Supplemental Data

Virus Binding to a Plasma Membrane Receptor

Triggers Interleukin-1α-Mediated

Proinflammatory Macrophage Response In Vivo

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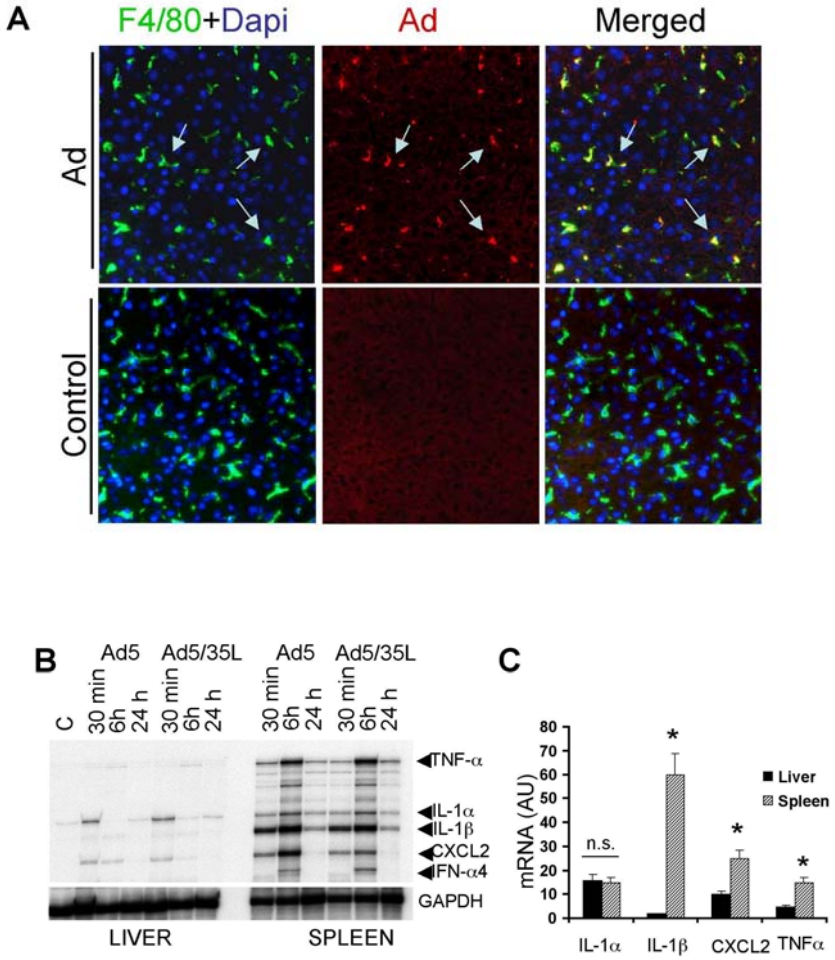

Figure S1. Interaction of Ad with Kupffer cells and transcriptional activation of pro-inflammatory cytokines and chemokines in the liver and spleen after intravenous Ad injection. (A) Ad was injected intravenously and 1 hour later, liver tissue was harvested, frozen in OCT compound and sections were stained with Ad-specific antibody (red) and macrophage F4/80-specific antibody (green) to detect Kupffer cells. In control settings (Control), mice were injected with saline. After staining of the liver sections with antibodies, the liver sections were analyzed

using conventional fluorescent microscopy. Kupffer cells, co-localized with Ad-specific staining are indicated by arrows. (B) Mice were injected with either Ad5 or fiber-chimeric Ad5/35L virus, that is unable to bind its primary cell attachment receptor in mice. At the indicated time points, spleens and livers of mice were harvested, total RNA was purified and processed by using RNAse protection assay. C – negative control RNA sample from a mouse injected with saline. Each line represents an individual mouse injected with either indicated virus or saline. For each time point the experiment was done in triplicate. Representative pictures are shown. (C) Quantitative representation of mRNA levels from the gel shown in (A) after phosphorimager analysis. Statistically significant differences between individual cytokine or chemokine mRNA levels in the liver and spleen are indicated by a star. * - $P < 0.01$. n.s.- no statistically significant differences between groups were identified ($P > 0.05$). AU – arbitrary units. Graphs show mean ± s.d.

