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Antiviral Antibodies Target Adenovirus to Phagolysosomes and Amplify the Innate Immune Response


Adenoviruses are nonenveloped dsDNA viruses that activates intracellular innate immune pathways. In vivo, adenovirus-immunized mice displayed an enhanced innate immune response and diminished virus-mediated gene delivery following challenge with the adenovirus vector AdLacZ suggesting that antiviral Abs modulate viral interactions with innate immune cells. Under naive serum conditions in vitro, antiviral binding and internalization in macrophages and the subsequent activation of innate immune mechanisms were inefficient. In contrast to the neutralizing effect observed in nonhematopoietic cells, adenovirus infection in the presence of antiviral Abs significantly increased FcR-dependent viral internalization in macrophages. In direct correlation with the increased viral internalization, antiviral Abs amplified the innate immune response to adenovirus as determined by the expression of NF-κB-dependent genes, type I IFNs, and caspase-dependent IL-1β maturation. Immune serum amplified TLR9-independent type I IFN expression and enhanced NLRP3-dependent IL-1β maturation in response to adenovirus, confirming that antiviral Abs specifically amplify intracellular innate pathways. In the presence of Abs, confocal microscopy demonstrated increased targeting of adenovirus to LAMP1-positive phagolysosomes in macrophages but not epithelial cells. These data show that antiviral Abs subvert natural viral tropism and target the adenovirus to phagolysosomes and the intracellular innate immune system in macrophages. Furthermore, these results illustrate a cross-talk where the adaptive immune system positively regulates the innate immune system and the antiviral state. The Journal of Immunology, 2009, 182: 7058–7068.
cells, the direct interaction between adenovirus and macrophages is poorly understood. Leukocytes lack the Coxsackievirus adenovirus receptor (CAR)\(^9\) used by adenovirus for binding to epithelial-derived cells and consequently virus attachment to hematopoietic cells occurs at a much lower affinity (22). Given that adenovirus internalization in leukocytes is key to the host detection of viral infection and the activation of intracellular innate receptors, these observations suggest that other mechanisms must exist that target viruses to the cellular compartments necessary to mount an efficient antiviral host response. Our previous studies demonstrate that opsonins such as complement and antiviral Abs play a significant role in altering natural viral tropism and targeting the adenovirus to neutrophils (14). Similarly, macrophages and dendritic cells efficiently interact with and internalize adenovirus-Ab immune complexes (10, 23). Since viral-sensing receptors are intracellular, it is likely that Ab-mediated viral entry in macrophages has an impact on the innate immune response to adenovirus.

In this study, we demonstrate that mice immunized with adenovirus display enhanced innate immune responses following a second adenoviral challenge. Antiviral Abs increase adenovirus internalization via FcRs in macrophages. Unlike adenovirus entry in nonhematopoietic cells that is typified by early endosomal escape, Ab-dependent viral entry in macrophages targets the adenovirus to the phagolysosome, an event that is associated with amplification of intracellular innate pathways including the NLRP3 inflammasome and TLR9-independent type I IFN expression.

Materials and Methods

Adenovirus

The type 5, E1-deleted, E3-defective adenovirus encoding the GFP or the LacZ transgene under the control of a CMV promoter was generated using an Ad-EASY system (Strategene), propagated on human embryonic kidney 293 cells, and purified as previously described (24). Wild-type adenovirus serotype 5 was propagated on 293 cells and purified as above. The particle titer was determined by measuring the OD at 260 nm described as particle per cell (part/cell). PsAd+ is AdGFP, which was rendered transcription defective by UV/psoralen treatment as previously described (25). AdLFB+ and AdLFB* are tropism-modified E1-E3-deleted Ad5-based vectors carrying the luciferase transgene in the E1 region under the control of the CMV promoter (13). Adenovirus and vectors were labeled with the green fluorescent dye Cy2 as described previously (14) using an Ab labeling kit (GE Healthcare) according to the manufacturer’s instructions. Low endotoxin tissue culture reagents and buffers were used for virus/vector production and experiments. Adenovirus and vectors were routinely tested for the presence of endotoxin using an Limulus Ameocyte Lysate Kit. All viruses/vectors contained <0.1 endotoxin units/ml.

Cell culture

Human embryonic kidney cells (HEK 293 cells) and HeLa cells were maintained in DMEM containing 10% FBS and 1% penicillin-streptomycin. Human THP-1 monocytic leukemia cells were grown in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin, 1% MEM, 100 mM sodium pyruvate, and 0.01% 2-ME. THP-1 cells were differentiated with 100 nM PMA overnight before all experiments. Cells were periodically tested for contamination. Viral infections or viral vectors contained ≥0.1 focus-forming units/ml (vector plus vehicle). Control animals received vehicle alone (3% sucrose, 50 mM NaCl, pH 7.50, and 1 mM MgCl\(_2\)). Animals were allowed to recover and then sacrificed at 6 h and the livers and sera were harvested for analysis. Some animals were immunized with 1 × 10\(^7\) particles/mouse AdGFP or injected with vehicle 10 days before subsequent adenovirus administration.

