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The Second Receptor for C5a, C5aR2, is Detrimental to Mice during Systemic Infection with *Listeria monocytogenes*

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Abstract

Infection with *Listeria monocytogenes* is acquired through ingestion of contaminated foods and may lead to systemic infection and possible death, with an overall 20% mortality rate. Our previous work using C5aR1−/− mice and C3aR−/− mice demonstrated that C5aR1 and C3aR both play powerful anti-inflammatory and pro-survival roles during systemic infection with *L. monocytogenes*. In our current study, we have examined the role of the third anaphylatoxin receptor, C5aR2, in the host immune response to systemic *L. monocytogenes* infection. C5aR2−/− mice had significantly lower bacterial burdens in the spleens and livers on both days 1 and 3 post-infection compared to C5aR2+/+ mice. The decreased bacterial burdens in the C5aR2−/− mice correlated with less liver damage and with improved survival of $CD4^+$ and $CD8^+$ T cells in the spleen on day 3 post-infection compared to C5aR2+/+ mice. C5aR2−/− mice also produced significantly less G-CSF, IL-6, and MCP-1 in the serum, spleen, and liver on day 1 post-infection compared to C5aR2+/+ mice. C5aR2−/− and C5aR2+/+ mice produced similar amounts of IFN-γ in their spleens on day 1 post-infection. Purified naïve splenocytes from C5aR2−/− mice produced significantly more IFN-γ and IL-12p70 during *in vitro* infection with *L. monocytogenes* compared to splenocytes from C5aR2+/+ mice in a NF-kB-dependent manner. Induction of IL-12 and IFN-γ early during infection with *L. monocytogenes* is protective to the host, and we believe this innate increased ability to produce more IL-12 and IFN-γ provided early protection to the C5aR2−/− mice.

Introduction

Listeria monocytogenes (LM) is a Gram-positive intracellular bacterium that lives in soil and water and is transmitted to humans via contaminated food, such as uncooked meats and produce, processed meats such as hot dogs and deli meats, and unpasteurized milk and cheeses. Infection with this bacterium can lead to sepsis and meningitis, and serious infection occurs mostly in those over 65, those with a compromised immune system, and those who are pregnant and their newborns. Outbreaks caused by this pathogen have a

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mortality rate of approximately 20%, which is much higher than the mortality rate for other foodborne-bacteria (1).

Infection with LM rapidly triggers an innate immune response that is essential for host protection and resistance. Several cytokines and chemokines are known to be important for a protective immune response. Mice deficient in IFN-γ or the IFN-γ receptor are extremely susceptible to LM infection (2–4), as IFN-γ is necessary for activating macrophages to kill LM. Similarly, mice deficient in G-CSF (5), IL-6 (6, 7), TNF-α (8–10), or MCP-1(11) or their receptors are also more susceptible to LM infection than WT mice since these cytokines and chemokines are necessary for recruitment of neutrophils, monocytes, and macrophages to the site of infection. Infection with LM also induces the production of type I interferons, known as IFN-α/IFN-β. These interferons, unlike IFN-γ, are detrimental to the host (12–14) and induce cell death of lymphocytes (13) and macrophages (15). IFN-α/β also reduces the responsiveness of macrophages to IFN-γ during LM infection (16). Mice deficient in the receptor for IFN-α/β are more resistant to LM infection, having reduced apoptosis in the spleen, decreased bacterial burdens in the spleen and liver, and lower cytokine levels compared to WT mice (12–14).