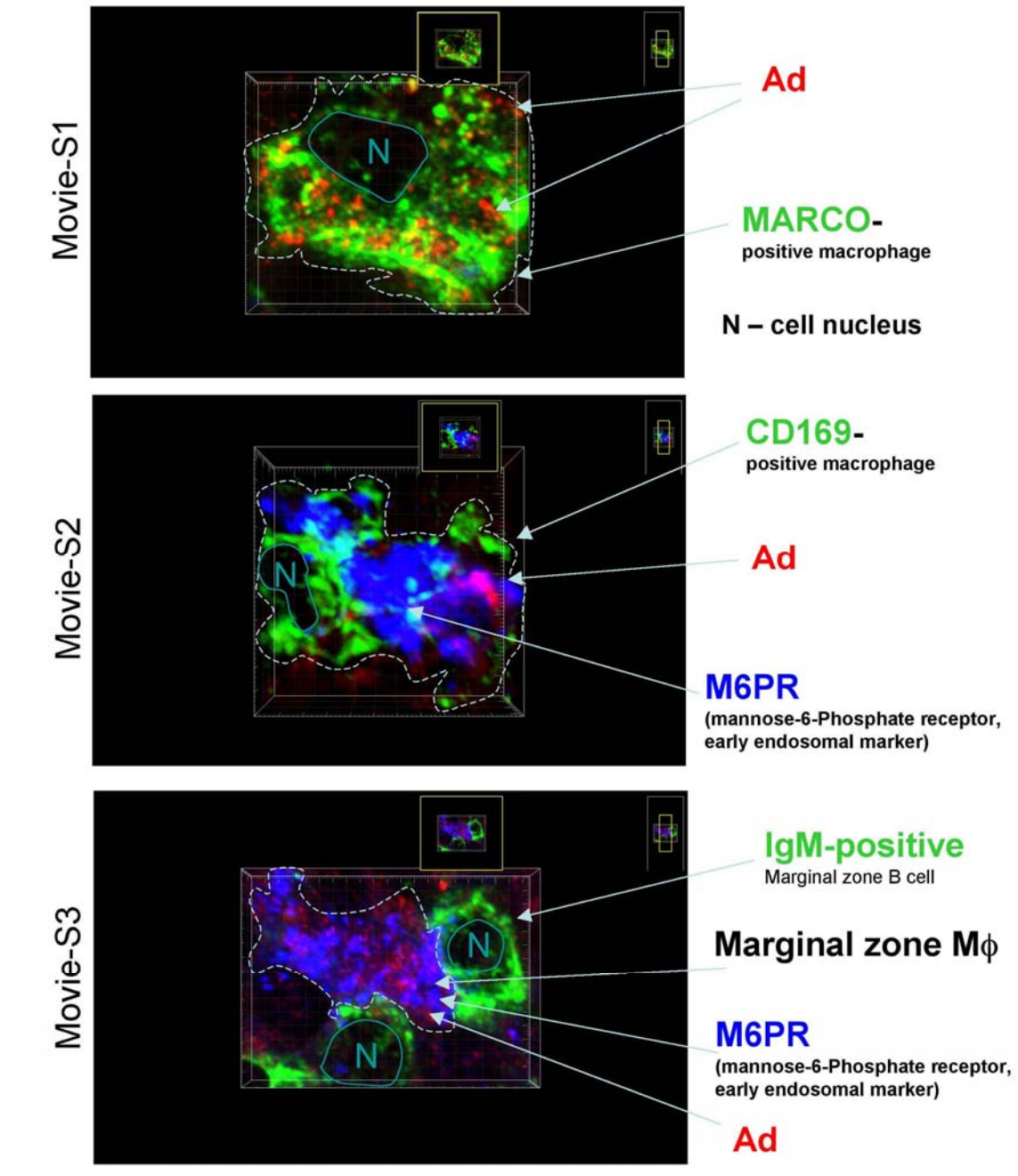

Figure S2. Color annotation to supplemental movies 1-3.

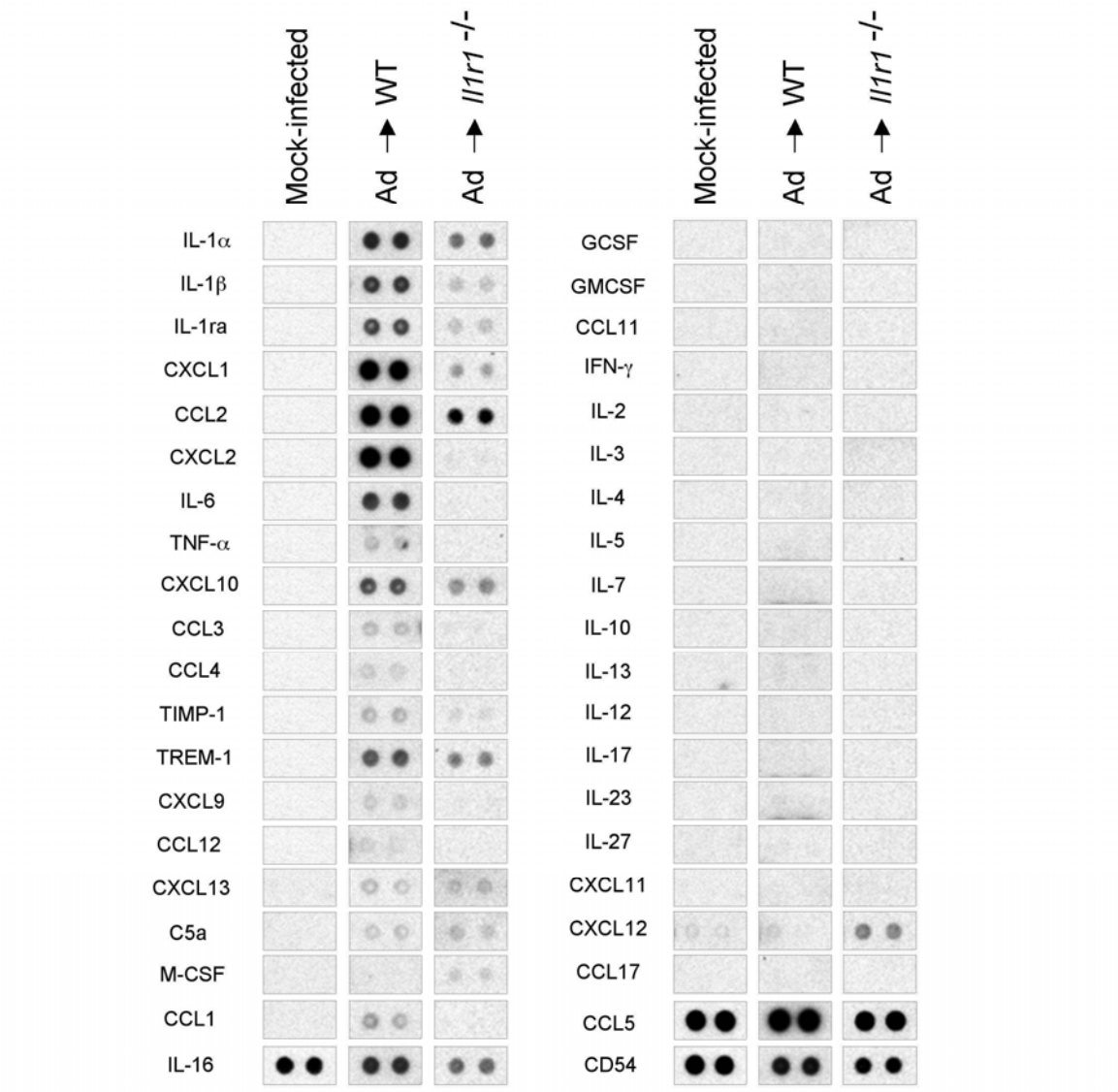

Figure S3. Activation of cytokine and chemokine expression in the spleen after Ad injection into control C57BL/6 (a) or *Il1r1*$^{-/-}$ mice, determined by the Proteome Profiler antibody array. Mice were injected with identical doses of Ad and 1 hour later spleens were harvested and analyzed for cytokine and chemokine expression using antibody array. Control mock-infected mice were injected with saline. All analyses were done in quadruplicate. Representative pictures are shown.

