significance was set as follows: *P < 0.05, **P < 0.01, and ***P < 0.001.

**Study approval.** All experiments and procedures complied with rules and regulations of the IACUC at the University of Virginia.

**Author contributions**
JCC, TJB, and JK designed the research. JCC, JH, TSK, AL, EKM, AKS, and IS performed the research. KST and TJB analyzed the histology. JCC, TJB, and JK wrote the paper.

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