Another part of the innate immune response is the complement system, which is comprised of more than 30 serum and cell surface proteins. During bacterial infections, the complement system is activated to generate the anaphylatoxins C3a and C5a, which are 77- and 74-amino acid peptides, respectively. C3a and C5a have both pro-inflammatory and anti-inflammatory functions, depending on the type of infection (or insult) and the site of infection (17). The three anaphylatoxin receptors are C3aR, C5aR1, and C5aR2. C3aR and C5aR1 are seven transmembrane G-protein-coupled receptors and are expressed on lymphoid and myeloid cells, as well as on nonimmune and tissue cells (17). In our previous studies using WT and C3aR−/− mice (18), we demonstrated that during LM infection, C3aR is protective to the host by dampening the inflammatory response and by protecting immune cells from apoptosis caused by LM. C3aR−/− mice had greater mortality, higher bacterial burdens in the livers and spleens, and excessive production of multiple cytokines and chemokines, including IFN-γ, TNF-α, IL-6, G-CSF, and MCP-1, post-infection compared to WT mice. In addition, C3aR−/− mice had more liver damage and increased apoptosis in the spleens compared to WT mice. Pre-treatment of C3aR−/− mice with an apoptosis inhibitor, Z-VAD-FMK, was able to rescue these mice by decreasing bacterial burden, improving liver pathology, decreasing apoptosis in the spleen, and reducing cytokine and chemokine levels, demonstrating the importance of C3aR in protecting immune cells from apoptosis (18). In addition, we have also demonstrated previously the importance of C5aR1 in protecting the host by modulating type I interferon production during LM infection (19). Similarly to C3aR −/− mice, C5aR1−/− mice had increased mortality, greater bacterial burdens in the liver and spleen, increased apoptosis in the spleens, and excessive inflammatory cytokine and chemokine production, including IFN-α/β, compared to WT mice. C5aR1−/− mice pre-treated with an antibody to the type I interferon receptor were protected from LM-induced mortality (19), demonstrating the importance of C5aR1 in suppressing type I interferon production in this model. In our subsequent studies, we determined that both C3a and C5a can directly suppress IFN-β production from bone marrow derived dendritic cells (BMDCs) in response to LM and cyclic dinucleotides by inhibiting the cyclic dinucleotide-activated

cytosolic surveillance pathway (20), thereby directly protecting the host from the harmful effects caused by excessive production of IFN-β during LM infection.

C5aR2, also known previously as C5L2, is the most recently discovered anaphylatoxin receptor and binds to C5a and C5a des-Arg with high-affinity (21). The genes for C5aR1 and C5aR2 are adjacent to each other, and these receptors are generally expressed in similar tissues and cells, which include macrophages, dendritic cells, neutrophils, eosinophils, NK cells, lymphocytes, as well as a variety of nonimmune cells and parenchymal cells in the spleen, liver, brain, heart, lung, and kidney (22–24). Unlike C3aR and C5aR1, which are mainly expressed on the cell surface of most cell types, C5aR2 is predominantly an intracellular receptor. For example, C5aR2 has been found mostly inside human (25) and mouse (23) neutrophils, human peripheral blood monocytes, human THP-1 monocytic cells, mouse peritoneal macrophages (26), and human NK cells (27). C5aR2 is detected on the surface of some cells at a low level, and this expression may change during infection or disease (26). Like C3aR and C5aR1, C5aR2 has been shown to have both protective and detrimental properties, depending on the disease model [reviewed in (28)]. In the case of cecal ligation and puncture-induced sepsis (29), experimental allergic asthma (22, 30), dextran sulfate sodium-induced colitis (31), and renal ischemia reperfusion injury (32, 33), C5aR2 is detrimental since C5aR2−/− mice were more protected than WT mice in these models. C5aR2 has been shown to be protective in models of LPS-induced sepsis (22) and allergic contact dermatitis (34), as C5aR2−/− mice were more susceptible than WT mice in these disease models. There is conflicting evidence on the role of C5aR2 during acute lung injury, with one group showing that C5aR2−/− mice were less susceptible to LPS-, immune complex-, and C5a-induced acute lung injury (35), while other groups demonstrated that C5aR2−/− mice were more susceptible to LPS-induced (36) and immune complex-induced acute lung injury (37).

In the studies reported here, we sought to determine if C5aR2, like C5aR1 and C3aR, is protective in a systemic infection model of LM, or if C5aR2 is detrimental during infection with this intracellular pathogen. Accordingly, we subjected C5aR2+/+ and C5aR2−/− mice to a model of systemic infection with LM. Our results demonstrate that an absence of C5aR2 leads to a protective phenotype, which demonstrates that C5aR2 is harmful during systemic infection with this bacterium.