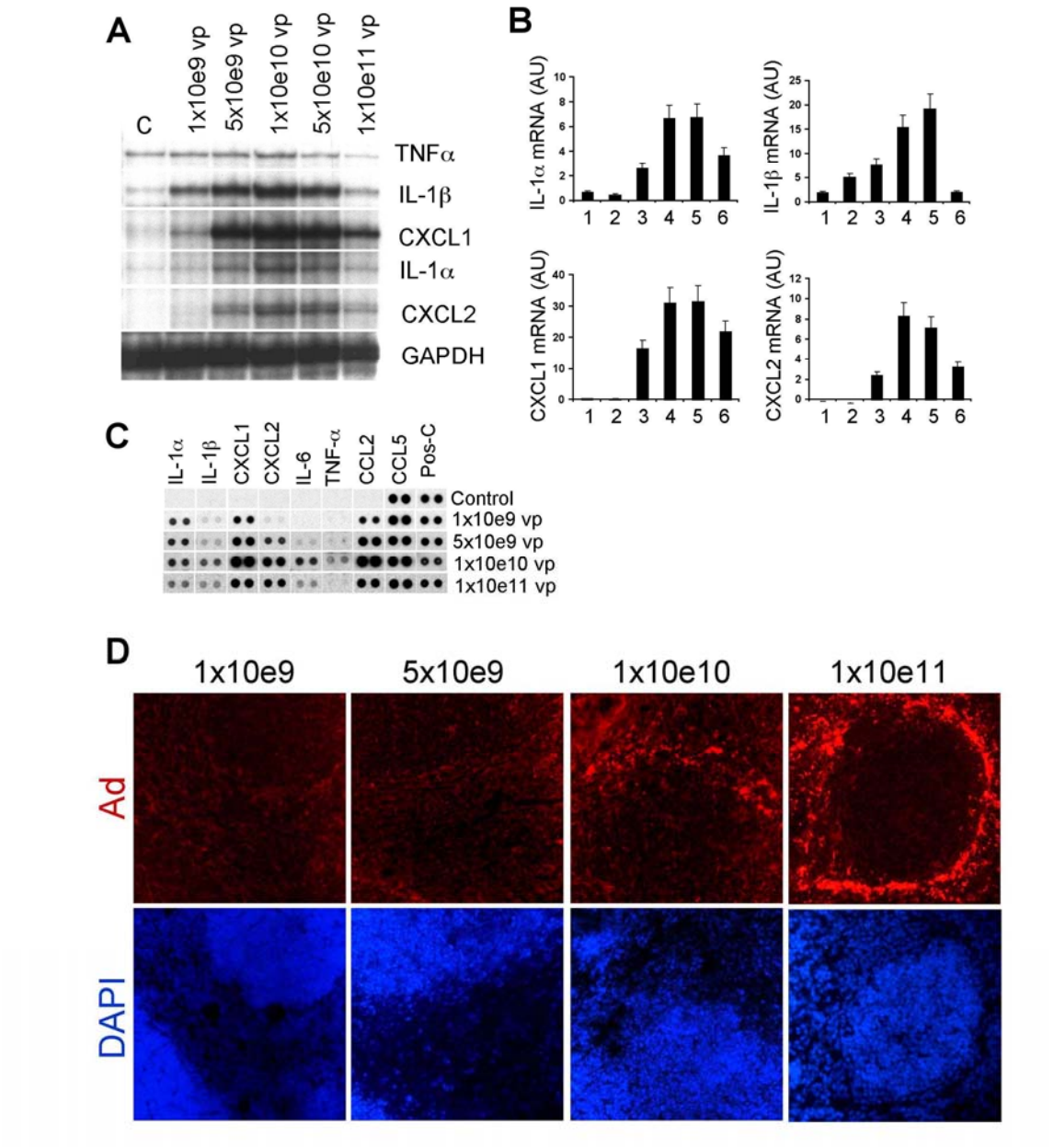

Figure S4. Dose-dependent activation of pro-inflammatory cytokines and chemokines after intravenous Ad injection in mice. (A) Mice were injected with Ad at indicated doses in virus particles (vp) per mouse and 30 min later spleens were harvested, and total RNA was purified and analyzed using RNAse protection assay. C – negative control RNA sample, obtained from a mock-infected mouse injected with saline. Representative pictures of RNA gels obtained from three independent experiments are shown. (B) Quantitative representation mRNA expression from the gel shown in (A) after phosphorimager analysis. AU – arbitrary units. 1 – negative control; 2 – mice injected with 10^9 vp of Ad; 3 – $5x10^9$ vp; 4 – 10^{10} vp; 5 – $5x10^{10}$ vp; 6 – 10^{11} vp of Ad. Graphs show mean ± s.d. n=3. (C) Analysis of protein expression for proinflammatory cytokines and chemokines in wild type mice injected with indicated doses of Ad per mouse. One hour after Ad injection, spleens were harvested and analyzed for proteins using mouse Proteome Profiler

antibody arrays as described in Experimental procedures. The representative pictures, obtained from three independent experiments, are shown. Control – negative control mice injected with saline. Pos-C are dots that show the manufacturer's internal positive control samples on the membrane. (D) Dose-dependent accumulation of Ad particles in marginal zone cells determined by immunofluorescence. Wild type (WT) mice were injected intravenously with indicated doses of Ad (in virus particles per mouse), and 1 hour later, spleens were harvested, frozen in OCT compound, sectioned and stained with anti-Ad hexon antibody (the major capsid protein of Ad particle). Ad-specific staining appears in red. To determine the localization of splenic germinal centers, sections were counter-stained with DAPI. N = 4. Representative pictures are shown.

