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Cutting Edge: Stealth Influenza Virus Replication Precedes the Initiation of Adaptive Immunity

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A timely immune response is crucial for the effective control of virus infection. The influenza virus NS1 protein interferes with the expression of key proinflammatory cytokines from infected cells in vitro. To investigate the effect of NS1 during the onset of immunity in vivo, we systematically studied the early events that occur in the lungs and draining lymph nodes upon infection with influenza virus. Strikingly, no sign of innate immunity was detected in the lungs for almost 2 days after infection until a sudden inflammatory burst, including IFNs, cytokines, and chemokines, occurred. This burst preceded the robust dendritic cell migration and T cell activation in the lymph nodes. An NS1-deficient virus triggered rapid inflammation in the lungs whereas a wild-type virus did not. Thus, we demonstrate that, in vivo, influenza virus uses the NS1 protein to replicate for almost 2 days after infection before detection by the immune system. The Journal of Immunology, 2009, 183: 3569–3573.

Initiation of adaptive immunity to influenza virus requires professional APCs such as dendritic cells (DCs) that mature in response to virus stimuli or to cues produced by infected cells (1, 2). Matured DCs migrate to the regional lymph nodes where they trigger the proliferation of naive T cells and their differentiation into effectors cells (3, 4). However, the stimuli and conditions required for in vivo DC activation during influenza virus infection remain controversial.

Studies using mouse and human DCs in vitro demonstrate that upon infection influenza virus blocks many features of DC maturation, including synthesis of the antiviral cytokine type I IFN together with a broad range of inflammatory cytokines (5–7). Moreover, virus-infected DCs fail to up-regulate surface MHC class II and costimulatory molecules such as CD80 and CD86 that are pivotal for T cell stimulation. This inhibition is mediated by the virus-encoded NS1 protein, which is critical for the fitness of the virus because recombinant influenza viruses lacking the NS1 protein (Δ-NS1 virus) are incapable of replicating efficiently in immunocompetent animals (8). However, despite NS1 antagonism, antiviral immunity and viral clearance are successfully accomplished during influenza infection (9–11). Thus, we set out to investigate this apparent contradiction.

In the present study, we analyzed the progression of early events occurring in the lungs of mice infected with aerosolized influenza virus that lead to the onset of immunity. In this model, virus particles are delivered to the lower respiratory tract where they establish an infection that progresses to an acute lethal disease that mimics a natural infection. Our data demonstrate that the initiation of lung inflammation does not begin until almost 2 full days after infection, when virus replication reaches its apex. The migration of lung DCs to lymph nodes and the subsequent priming of naive T cells are similarly subject to this delay. We demonstrate that the delay in the generation of immediate lung inflammation is mediated by the influenza NS1 protein. We propose that the virally encoded NS1 protein establishes a time-limited “stealth phase” during which the replicating influenza virus remains undetected, thus preventing the immediate initiation of innate and adaptive immunity.

Materials and Methods

Mice, viruses, and infection

C57BL/6, OT-I, and OT-II mice were purchased from Taconic Farms and experiments were conducted under institutional guidelines from the Mount Sinai School of Medicine Institutional Animal Care and Use Committee (New York, NY). Mice were infected in an aerosol infection chamber (model A4212; Glass-Col) with a solution consisting of 10^7.9 virus particles in 12 ml of PBS that was aerosolized for 30 min. Viruses used in these studies include the influenza virus A/PR8 strain, two recombinant strains, A/PR8-OT-I (12) and A/PR8-OT-II (13), an A/PR8 virus that lacks the NS1 gene (Δ-NS1) (8), Sendai virus (SeV) Cantell (SeV-C), Sendai virus 52 (SeV-52), and Newcastle disease virus (NDV).

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3 Abbreviations used in this paper: DC, dendritic cell; BM, bone marrow; dpi, days postinfection; hpi, hours postinfection; MLN, mediastinal lymph node; NDV, Newcastle disease virus; qPCR, quantitative RT-PCR; SeV, Sendai virus; SeV-C, SeV Cantell; CCH, cholera hemocyanin; C-488, CCH labeled with Alexa Fluor 488; NS1, nonstructural protein 1; Δ-NS1, absence of NS1.

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To investigate whether influenza virus was capable of ablating the initiation of immunity in vivo, we analyzed the lungs of mice at different time points after infection for evidence of inflammation. As shown in Fig. 2A, qPCR of total lung mRNA shows that up-regulation of proinflammatory genes was undetectable for the first 2 days postinfection (dpi). However, at 48 hpi a sudden inflammatory burst could be observed in which the expression of type I and II IFNs, cytokines, and chemokines was strongly up-regulated. mRNA encoding for the type I IFN-responsive antiviral proteins Mx-1 and ISG54 was only detected after 48 hpi, demonstrating that not even trace amounts of IFN protein were present at earlier time points. Moreover, as depicted in Fig. 2B, multiplex ELISA quantification of protein production from lung lysates of infected mice shows detectable type I IFNs and proinflammatory cytokines only after 48 hpi, corresponding with the timing and magnitude of the qPCR data. Immunohistochemical analysis of lung samples shows that leukocyte infiltration begins after 48 hpi and drastically increases by 72 hpi (supplemental Fig. 2). Serum samples from infected animals were also analyzed by ELISA (supplemental Fig. 3) and inflammatory mediators were only demonstrable at 48 hpi. As shown in Fig. 2 and supplemental Figs. 2 and 3, the initiation of local lung inflammation, the subsequent systemic spread of inflammatory mediators, and leukocyte infiltration correspond with the peak of virus replication.