Immunoblotting and ELISA

For immunoblotting of IL-1β protein, cells were lysed with buffer (10 mM Tris (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, and protease inhibitor mixture), then proteins in cell lysate or tissue culture supernatant were separated on SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were blocked with 5% milk proteins in 1× PBS and 0.5% Tween 20 and then probed with primary Abs as follows: polyclonal rabbit anti-human-cleaved IL-1β (D116, Cell Signaling), polyclonal rabbit anti-human IL-1β (Cell Signaling), or polyclonal sheep anti-mouse IL-1β. Appropriate HRP-conjugated secondary Abs were used and proteins were detected using ECL reagent (Amersham Biosciences). IL-1β protein in mouse serum was quantified using an ELISA against IL-1β protein as per the manufacturer’s protocol (BD Biosciences). ODs were determined spectrophotometrically at 450 nm.

Microscopy and flow cytometry

To visualize GFP transgene expression and cell morphology, cells were examined by phase contrast and fluorescent microscopy using an Olympus IX70 inverted epifluorescence microscope (Olympus). Digital images were obtained and analyzed using OpenLab software (Improvision). Flow cytometry was performed on formalin-fixed cells using a FACScan instrument and analyzed with CellQuest Pro software (BD Biosciences). For the FcR-blocking experiments, THP-1 cells were pretreated with 30 μg/ml anti-human CD64 (hFcγRI) (BD Pharmingen) and the hFcγRIIA-blocking Ab IV.3 (American Type Culture Collection) or isotype control Abs for...
10 min at 37°C. Cells were then infected with Cy2-labeled adenovirus for 90 min and analyzed by flow cytometry as described above.

An analysis of the distribution of the virus within cells was performed using an Olympus Flouview 1000 inverted confocal microscope with a ×63/1.42 aperture or a ×60/1.20 aperture objective. Stacks in the z direction were acquired at 0.2-µm intervals. Cells (THP-1 or 293) were grown on coverslips contained in standard 6-well plates. For live cell imaging, cells were pulsed with Alexa Fluor 594-labeled 40-kDa dextran and incubated overnight. Cells were then incubated with Cy2-labeled adenovirus in the presence of 5% pooled human or FBS and incubated for 1 h followed by imaging. For LAMP1 staining, cells were fixed in 0.2% glutaraldehyde/PBS supplemented with 0.1 M EDTA and 1 M MgCl₂. After washing with PBS/1 M MgCl₂/2% Nonidet P-40, sections were incubated in a solution containing 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for 1 h at 37°C.

**DNA isolation and Southern blotting**

To obtain total DNA, cells were trypsinized for 5 min and washed several times with PBS to remove bound virus from the cell surface. The cell pellet or mouse liver was subjected to processing for total DNA using a DNeasy kit (Qiagen) according to the manufacturer’s protocol. For Southern analysis, 1–3 µg of DNA was digested with HindIII and separated on a 1% agarose gel. The gel was washed in 0.25 M HCl followed by another wash in a 0.4 M NaOH solution. The DNA was then alkali transferred onto a positive charged Hybond XL nylon membrane (Amersham Biosciences). A full-length cDNA fragment of the adenovirus 5 fiber gene (332 bp) was labeled with [32P]dCTP using a Redprime random primer labeling system (Amersham Biosciences). Hybridization was performed by using 2 ng of the labeled DNA probe per 5 ml of ExpressHyb Solution (BD Clontech) at 60°C for 1 h. The membrane was then washed three times for 15 min in 2×...
Semiquantitative PCR was performed to probe for the and expressed as percent induction relative to vehicle-treated controls. Amplification was performed in MicroAmp Fast Optical 96-well reaction sequence fragments of the murine IFN- primers and probes were designed using Primer3 software to amplify MCP-1, TCA-3, and MIP-1 analyzed. As expected in naive mice, AdLacZ induced the expression of the IFN- gene in liver DNA using forward primer, 5'-AAGGACAGGAGATTGGTTGATT-3' and reverse primer, 5'-GAGGCTTCTTGGATCTGTTGGTT-3'; probes, miIFN-4 forward primer, 5'-CGGCAAGGCAGGTAACTTTCTC-3' and reverse primer, 5'-ATGGCAAAAGGCAGGTAACTTCTC-3'; and probe, 6FAM-TGACGGAGAATGC-MGBNFQ. The 20X Mouse GAPD (GAPDH) FAM/MGB Probe (Applied Biosystems) was used as the endogenous control. Target gene reactions were performed in a total reaction volume of 25 μl using 12.5 μl of 2X TaqMan Universal PCR master mix (Applied Biosystems), 900 nM of each primer, 200 nM probe, 2.95 μl of RNase/DNase-free distilled water, and 5 μl of cDNA template (diluted 1/5 from original). Amplification was performed in MicroAmp Fast Optical 96-well reaction plates (Applied Biosystems) using the 7900HT Fast Real-Time PCR System (Applied Biosystems). Results were normalized to GAPDH transcripts and expressed as percent induction relative to vehicle-treated controls. Semi-quantitative PCR was performed to probe for the β-galactosidase (LacZ) gene in liver DNA using forward primer, 5'-GATCGATC CCTGCTTGTTTACAACTG-3' and reverse primer, 5'-ATCGATTGCT GTTGACTGTAGCCGC-3'. Results were analyzed by densitometry using the Quantity One software (Bio-Rad) and normalized to GAPDH.