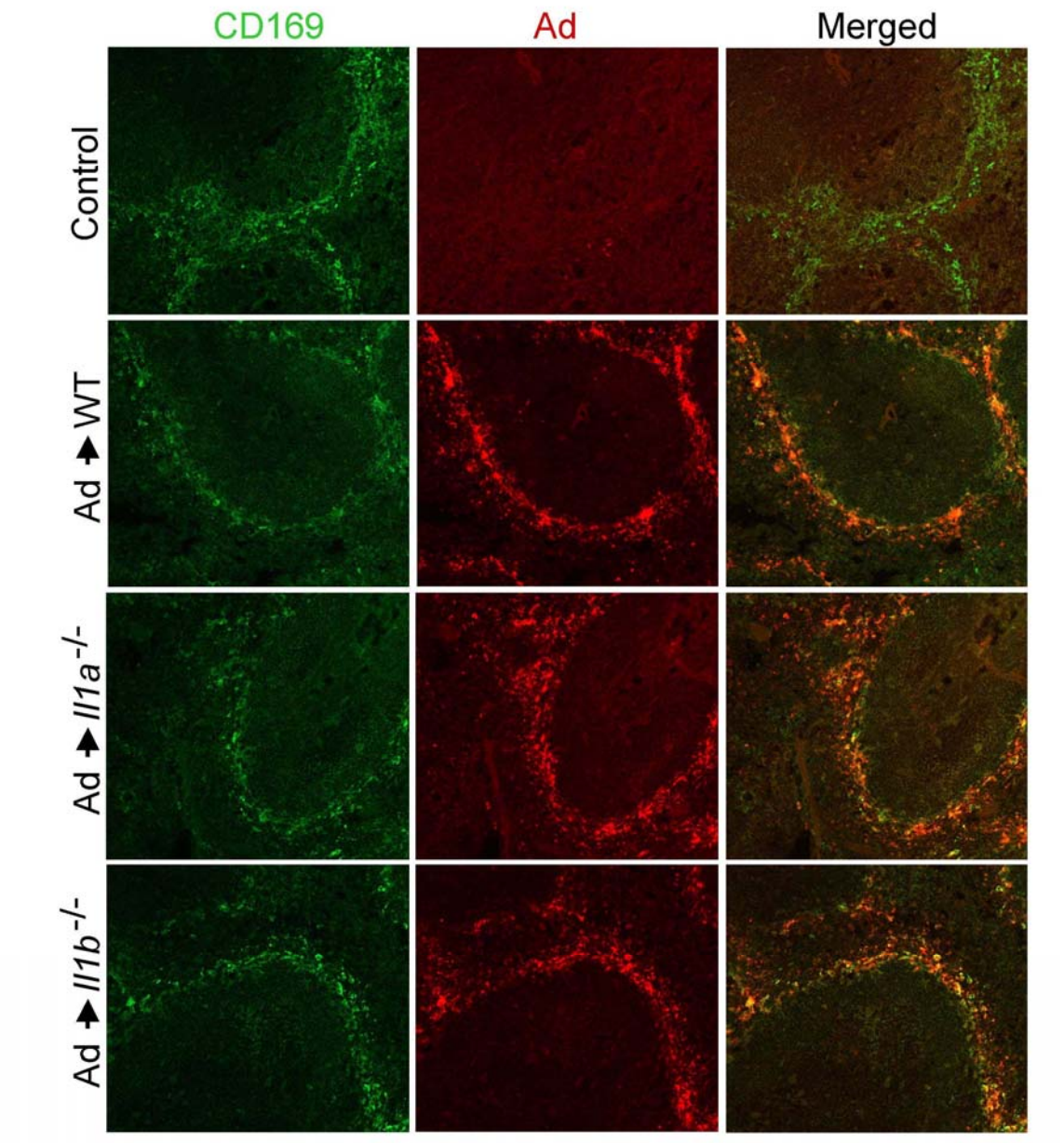

Figure S5. Immunofluorescent analysis of Ad accumulation in marginal zone cells in wild type mice and mice deficient for IL-1α and IL-1β after intravenous virus injection. Wild type mice (WT) or mice deficient for IL-1α or IL-1β were injected with equal doses of Ad and 1 hour later spleens were harvested, frozen in OCT compound, sectioned and stained with Ad-specific antibody (red) or CD169-specific antibody (green) to determine the localization of Ad particles and marginal zone macrophages. Images obtained in green and red channels were superimposed and the co-localization of Ad and marginal zone macrophages was evaluated. In control settings (Control) mice were mock infected by saline injection. At least 5 sections of spleens obtained from three individual mice injected with Ad were analyzed. Representative pictures are shown.