Considering the delay in the initiation of lung inflammation, we further evaluated whether the migration of lung DCs to MLNs correlated temporally with the onset of lung inflammation. In a previous study, endotoxin-free OVA was administered once by aerosol to groups of influenza virus-infected mice at 24-h intervals, beginning at the time of infection (3). Noticeably, only when the protein was given at or after 48 hpi did mice exhibit strong Ab responses to OVA (3). If, as we suspected, the virus was triggering DCs that carry the OVA to the draining MLNs, then the migration did not begin until 2 dpi (3). Revisiting these experiments, we used an endotoxin-free, high m.w. protein known as CCH (15) labeled with Alexa Fluor 488 (C-488) to track the migration of lung DCs to the draining MLNs during PR8 virus infection. This protein is not inflammatory to DCs (supplemental Fig. 4), and its large molecular size minimizes leakage to the MLNs (supplemental Fig. 5); thus, C-488 can unambiguously be used to track cell-associated Ag transport from the lungs to the lymph nodes. Influenza-infected mice were given one dose of C-488 intranasally at different time points after infection, and C-488 was tracked by guest on April 13, 2017 http://www.jimmunol.org/ Downloaded from
This was corroborated by staining CD11c+/H11001 positive cells in the MLN for the presence of viral proteins. As shown in Fig. 3C, viral protein-bearing DCs can be shown to arrive in the MLNs with kinetics identical with those of the C-488 positive cells. These observations indicated that lung inflammation began at 48 hpi, increased thereafter, and the migration of DCs to the MLNs closely followed these kinetics. We hypothesized that the absence of inflammation before this threshold time (48 hpi) was an effect of NS1 antagonism. To test this hypothesis, we intranasally infected naive mice with an A/PR8-H9004-NS1 virus and compared the onset of lung inflammation with that induced by wild-type A/PR8 at various doses. As shown in Fig. 4A and B, and supplemental Fig. 7, infection with as few as 10⁵ TCID₅₀ of H9004-NS1 virus triggered lung inflammation as early as 8 hpi. In contrast, doses of A/PR8 virus 100 times higher than the doses of H1004-NS1 virus (and equivalent to titers observed in the lungs of infected mice at 48 hpi) were not enough to set off the cytokine response. Moreover, measurements of virus replication in the lungs of these animals at these early time points by PCR showed that both viruses had replicated to equivalent levels (influenza M protein; Fig. 4D). These results demonstrate that in vivo the NS1 protein effectively blocks the production of inflammatory cytokines and the establishment of an immediate antiviral state.

Discussion

The initiation of adaptive immune responses to influenza virus is a multistep process that begins with the capture of viral Ag in the lungs by DCs and their subsequent migration to the draining lymph nodes where they initiate T cell responses. Any intervention by the virus in this process will allow prolonged replication without the involvement of the immune system. In our studies, virus replication was almost at peak levels before any sign of immune activation was observed. The virus remained inconspicuous to innate immune detection (stealth phase) for almost 2 dpi because of its effective NS1 antagonism. Subsequently, a sudden burst of lung inflammation occurred, leading to the initiation of adaptive immunity by migratory lung DCs. The efficiency of the NS1 suppression is clearly shown by the lack of a response in the lungs of mice directly inoculated with wild-type virus in contrast to the strong inflammatory response observed with 100 times less Δ-NS1 virus. This was true even though the viruses have replicated to equivalent levels in the lungs of the mice at this time.

The stimulus that eventually overcomes this antagonism and promotes the inflammatory burst is unknown. The eventual triggering may result from virus-derived molecular patterns occurring at high multiplicity of infection, massive host cell death, or an as yet undiscovered event (16, 17). DCs, macrophages, plasmacytoid DCs, or other immune cells present in the lung...
may sense these ligands and initiate the abrupt inflammatory cascade (18).

Directly infected APCs (DCs and macrophages) are subject to the inhibitory effect of the viral NS1. However, DCs or monocytes exposed to type I IFNs and other inflammatory cytokines are protected against uncontrolled virus infection and gain the capacity to respond vigorously to the virus even in the presence of NS1 (19, 20). Thus, the concentration of inflammatory cytokines in the lungs and bloodstream of infected animals is rapidly increased when the preactivated cells respond to virus. This cytokine exposure may well be crucial to “preprogram” DCs or their precursors that are recruited to or residing in the lung during influenza infection. Therefore, the observed blockade of the induction of type I IFNs in the lungs may serve to delay the physiological response by preventing the preactivation of DCs with enhanced APC capabilities.