RNase protection assay and real-time PCR

Total RNA from cells and tissue was isolated using a RNeasy kit (Qiagen) according to the manufacturer’s protocol. RNase protection assays were conducted using the RiboQuant MultiProbe RNase protection assay system and the mCK3-mCK5c-mCK2b multiprobe template sets for mouse or hCK5-hCK2b sets for human (BD Pharmingen) as per the manufacturer’s protocol. Gels were visualized by autoradiography and quantified using a PhosphoImager Molecular Imager and the Quantity One software (Bio-Rad). The signal intensity of individual genes was normalized to the intensity of the housekeeping gene GAPDH within the same sample.

Reverse transcription was conducted using 500 ng of RNA, random hexamers (3 μg/μl), and Moloney mouse leukemia virus reverse transcriptase (200 U/μl; Invitrogen) per the manufacturer’s protocol. Real-time PCR primers and probes were designed using Primer3 software to amplify sequence fragments of the murine IFN-α4 and IFN-β genes. They were as follows: miIFN-α4 forward primer, 5'-AAAGGACAGGAGATTGGTTGATT-3' and reverse primer, 5'-GAGGCTTCTTGGATCTGTTGGTT-3'; probes, miIFN-4 forward primer, 5'-CGGCAAGGCAGGTAACTTTCTC-3' and reverse primer, 5'-ATGGCAAAAGGCAGGTAACTTCTC-3'; and probe, 6FAM-TGACGGAGAATGC-MGBNFQ. The 20X Mouse GAPD (GAPDH) FAM/MGB Probe (Applied Biosystems) was used as the endogenous control. Target gene reactions were formed in a total reaction volume of 25 μl using 12.5 μl of 2X TaqMan Universal PCR master mix (Applied Biosystems), 900 nM of each primer, 200 nM probe, 2.95 μl of RiboQuant MultiProbe RNase protection assay system according to the manufacturer’s protocol. RNase protection assays were conducted using human CK5-hCK2b sets for human (BD Pharmingen) as per the manufacturer’s protocol. Gels were visualized by autoradiography and quantified using a PhosphoImager Molecular Imager and the Quantity One software (Bio-Rad). The signal intensity of individual genes was normalized to the intensity of the housekeeping gene GAPDH within the same sample.

Statistical analysis

Statistical analyses were performed using GraphPad Instat version 3.01. All data are expressed as mean ± SD. The results were analyzed for statistical variance using an unpaired Student’s t test or one-way ANOVA as appropriate. Results were considered significant when p < 0.05.

Results

Immunized mice display enhanced innate immune responses to adenovirus

The majority of humans have been exposed to common adenovirus serotypes and carry neutralizing antiviral Abs. To determine the impact of preexisting immunity on the innate immune response to adenovirus, C57BL/6 mice were immunized with AdGFP (1 × 10¹¹ particles i.v.) or vehicle control 10 days before a second challenge with the adenovirus vector AdLacZ (2 × 10¹¹ particles i.v.). At 6 h following AdLacZ administration, mice were sacrificed and analyzed. As expected in naive mice, AdLacZ induced the expression of numerous inflammatory genes in the liver including IP-10, MCP-1, TCA-3, and MIP-1β (Fig. 1, A and B). In the serum, IL-1β levels were also elevated following AdLacZ administration as determined by ELISA (Fig. 1C). In contrast, immunized mice were not protected from a second adenovirus challenge and displayed significantly higher levels of inflammatory gene expression in the liver and IL-1β levels in the serum (Fig. 1, A–C). Small increases in IFN-α4 mRNA could be detected in the livers of naive mice receiving AdLacZ that was enhanced by prior immunization, although the differences did not reach statistical significance (Fig. 1D).

To assess the effect of increased inflammation on adenovirus-mediated LacZ expression, livers were analyzed histologically for transgene expression. At 6 h, LacZ expression was clearly evident...
in ~30% of hepatocytes in naive mice. In contrast, absolutely no
LacZ expression could be detected in immunized mice re-
ceiving AdLacZ (Fig. 1E). The lack of transgene expression was
associated with a significant reduction in AdLacZ genomes in the
liver determined by PCR for the LacZ transgene reflecting a sig-
nificant alteration in viral tropism, increased viral clearance, or
both in immunized mice (Fig. 1F). Since the majority of i.v. ad-
ministered adenovirus targets to the liver in naive mice, collec-
tively these results suggest that immunization modulates adenovi-
rus interaction with the innate immune system in vivo.