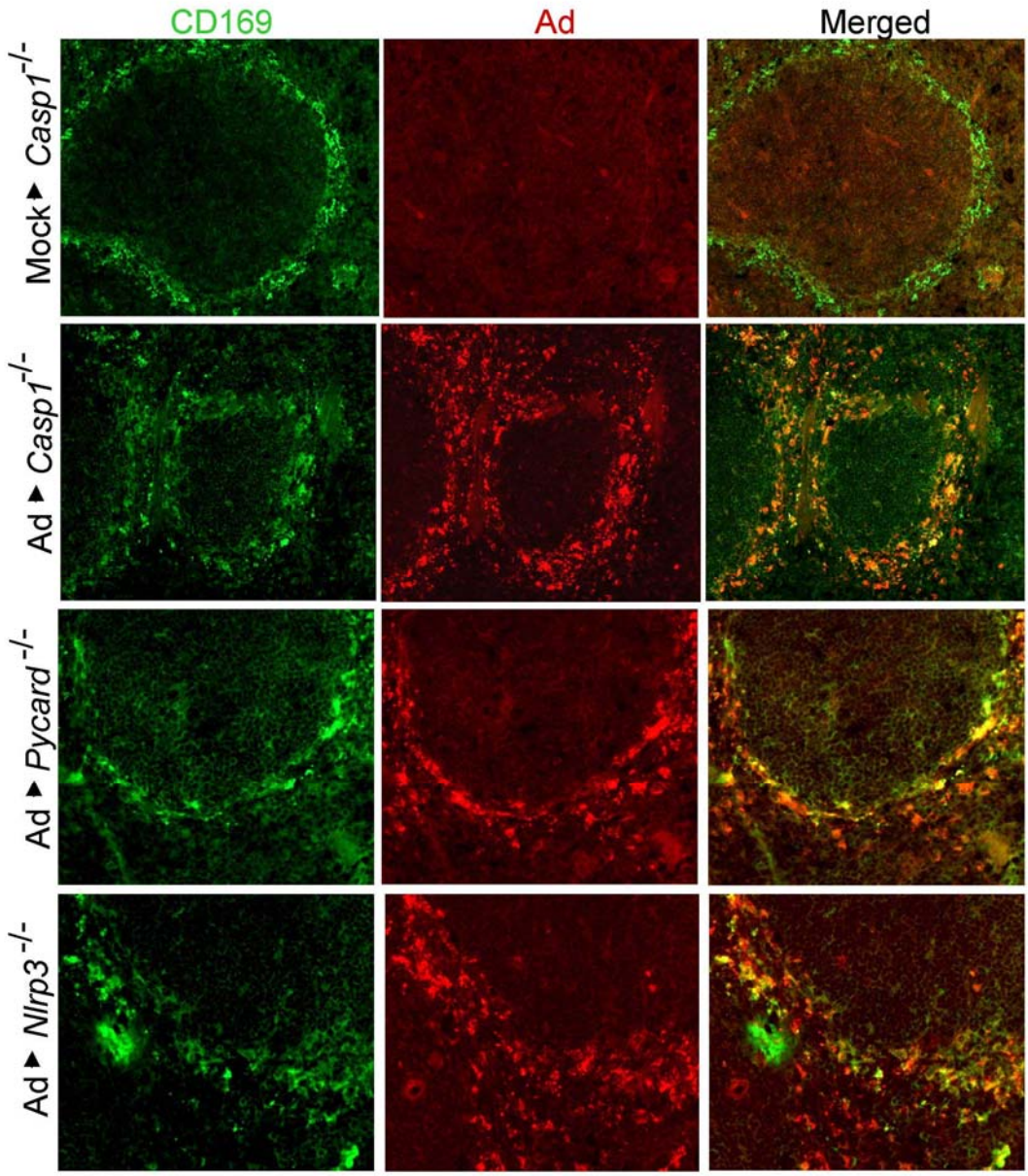

Figure S6. Immunofluorescent analysis of Ad accumulation in marginal zone cells of *Casp1*$^{-/-}$, *Pycard*$^{-/-}$, and *Nlrp3*$^{-/-}$ mice after intravenous virus injection. *Casp1*$^{-/-}$, *Pycard*$^{-/-}$, *Nlrp3*$^{-/-}$ mice were injected with equal doses of Ad and 1 hour later spleens were harvested, frozen in OCT compound, sectioned and stained with Ad-specific antibody (red) or CD169-specific antibody (green) to determine the localization of Ad particles and marginal zone macrophages. Images obtained in green and red channels were superimposed and co-localization of Ad and marginal zone macrophages was evaluated. In control settings (Mock) *Casp1*$^{-/-}$ mice were injected with saline. At least 5 sections of spleens obtained from three individual mice injected with Ad were analyzed. Representative pictures are shown.