What additional advantage does NS1 inhibition of immunity bestow on the pathogen? Experimental aerosol infection in humans has shown that the transmission of influenza virus is maximal before the onset of symptoms that appear ~2 dpi (21). Moreover, a comparable time course is observed in the mouse and guinea pig models of infection and transmission (22, 23). It is noteworthy that the onset of symptoms in humans occurs ~2 dpi and correlates with the appearance of inflammatory cytokines and a drop in virus titers in nasal fluids (24, 25). Thus, a stealth phase may also occur in humans and probably functions to maximize the probability of transmission before cytokines such as type I IFNs hamper the normal replicative cycle of influenza virus.

The stealth phase concept is not only applicable to influenza virus but can probably be extended to virtually all “real” human viral pathogens that have been shown to have an asymptomatic incubation time. For example, measles and varicella zoster viruses have a substantially prolonged evasion period that can last up to 2 wk (26, 27). During this asymptomatic phase, these viruses also transmit to other susceptible hosts. Research aimed at interfering with the stealth phase may lead to the development of novel modulators as preventive treatments that target this early immune evasion mechanism.

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Disclosures
The authors have no financial conflict of interest.

References
Supplemental Data.

DC upregulation of costimulatory molecules and MHC-II after influenza virus infection, immunohistochemical analysis of influenza infected mice, blood cytokines after influenza infection, C-488 as a tracker of lung DC migration in vivo, and lung cytokine production after delta-NS1 infection compared to PR8.

Stealth influenza virus replication precedes the initiation of adaptive immunity
Bruno Moltedo*, Carolina B. López*, Michael Pazos*, Maria I. Becker†,‡, Tamar Hermesh*, and Thomas M. Moran 2,*

Supplemental Figure 1: NS1 interferes with the upregulation of costimulatory molecules and MHC-II in influenza infected bone-marrow derived-DCs (BMDCs). A-C. BMDCs were infected either with PR8 or Delta-NS1 virus at an MOI=3 overnight. DCs were stained for CD80(A), CD86(B) and MHC-II(C) and the surface expression of these molecules was analyzed by flow cytometry.
Supplemental Figure 2: Lung leukocyte infiltration starts 48h after influenza virus infection and correlates with a delayed initiation of inflammation. Hematoxilin and Eosin staining of fixed-frozen lung sections were performed at different time points after infection (10x magnification). Detailed analysis of lung sections from infected mice shows no significant increase of infiltrating leukocytes between 0-32 hpi. Only at 48h, the first wave of infiltrating leukocytes could be seen (arrows), and by 72 h pervade an extensive surface of the lung. A magnified view of the leukocyte infiltrate at 48 hpi (60x, blue box) as indicated by the black arrow.
Supplemental Figure 3: The kinetics of the cytokine response in the blood to influenza virus follows the initiation of lung inflammation. Different groups of mice were administered one intranasal dose of LPS or infected via aerosol with A/PR8 virus. Serum was collected from mice at the given times after LPS administration or influenza infection and analyzed via Multiplex ELISA for A. Chemokines (IP-10, KC, MCP-1 and MIP-1α); B. Cytokines (IFN-γ, IL-10 and IL-6), and the growth factor G-CSF (C).
Supplementary Figure 4: C-488 does not cause maturation of DCs. BM-DCs were incubated either with Concholepas concholepas hemocyanin (CCH) or Alexa-Fluor-488-labeled CCH (C-488) for 18 h at different concentrations of protein (10-500 ug/mL). BM-DCs were also infected with Sendai (MOI=2), PR8(MOI=2) or mock infected with PBS. BM-DCs were also incubated with LPS (10 ug/mL) as a positive control. TNF-α and IL-12p40 were measured from the supernatants of these cultures by ELISA.
Supplementary Figure 5: C-488 does not cause migration of lung DCs in the absence of inflammation in vivo. Naïve C57BL/6 animals were given intranasally PBS, C-488 or C-488 spiked with LPS and mediastinal lymph nodes were collected 24 hr to assess for the migration of lung C-488+ DCs.

Supplementary Figure 6: Migratory C-488+DC bear viral antigens. PR8 infected mice at 48 hpi received a single intranasal dose of C-488 and at 72 hpi, C-488+ CD11c+ DCs were analyzed for the presence of intracellular viral antigens in the draining mediastinal lymph nodes. As shown, CD11c+ C-488+ gated DCs were analyzed for intracellular viral proteins. Isotype controls and positive HA+NP antibody staining are compared side by side.
Supplemental Figure 7: NS1 expression precludes the rapid production of pro-inflammatory cytokines in influenza-infected mice. Naive mice were mock infected (NI) or infected with different doses of PR8 or Delta-NS1 viruses. 8 hpi, lung homogenates were assayed for the production of (A) IFN-β, (B) IL-6, and (C) IL-1β.