Serum Ig increases adenovirus internalization in macrophages
The enhanced innate immune response to adenovirus in immu-
nized mice suggested that antiviral Abs may significantly alter vi-
ral interactions with innate effector cells. To examine the mecha-
nism of this observation further, pooled HS (due to the high
seroprevalence to adenovirus in the human population) was used
as a source of anti-adenovirus Abs for in vitro studies. First, the
ability of the pooled HS to neutralize adenovirus was tested. In-
fection of HeLa cells with $1 \times 10^4$ part/cell of an E1-deleted,
E3-defective, serotype 5-based adenovirus vector encoding GFP
(AdGFP) resulted in strong expression of the transgene as deter-
mined by fluorescent microscopy and FACS analysis at 24 h (Fig.
2A). In contrast, when the tissue culture medium was supple-
mented with 10% pooled HS, GFP transgene expression was al-
most completely abrogated (Fig. 2A). Southern blot analysis of
HeLa cell DNA and blotting for the adenovirus fiber gene showed
that internalized vector genomes correlated with GFP transgene
expression; significantly less adenoviral DNA could be detected
when the cells were infected in the presence of 10% pooled HS,
suggesting that AdGFP failed to enter the cells (Fig. 2B). These
results confirm that pooled HS neutralizes adenovirus entry and
infection in nonhematopoietic, epithelium-derived cells, consistent
with the presence of antiviral Abs.

Macrophages play a significant role in the innate immune re-
response to adenovirus in vivo (9–12). To determine the role of
serum components in macrophage-adenovirus interactions, THP-1
cells were infected with AdGFP in the presence of pooled HS and
the amount of intracellular vector genomes was analyzed by
Southern blot probing for the adenovirus fiber gene (Fig. 3A). In
the presence of normal tissue culture medium (FBS), medium containing 10% HS, or 10% HS-Ig, Quantitative analysis (right) of GFP transgene expression in
AdGFP-infected THP-1 cells determined by FACS analysis. VH, Vehicle. Values represent mean ± SD, n = 3. Cells infected in the presence of 10% HS or HS-HI showed significantly less GFP expression compared with cells infected in FBS or 10% HS-Ig (*** p < 0.001). Differences
between HS and HS-HI or between FBS and HS-Ig were not significant.

FIGURE 4. Adenovirus activation of human THP-1 cells. A, RNase protection assay of THP-1 cell RNA 6 h following infection with AdGFP in different
serum conditions. AdGFP induced expression of IP-10, MIP-1β, MIP-1α, MCP-1, and IL-8 mRNA is severalfold higher in the presence of 10% pooled
HS compared with FBS containing normal tissue culture medium, but returns to baseline activation when the HS is IgG depleted. B, TNF-α and IFN-β
mRNA is also induced in AdGFP-infected THP-1 cells in the presence of HS (RNase protection assay). C, Immunoblotting for pro-IL-1β in extracts and
mature IL-1β (D116) in supernatants of THP-1 cells infected with wild-type adenovirus (Ad5) in the presence of FBS or HS. Cells were also pretreated
with the pan-caspase inhibitor zVAD-fmk (50 μM). D, Fluorescent microscopy images of THP-1 cells 24 h after infection with AdGFP in the presence
of normal tissue culture medium medium, medium containing 10% HS, or 10% HS-Ig. Quantitative analysis (right) of GFP transgene expression in
AdGFP-infected THP-1 cells determined by FACS analysis. VH, Vehicle. Values represent mean ± SD, n = 3. Cells infected in the presence of 10% HS
or HS-HI showed significantly less GFP expression compared with cells infected in FBS or 10% HS-Ig (*** p < 0.001). Differences
between HS and HS-HI or between FBS and HS-Ig were not significant.
presence of HS-HI but not IgG-depleted HS or FBS consistent with Ab, but not complement-mediated adenovirus entry. To further confirm that antiviral Abs increased viral entry, THP-1 cells were infected with Cy2-labeled serotype 5 adenovirus (Cy2-Ad) in the presence or absence of HS and uptake was determined at 90 min by flow cytometry (Fig. 3B). Cy2-Ad uptake by THP-1 cells was significantly enhanced in the presence of HS compared with FBS.

THP-1 cells are capable of phagocytosing IgG-opsonized immune complexes by FcγRI (CD64) and FcγRIIa (CD32) (27). To assess the contribution of THP-1 cell-expressed FcγRs in