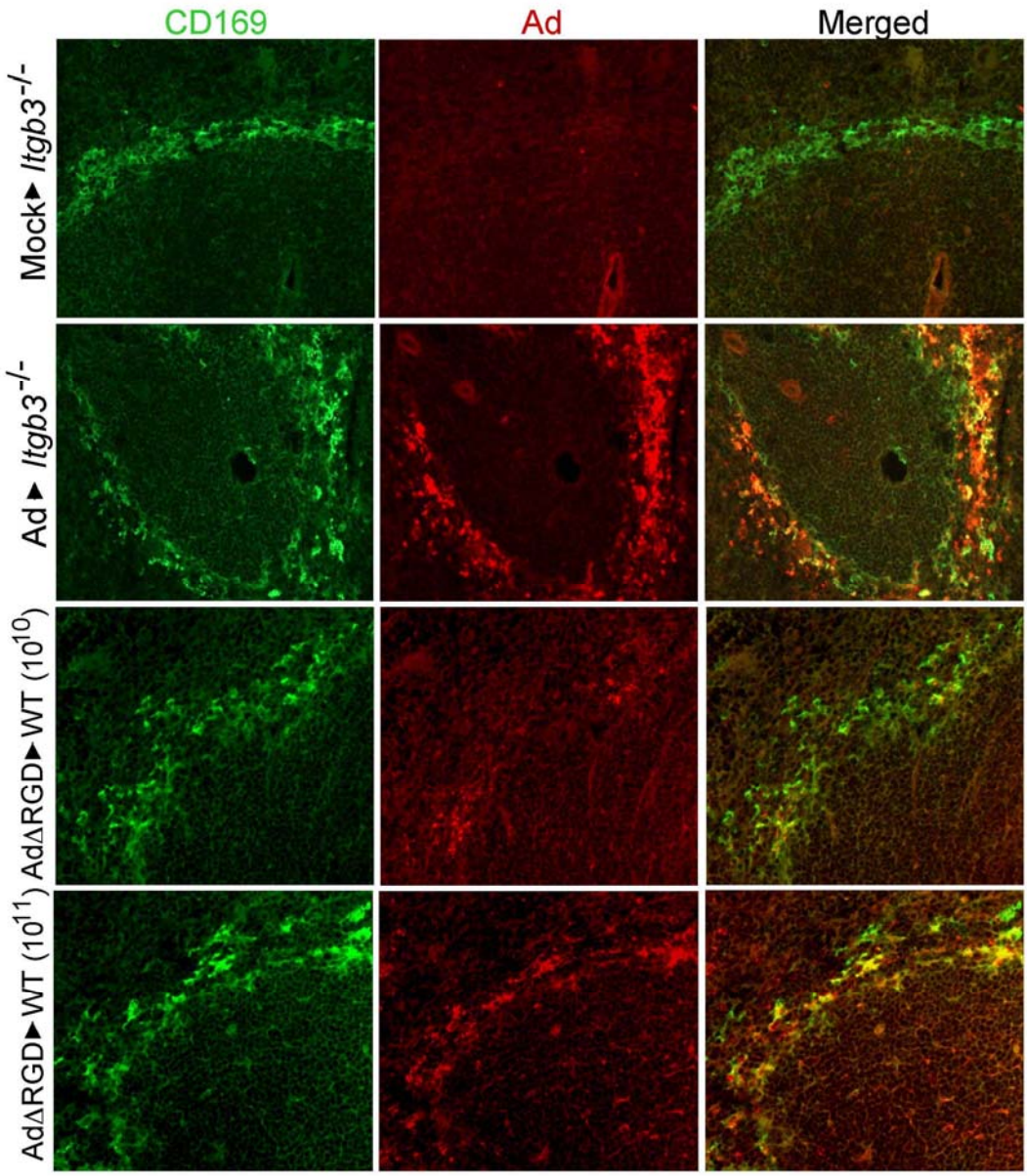

Figure S7. Immunofluorescent analysis of Ad accumulation in marginal zone cells of *Itgb3*$^{-/-}$ mice injected with Ad, and in wild type mice injected with AdΔRGD mutant. Wild type (WT) or *Itgb3*$^{-/-}$ mice were injected with Ad and 1 hour later spleens were harvested, frozen in OCT compound, sectioned and stained with Ad-specific antibody (red) or CD169-specific antibody (green) to determine the localization of Ad particles and marginal zone macrophages. Images obtained in green and red channels were superimposed and the co-localization of Ad and marginal zone macrophages was evaluated. In control settings (Mock) *Itgb3*$^{-/-}$ mice were injected with saline. At least 5 sections of spleens obtained from three individual mice injected with Ad were analyzed. Representative pictures are shown. Ad was injected in *Itgb3*$^{-/-}$ mice at a dose of 10^{10} virus particles per mouse. Doses (virus particles per mouse) of AdΔRGD mutant virus that were used for injection into wild type mice are shown in the legends to corresponding panels.

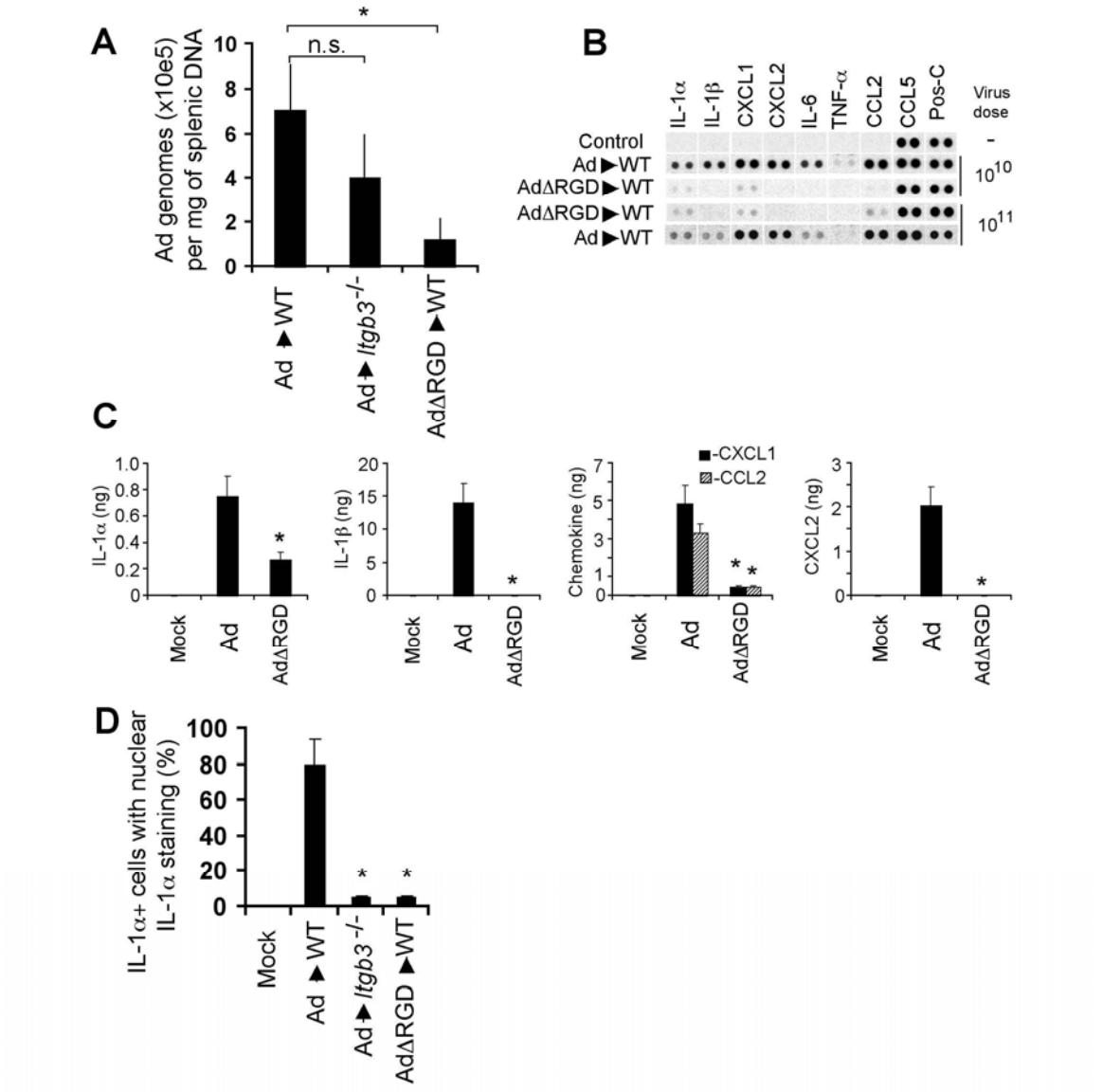

Figure S8. Interactions of Ad with splenic cells in *Itgb3*$^{-/-}$ mice and AdΔRGD mutant virus with cells in wild type mice. (A) Wild type (WT) or *Itgb3*$^{-/-}$ mice were injected with Ad as well as WT mice were injected with AdΔRGD mutant virus. One hour after virus injection, mice were sacrificed, spleens were harvested and total DNA was purified. The amount of Ad genomic DNA in spleens was analyzed by real-time PCR as described elsewhere. For each experimental setting, mice were injected with viruses in triplicates. Graphs show mean ± s.d. n.s. – statistically not significant. * - $P < 0.05$. (B) Analysis of protein expression for pro-inflammatory cytokines and chemokines in wild type mice (WT) injected with Ad or AdΔRGD at standard (10^{10} virus particles per mouse) or high (10^{11} virus particles per mouse) doses. Mice were injected with Ad at indicated doses and 1 h later spleens were harvested and analyzed for proteins using mouse Proteome Profiler antibody arrays. The representative pictures, obtained from three independent experiments, are shown. Mock – negative control mice injected with saline. Pos-C are dots that show the manufacturer's internal positive control samples on the membrane. (C) The amounts of