FIGURE 5. Adenovirus infection and activation of primary mouse macrophages. A, Increased virus vector uptake by primary mouse bone marrow macrophages in the presence of immunized MS. Southern blot analysis of internalized vector genomes 6 h after AdGFP infection of primary mouse bone marrow macrophages blotting for the adenovirus fiber gene and corresponding quantification (based on phosphor imaging). The tissue culture medium was serum-free (SF) or supplemented with 10% FBS, MS, MS-HI, or MS from mice that had been immunized with $1 \times 10^9$ part/mouse Ad-GFP 10 days earlier (referred to as immune serum, MS + Ig). Values represent mean density ± SD, n = 3. Viral vector uptake by primary macrophages was significantly higher in the presence of immune MS (MS + Ig) than in the presence of serum-free medium (**, p < 0.01), MS (+, p < 0.05), or MS-HI (***, p < 0.01). B, RNase protection assay of primary mouse bone marrow macrophage RNA 6 h after infection with AdGFP. Cells were infected in tissue culture medium supplemented with 10% MS, 10% immune MS (MS + Ig), or 10% MS-HI. AdGFP induced the expression of RANTES, MIP-1β, MIP-1α, MIP-2, TCA-3, and IP-10 above baseline. Ad-induced chemokine and cytokine expression was highest in the presence of immune serum (MS + Ig). C, Real-time PCR analysis of type I IFN expression (IFN-β; IFN-α) in bone marrow-derived murine macrophages at 6 h after infection with wild-type adenovirus. Infections were performed in the presence of 10% naive (MS) or adenovirus-immunized MS (MS + Ig). Values represent mean percent induction over vehicle-treated cells ± SD, n = 6 (MS vs MS + Ig; ***, p < 0.001). D, Fluorescent microscopy of primary murine bone marrow macrophages infected with AdGFP. Cells were infected with AdGFP in the presence of either FBS containing tissue culture medium (FBS), medium containing 10% MS, or 10% immune MS (MS + Ig). Immune serum abrogates transgene expression in AdGFP-infected primary mouse bone marrow macrophages. Quantification of GFP expression (left) in AdGFP-infected primary mouse bone marrow macrophages by FACS analysis. Values represent mean ± SD, n = 3. Cells infected in the presence of immune serum (MS + Ig) showed significantly less GFP expression compared with cells infected in the presence of MS or MS-HI (***, p < 0.001). E, RNase protection assay showing chemokine mRNA expression in primary mouse bone marrow macrophages 6 h after incubation with tropism-modified adenovirus vectors in the presence or absence of immune serum. Ps-Ad, transcription-defective adenovirus vector; AdL-PB, RGD-deleted adenovirus vector; AdL.F, fiber-modified, CAR-ablated adenovirus vector. Adenovirus-induced expression of chemokine and cytokine mRNA is increased in the presence of MS containing neutralizing Abs (+) compared with MS alone (−).
the uptake of adenovirus in the presence of HS, blocking experiments were performed with saturating amounts of anti-FcγR-blocking Abs or isotype controls (Fig. 3C). The uptake of Cy2-Ad in the presence of HS was reduced by preincubating THP-1 cells with FcγRII- or FcγRI-blocking Abs or both, suggesting a role for both receptors in serum IgG-mediated adenovirus uptake. Taken together, these results show that pooled HS contains Abs that neutralize adenovirus infection in epithelial cells but enhance FcR-mediated internalization in macrophages.

Abs amplify the antiviral innate immune response to adenovirus in macrophages

Macrophages are a major source of proinflammatory cytokines after infection with adenovirus (9–12) and viral internalization is a prerequisite for adenovirus induction of innate immune responses (9, 13). Therefore, experiments were performed to determine whether the Ab-dependent increase in adenovirus internalization in macrophages coincided with enhanced activation of the innate immune system. THP-1 cells were incubated with AdGFP in different serum conditions. Six hours following infection, total RNA was harvested and analyzed for the induction of chemokine and cytokine expression using a RNase protection assay. In FBS, AdGFP induced, albeit weakly, the expression of IP-10, MIP-1β, MIP-1α, MCP-1, and IL-8 in THP-1 cells (Fig. 4A), reflecting limited viral entry under these conditions. Viral infection in the presence of 10% pooled HS significantly increased adenovirus-induced cytokine/chemokine gene expression, a response that returned to baseline when the serum was IgG depleted (Fig. 4A). Consistent with the increase in NF-kB-dependent gene expression, TNF-α and IFN-β mRNA expression was also significantly increased in the presence of immune serum (Fig. 4B). Experiments were also performed to determine whether Ab-mediated adenovirus entry enhanced IL-1β maturation, a marker of inflammasome activation. Differentiated THP-1 cells were infected with 5 × 10⁵ part/cell of wild-type adenovirus and 6 h later the cell culture supernatant and cell extracts were analyzed for processed (17 kDa) IL-1β by immunoblotting. At this multiplicity of infection, adenovirus failed to activate IL-1β above baseline in the absence of HS (Fig. 4C). In the presence of HS that increases viral cell entry, adenovirus efficiently induced IL-1β processing and secretion of the 17-kDa cytokine at 6 h, an effect that was inhibited by the pan-caspase inhibitor zVAD-fmk (Fig. 4C). These results confirm that adenovirus Abs amplify the activation of innate immune pathways in macrophages that result in the expression of NF-kB-dependent genes, type I IFNs, and IL-1β maturation. Interestingly, the increase in adenovirus internalization and amplification of the innate immune response in the presence of Abs was associated with a significant decrease in virus-mediated GFP expression. This observation is consistent with increased type I IFN expression and enhancement of the antiviral state following Ab-dependent adenovirus internalization (Fig. 4D).

To confirm the findings in THP-1 cells, similar experiments were performed in primary bone marrow-derived murine macrophages (Fig. 5). AdGFP uptake in primary mouse bone marrow macrophages was significantly increased in the presence of serum from mice immunized with adenovirus (MS plus Ig) as determined by Southern blot, compared with naive MS alone, heat-inactivated mouse serum (MS-HI), serum-free medium, or FBS (Fig. 5A). The Ab-dependent increase in AdGFP internalization in primary macrophages was also associated with amplified RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, and type I IFN expression (Fig. 5, B and C). Similar to the results obtained in THP-1 cells and consistent with enhancement of the antiviral response, AdGFP infection resulted in substantially less GFP expression despite increased viral uptake in the presence of adenovirus-immunized MS (Fig. 5D). Together, these data show that immune serum enhances adenovirus internalization and activation of the antiviral innate immune response in primary macrophages. Again, complement inactivation did not affect the entry or activation of macrophages induced by AdGFP, confirming that complement plays little role in mediating adenovirus-macrophage interactions in vitro.

**FIGURE 6.** Ab-dependent amplification of the NLRP3 inflammasome and TLR9-independent IFN pathway. A, Immunoblotting for IL-1β in cell extracts (ext) or supernatants (sup) from LPS-primed wild-type (WT), NLRP3−/−, or ASC−/− peritoneal macrophages 6 h following infection with wild-type adenovirus (Ad5) in the presence of 10% MS or 10% immune MS (MS + Ig). Vehicle-treated cells served as negative control. The pan-caspase inhibitor zVAD-fmk (50 μM) was added to cells before infection. Adenovirus-induced IL-1β processing is enhanced in the presence of immune serum, but completely absent in ASC−/− and NLRP3−/− macrophages. B, Real-time PCR analysis of type I IFN expression (IFN-β, IFN-α) in TLR9+/+ (□) and TLR9−/− (■) bone marrow-derived murine macrophages at 6 h after infection with wild-type adenovirus. Infections were performed in the presence of 10% naive (MS) or adenovirus-immunized MS (MS + Ig). Values represent mean percent induction over vehicle-treated cells ± SD, n = 4 (TLR9+/+ vs TLR9−/−, p > 0.05).
The previous results suggest that adenovirus-macrophage interactions differ considerably from the classic paradigm of viral entry in epithelial cells that depend on binding to CAR and αV integrins. To confirm that the Ab-dependent enhancement of viral entry and innate activation in macrophages occurred independent of viral transcription or capsid domains that mediate adenovirus binding to αV integrins and CAR, experiments were performed using the modified vectors Ps-Ad, AdL.PB*, and AdL.F* (13). Ps-Ad is an AdGFP vector, which was rendered transcription defective by UV-psoralen treatment. AdL.F* and AdL.PB* have mutations in the fiber gene (AdL.F*) and in the RGD peptide in the penton base (AdL.PB*), which ablates their interaction with the adenovirus receptor CAR and coreceptor integrins for internalization, respectively (13). Tropism-modified and transcription-defective adenovirus vectors all induced a similar degree of chemokine mRNA expression in primary macrophages. Not surprisingly and consistent with the previous data demonstrating the retargeting of adenovirus to FcRs, macrophage activation was significantly increased in all cases by the presence of immune serum (Fig. 5E). These results show that viral gene transcription, CAR-binding and capsid RGD domains are dispensable for the Ab-dependent amplification of the innate immune response to adenovirus.

**Ab-dependent amplification of the innate immune response to adenovirus is dependent on the NLRP3 and TLR9-independent pathways in macrophages**

Macrophages and monocytes express a wide range of intracellular TLR and NLR proteins that recognize a vast array of pathogen-associated molecular patterns and nonpathogen-associated danger signals (28). The correlation of internalized vector genomes with cellular activation suggests that adenovirus directly activates an intracellular innate receptor system, an effect that is increased by antiviral Abs. TLR9, NLRP3, and a TLR-independent DNA sensing pathway have all recently been shown to detect intracellular adenovirus virions and/or DNA (15–17, 29). To confirm that Ab-dependent amplification of the innate immune response to adenovirus was mediated specifically via intracellular innate pathways, we first assessed the role of the NLRP3 inflammasome directly using NLRP3−/− and ASC−/− macrophages. Consistent with the previous findings, caspase-dependent IL-1β maturation was amplified in LPS-primed murine peritoneal macrophages stimulated with adenovirus in the presence of immune serum (Fig. 6A). In contrast, IL-1β processing and secretion was completely absent in NLRP3- and ASC-deficient macrophages confirming that antiviral Abs specifically enhance activation of the NLRP3 inflammasome in response to adenovirus (Fig. 6A). In macrophages, adenovirus induction of type I IFNs occurs primarily via the TLR-independent DNA-sensing pathway, whereas TLR9 predominates in dendritic cells (16, 17). The role of the TLR-independent pathway in the Ab-dependent amplification of type I IFNs was therefore assessed indirectly using TLR9−/− macrophages. Not unexpectedly, TLR9 was not required for type I IFN expression in response to adenovirus in the presence of immune serum. Adenovirus-induced IFN-β and IFN-α expression was similar in peritoneal macrophages (data not shown) and bone marrow-derived macrophages isolated from TLR9−/− and wild-type mice (Fig. 6B).