proinflammatory cytokines and chemokines in the spleens of mice 1 h after Ad injection. The amounts of analytes shown on y-axis were determined for 50 mg of splenic tissue. The amounts of indicated cytokines and chemokines were determined from three independent experiments. Mock – negative control mice injected with saline. * - $P < 0.01$. (D) Semi-quantitative presentation of the proportion of IL-1α-expressing marginal zone cells with IL-1α-positive nuclei in mock-injected and virus-injected mice. The nuclear IL-1α-positive staining was analyzed in two hundred IL-1α-positive cells on spleen sections of virus-injected groups. No IL-1α-positive nuclei were found on spleen sections of mock (saline)-injected animals. N=6. * - $P < 0.01$.

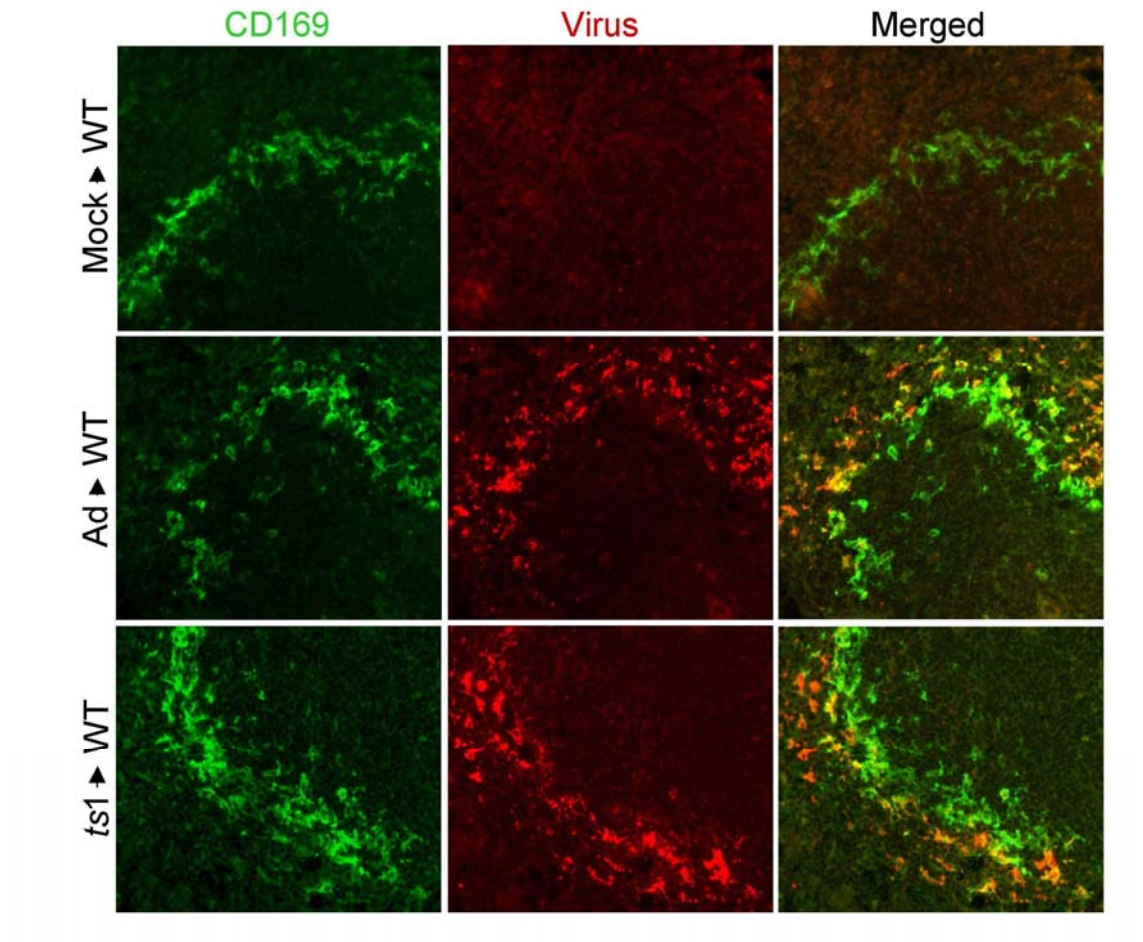

Figure S9. Immunofluorescent analysis of virus accumulation in marginal zone cells of wild type mice mock-injected, or injected with Ad or *ts*1 mutant virus. Wild type mice (WT) were injected with Ad or *ts*1 mutant virus and 1 hour later spleens were harvested, frozen in OCT compound, sectioned and stained with Ad-specific antibody (red) or CD169-specific antibody (green) to determine the localization of Ad particles and marginal zone macrophages. Images obtained in green and red channels were superimposed and the co-localization of Ad and marginal zone macrophages was evaluated. In control settings (Mock) mice were injected with saline. At least 5 sections of spleens obtained from three individual mice injected with viruses were analyzed. Representative pictures are shown. Viruses were injected into mice at a dose of 10^{10} virus particles per mouse.

Supplemental Experimental Procedures.

Viruses. For Ad amplification, 293 cells were infected under conditions that prevented cross-contamination. Viruses were banded in CsCl gradients, dialyzed and stored in aliquots as described earlier (Shayakhmetov et al., 2000). Ad genome titers were determined by OD_{260} measurement. For each Ad used in this study, at least two independently prepared virus stocks were obtained. Each produced virus stock was tasted for endotoxin contamination using *Limulus* amebocyte lysate Pyrotell (Cape Cod Inc, Falmouth, MA). For *in vivo* experiments, only virus preparations confirmed to be free of endotoxin contamination were used.