**FIGURE 7.** Phagocytosis and adenovirus activation of the innate immune response. A, CCB (10 µg/ml) blocks THP-1 cell-adenovirus uptake. Cy2-Ad was incubated with THP-1 cells in the presence or absence of HS and CCB. Cells were washed and analyzed by flow cytometry at 90 min. Values represent mean fluorescence ± SD, n = 3–6. CCB significantly decreases Cy2-Ad uptake in the presence of HS; ***, p < 0.001. B, Immunoblotting for mature IL-1β (D116) in cell extracts (ext) or supernatants (sup) in PMA-differentiated THP-1 cells 6 h after infection with adenovirus. Immunoblotting of cell extracts and supernatants demonstrates increased mature IL-1β in the presence of HS, which is reduced in the presence of CCB, confirming that viral entry is required to activate the inflammasome. C, Immunoblotting for pro-IL-1β in cell extracts or mature IL-1β (D116) in supernatants from PMA-differentiated THP-1 cells 6 h after infection with wild-type adenovirus in the presence of HS. Increased IL-1β maturation is observed in the presence of HS, which is reduced in the presence of bafilomycin A1 (BafA1; 100 nM). D, Real-time PCR analysis of type I IFN expression bone marrow-derived murine macrophages at 6 h following infection with wild-type adenovirus. Infections were performed in the presence of 10% adenovirus-immunized MS with or without bafilomycin A1 (100 nM) or CCB (10 µg/ml). Values represent mean percent induction over vehicle-treated cells ± SD, n = 3 (untreated cells vs bafilomycin A1 or CCB-treated cells; *, p < 0.05 and **, p < 0.01).
Taken together, these data are consistent with Ab-dependent amplification of the TLR-independent DNA-sensing pathway following adenovirus infection in macrophages.

**Antiviral Abs target the adenovirus to phagolysosomes in macrophages**

Recent studies show that the activation of several innate pathways is linked to phagosomes and phagosome maturation in macrophages (20, 21). In epithelial cells, >90% of adenovirus escapes the early endosome during viral entry (30). Little is known regarding viral trafficking of adenovirus in macrophages. In the presence of opsonins though, it is likely that the adenovirus is subjected to entry and trafficking based on the properties of the respective opsonin receptor. In the context of antiviral Abs, adenovirus likely undergoes FcR-mediated phagocytosis. Given the robust activation of the innate immune response in this setting, it is possible that this mode of viral internalization targets the adenovirus to phagosomes and phagolysosomes that are implicated in the activation of the innate immune system (20, 21). To test this possibility, functional experiments were first performed using CCB and bafilomycin A1 to block phagocytosis and endosomal acidification, respectively, during adenovirus infection. At 6 h following adenovirus infection in THP-1 cells and primary mouse macrophages, both CCB and bafilomycin A1 diminished Ab-mediated amplification of type I IFN expression and IL-1β maturation (Fig. 7). These results suggest that phagocytosis and phagosome maturation are an important component of the Ab-mediated amplification of the innate immune response to adenovirus.

Next, to determine the effect of antiviral Abs on adenovirus localization in macrophages, confocal microscopy was performed. To stain lysosomes, HEK 293 cells and differentiated THP-1 cells were pulsed with Alexa Fluor 594-labeled 40-kDa dextran and chased overnight. Cells were then infected for 1 h with 1 × 10⁴ part/cell of Cy2-labeled adenovirus and images captured in living cells. In the absence of human serum, Cy2-Ad entry in 293 cells was efficient and easily detectable (Fig. 8A) but did not colocalize significantly with dextran-containing vesicles. Similarly, in the presence of human serum and despite less viral entry, little lysosome colocalization was found. In line with the previous studies, viral entry was found to be inefficient in THP-1 cells infected with adenovirus in the presence of FBS, as few cells demonstrated Cy2 positivity (Fig. 8B). Occasional virions however did colocalize with dextran-labeled vesicles when infection were conducted in FBS. In contrast, THP-1 cells infected with adenovirus in the presence of HS demonstrated considerable virus uptake at 1 h that was localized to dextran-labeled vesicles indicative of lysosomes (Fig. 8C and D). To confirm that adenovirus localization was in fact with late phagosomes/phagolysosomes under these serum conditions, staining for the late phagosome marker LAMP1 was performed. Consistent with the dextran studies, adenovirus strongly colocalized with LAMP1 following infection in the presence of HS (Fig. 8C). Importantly, quantification studies revealed that HS not only increased viral entry but also the relative proportion of virions that colocalized to LAMP1-positive phagosomes. In the presence of FBS, <10% of internalized adenovirus particles were found within LAMP1-positive phagosomes compared with >50% of internalized virions in the presence of HS (Fig. 8D). Therefore, these results demonstrate that Ab-dependent amplification of the innate immune response is associated with adenovirus targeting to phagolysosomes in macrophages.

**Discussion**

The sensing of infectious agents is an essential component of first-line host defense. After recognition of viral components by host
pattern recognition receptors, immune cells initiate an antiviral response that consists of cytokine and type I IFN production creating an antiviral state and promoting the development of adaptive immunity (28, 31). In macrophages and dendritic cells, the innate machinery that recognizes adenovirus is intracellular and recently identified as TLR9, a TLR-independent DNA-sensing pathway that activates IFN regulatory factor 3 and the NLRP3 inflammasome (15, 17, 29). However, the adenovirus lacks a specific tropism for macrophages, suggesting that other host mechanisms are in place to target virions to the innate immune system. In this study, we demonstrate that the adaptive immune system and antiviral Abs in particular subvert the natural tropism of the adenovirus to target virions to phagolysosomes in macrophages. The Ab-dependent increase in viral entry results in the specific activation of the NLRP3 inflammasome and the TLR9-independent type I IFN pathway revealing a novel function for antiviral Abs in positively regulating the intracellular innate immune system.

Our results emphasize the importance of internalization for the induction of the innate immune responses to adenovirus (9, 13). Therefore, it is not surprising that the immune system has evolved an adaptive mechanism to target virions to the cellular compartments required to mount an optimal antiviral response. We show that the Ab-dependent amplification of the innate immune response to adenovirus was associated with viral targeting to the phagolysosome. Although the exact mechanism of NLRP3 activation is only recently emerging, a link to endosomal compartments has been suggested in macrophages. Recent studies demonstrate that phagolysosome injury is a prerequisite for NLRP3 activation in response to silica crystals (20). Furthermore, M. tuberculosis actively inhibits phagosome maturation, progression to a phagolysosome, and subsequent inflammasome activation (21). Our results strengthen the view that NLRP3 activation is linked to phagolysosome stress. The amplification of type I IFN expression was also associated with increased adenovirus internalization and localization to the phagolysosome; however, the response in macrophages was not dependent on TLR9. Recent studies show that TLR-independent DNA sensing is the major mechanism of type I IFN expression in response to adenovirus in macrophages, while TLR9 mediates the response in plasmacytoid dendritic cells (15, 17). Consistent with this observation and our data, a recent study demonstrates that Ab-dependent amplification of innate responses to adenovirus in dendritic cells occurs via TLR9 (32). Unlike TLR9 however, little is known regarding the actual receptor and cellular localization of the TLR-independent DNA-sensing pathway in macrophages, although it is widely believed to be cytosolic. Although antiviral Abs increased viral targeting to LAMP1-positive phagolysosomes, our data also show that only 50% of internalized virions localized to these endosomes, indicating significant viral escape into the cytosol where activation of the TLR-independent DNA-sensing pathway could occur. This premise is consistent with the reduction in TLR9-independent type I IFN expression mediated by bafilomycin A1 since adenovirus requires endosomal acidification to penetrate and gain entry to the cytosol. Regardless of the receptor activated, our data show that antiviral Abs specifically target virions to intracellular compartments that facilitate the activation of the innate immune system. The diverse repertoire of the humoral immune system therefore confers a capability for the host to activate intracellular innate pathways such as TLR9 and the NLRP3 inflammasome in response to a broad range of pathogens.

Our findings also suggest that the antimicrobial role of the innate immune system does not end at the onset of adaptive immunity. The activation of a specific innate receptor that is facilitated by the humoral adaptive immune system displays a novel “inverse” cross-talk between adaptive and innate immunity. Our results show that antiviral Ig has functions beyond neutralizing virus and preventing infection in target tissues such as epithelia. Antiviral Abs modulate viral tropism to nontarget FcR-bearing cells such as macrophages where innate immune mechanisms reside, causing an up-regulation of antiviral immune responses. Moreover, the adenovirus has evolved to escape from the early endosome during cellular entry, a viral characteristic that may allow the virus to evade host innate mechanisms in the initial stages of an infection. The ability for the adaptive immune system to subvert the natural viral characteristics and rescue the antiviral capability of the innate immune system is illustrated by our studies. Despite relatively limited adenovirus infection under naive conditions, the adenovirus-expressed transgene was high in the face of a diminutive antiviral innate response, an effect that was reversed by the presence of antiviral Abs.

Collectively, we show that the Ab- and FcR-mediated internalization of viruses represents a mechanism by which the intracellular innate immune system might be activated. Since the majority of viruses do not exhibit a natural tropism for effector leukocytes of the innate immune system, the ability of virus-specific Abs to target infectious virions to the appropriate cellular compartments provides a mechanism to increase the efficiency of the innate immune system and the development of the antiviral state. This effect is illustrated by our in vivo studies where immunized mice received a second challenge with AdLacZ. The enhanced activation of the innate immune response in this setting was associated with a dramatic reduction in adenovirus-mediated gene transfer to the liver. Based on our in vitro studies that show increased targeting to macrophage phagolysosomes, the significant decline in AdLacZ genomes in the livers of immunized mice can be explained not only by a change in viral tropism but also by enhanced viral degradation. In most cases, this would provide an advantage to the host and expedite viral clearance. However, there are some instances where preexisting immunity can increase an innate immune response to subsequent viral infection that is detrimental. The administration of high-dose recombinant adenovirus for oncolytic or gene therapies to humans and other hosts with preexisting immunity results in increased and sometimes fatal toxicity (5, 33–35). Similarly, Ab-dependent enhancement also occurs in the setting of Dengue virus infection where heterotypic infection with related serotypes can trigger increased viral replication and a fatal inflammatory response (36). Finally, a recent study has shown that Ab-dependent amplification of the innate immune response to adenovirus vectors in dendritic cells can enhance HIV replication (32).

Our results shed new light on the function of antiviral Abs in the host response to viral infection. Not only is an Ab response important to neutralize infection of target tissues, but they play a role in targeting virions appropriate cellular compartments that enhance host defense. In this regard, our results show that the adaptive immune system has an ongoing cross-talk with the innate immune system that positively regulates the activation of the intracellular innate immune system that is essential in the host response to viruses.

Disclosures
The authors have no financial conflict of interest.

References


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uation of toxicity from high-dose systemic administration of recombinant ade-