Ablation of β1 integrin expression in hematopoietic cells. β_1-integrin deletion is lethal in mice (Bouvard et al., 2001). To evaluate the contribution of β_1 integrin in the RGD motif-mediated activation of IL-1α after Ad injection in mice, we crossed *Itgb1*$^{flox/flox}$ mice with *Mx1*-CRE mice to obtain F_2 progeny with *Mx1*-CRE/*Itgb1*$^{flox/flox}$ and *Mx1*-CRE/*Itgb1*$^{flox/+}$ genotypes. The littermate mice with these genotypes were injected with polyI:C to induce activation of CRE expression and excision of β_1 integrin gene surrounded by *flox* sites. The resultant mice were either ablated for β_1-integrin expression (*h-Itgb1*$^{-/-}$) or were heterozygotes (*Itgb1*$^{-/+}$), primarily in hematopoietic compartment (Bouvard et al., 2001). The β_1 integrin ablation efficiency in hematopoietic cells was confirmed by flow cytometry analysis of surface β_1 integrin expression on peripheral blood monocytes. For our studies, only mice with β_1-integrin expression levels below 10%, compared to wild type mice, were used. The polyI:C-treated mice of *Itgb1*$^{-/+}$ genotype expressed β1 integrin

levels comparable to those observed in wild type mice and were used as matching controls in the analysis of macrophage response to Ad *in vivo*.

Adenovirus delivery and estimation of the actual virus dose *in vivo*. Previously published data shows that the frequency of ERTR9-positive marginal zone macrophages in the mouse spleen is 0.2-0.3% of splenocytes, and the frequency of CD169 (MOMA-1)-positive macrophages is 5.1% of splenocytes [see Figure 1 in (Ciavarra et al., 2006)]. According to our data on the absolute amount of Ad particles trapped in the spleen (**Figure S8**), we estimated that if mice are injected with a dose of 10^{10} virus particles, each marginal zone macrophage will receive between 50 and 100 virus particles per cell. We experimentally determined that an average mouse spleen possesses $2.5x10^8$ nucleated cells and yields on average 1 mg of total genomic DNA. Considering that the cumulative number of MZMϕ is around 5% (Ciavarra et al., 2006), the total average number of MZMϕ cells in a mouse spleen is approximately $1.25x10^7$. Our quantitative real-time PCR analysis shows that upon the injection of mice with Ad at a dose of 10^{10} virus particles per mouse, the total number of Ad genomes accumulated in the spleen 30 min after virus injection is $7x10^5$ per μg of splenic DNA **(Figure S8A)**, or $7x10^8$ per whole spleen. By dividing $7x10^8$ (the number of Ad genomes per whole spleen) by $1.25x10^7$ (the average number of MZMϕ) we find a maximum actual dose of around 50 Ad particles per each MZMϕ cell. Accordingly, if the dose of injected virus is 10^9 virus particles per mouse, then each marginal zone macrophage receives between 5 and 10 virus particles per cell. If the dose of the injected virus is 10^{11} virus particles per mouse, then each macrophage can accumulate between 500 and 1000 virus particles.

RNAse protection assay. Total RNA was extracted from tissues using the "RNAqueous-Midi kit" (Ambion Inc., Austin, TX). Ten μg of RNA were hybridized with a mix of ^{32}P-labeled RNA probes. The ^{32}P-labeled RNA probe mix was prepared by *in vitro* transcription using the "*In vitro* transcription kit", CK-3, and custom template sets were provided by BD Biosciences/Pharmingen (San Diego, CA). The hybridized RNAs were treated with RNAse, using the "RNAse protection Assay kit" (BD Biosciences), precipitated and the protected fragments were resolved on vertical sequencing (10% acrylamide) gels. Following electrophoresis, the gels were dried and exposed to X-ray film (Kodak-X-Omat) and PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). The signals on the screen were analyzed by PhosphorImager Image-Quant software. The RNAse protection assay was performed using RNA samples of at least 3 to 5 individual mice per each virus. At least two independently prepared virus stocks were used for RNA levels analysis.

Immunohistochemical and immunofluorescence stainings.

Mice were anaesthetized, and spleens and livers were collected, frozen in O.C.T. compound and stored at -80°C until processed. Six to eight micron sections were cut, air dried, fixed for 10 minutes in acetone at -20°C, air dried for at least 4 hours, re-hydrated in TBS for one hour, blocked in 2% N.S. for 1 hour and incubated with primary antibodies overnight at 4°C with or without 0.1% saponin depending on the antigen. Then, sections were incubated with HRP-labeled secondary antibodies for 1 hour. Slides were developed with ImmPact DAB or NovaRed substrates (Vector Laboratories), air dried, mounted, and analyzed on a Leica microscope. For immunofluorescence stainings, slides were immediately mounted after washing the secondary antibodies. Confocal imaging was done on a Zeiss 510 Meta Confocal microscope.

Proteome Profiler antibody arrays.

For quantitative evaluation of IL-1α, IL-1β, KC, MIP-2, and MCP-1 proteins in the spleen, the purified recombinant proteins were purchased from R&D Systems (Minneapolis, MN) and spiked with spleen samples of mock-injected animals at two-fold increasing concentrations and processed in the same way as experimental samples. The intensity of dots for individual cytokines and chemokines of known concentrations were analyzed using Adobe Photoshop densitometry function and standard curves were obtained for each individual analyte. The obtained standard curves were used to determine the amount of each of the proteins in experimental samples. For quantitative analyses of splenic cytokines and chemokines, Ad was injected into 3 to 5 mice per experimental group.

Supplemental References:

Bouvard, D., Brakebusch, C., Gustafsson, E., Aszodi, A., Bengtsson, T., Berna, A., and Fassler, R. (2001). Functional consequences of integrin gene mutations in mice. Circ Res *89*, 211-223.

Ciavarra, R. P., Stephens, A., Nagy, S., Sekellick, M., and Steel, C. (2006). Evaluation of immunological paradigms in a virus model: Are dendritic cells critical for antiviral immunity and viral clearance? Journal of Immunology *177*, 492-500.

Shayakhmetov, D. M., Papayannopoulou, T., Stamatoyannopoulos, G., and Lieber, A. (2000). Efficient gene transfer into human CD34(+) cells by a retargeted adenovirus vector. J Virol *74*, 2567-2583.