Materials and Methods

Mice

C5aR2+/− mice on a C57BL/6 background were a kind gift from Craig Gerard and have been described previously (37). C5aR2+/− mice were intercrossed to generate C5aR2+/+ and C5aR2−/− mice, and then individual breeding colonies of C5aR2+/+ and C5aR2−/− mice were maintained. The C5aR1−/− mice used in these studies have been described previously (38), and C57BL/6 mice from our colony served as WT controls. All mice were housed in HEPA-filtered Tecniplast cages in a barrier facility. Male mice that were 10–13 weeks of age were used for these studies. All mouse protocols followed institutional and NIH guidelines for animal care and welfare.

Bacterial infection

Listeria monocytogenes ATCC strain 13932 (serotype 4b) (MicroBioLogics, Inc.) was used for all infection studies. Bacteria were cultured in Bacto brain heart infusion (BHI) broth at 37°C to mid-logarithmic phase, harvested by centrifugation, washed once in sterile PBS, and resuspended in sterile PBS. Mice were injected i.v. with either PBS or 1 X 10^5 bacteria in a volume of 100 μl. The number of bacteria present in the inoculum was verified by culturing serial dilutions of the inoculum on BHI agar plates.

Bacterial burden in the liver and spleen

Liver and spleen were aseptically removed from the mice either 1 day or 3 days post-infection, rinsed in PBS, and then placed in HBSS in 5 ml tubes. The organs were homogenized using a PRO200 homogenizer (ProScientific) on medium speed and were then placed on ice. Bacterial counts were obtained by plating serial dilutions of each homogenate on BHI agar plates. Data are expressed as mean CFU per organ (Log_{10}) ± SEM.

In vitro LM killing assay

C5aR2+/+ and C5aR2−/− mice were injected i.p. with 1 ml of 3% Proteose Peptone (Oxoid). Three days later the peritoneal cavity was lavaged with 5 ml of HBSS to collect the cells. For the infection, 10^6 peritoneal exudate cells were incubated with 10^4 LM in a volume of 1 ml of DMEM containing 5% autologous normal mouse serum at 37°C with 5% CO_2. Aliquots were removed immediately upon infection (0 h) and also at 2 h, 4 h, and 20 h, and serial dilutions were made and plated on BHI agar plates. The data is presented as mean CFU/ml (Log_{10}) ± SEM.

Liver histology and immunostaining

The large lobe of the liver was removed from uninfected control mice as well as from infected mice on days 1 and 3 post-infection, rinsed in PBS, and placed in 10% buffered formalin. The livers were dehydrated with increasing concentrations of ethanol, embedded in paraffin, cut into 5-μm sections, and stained with H&E (Fisher). The brightfield images were acquired using SPOT Advanced software (Diagnostic Instruments, Inc.) and a Zeiss Axioskop microscope (Carl Zeiss, Inc.) equipped with a SPOT-RT digital camera (Diagnostic Instruments, Inc.). The abscess area in the livers was quantitated using Image J software (NIH) on H&E images taken at 100X magnification. The outer edge of each abscess was outlined using the drawing tool to give the abscess area. Three to six abscesses per mouse were used to obtain a mean liver abscess area per mouse from 3–4 mice per group. The data are expressed as mean liver abscess area ± SEM. For immunofluorescence staining of the liver sections, tissue sections were deparaffinized, rehydrated, fixed using 2% paraformaldehyde in 1X PBS for 20 minutes, and then permeabilized with 0.3% triton-X-100 in 1X PBS for 10 minutes. Tissue sections were then blocked with 10% fetal calf serum containing DMEM for 1 h at room temperature. After washing the sections three times with 1X PBS-0.05% Tween20, primary antibodies for C5aR1 (clone 20/70 from BioLegend) or F4/80 (clone BM8 from BioLegend) were added overnight at 4°C. The sections were washed and then incubated with either FITC- or PE-conjugated goat anti-rat IgG secondary antibodies (Abnova) along with the DNA dye DAPI for 1 hour at room

temperature. The sections were washed and then mounted with FluorSave (EMD Millipore). Images were acquired using an inverted Nikon microscope equipped with fluorescence detection filters and UV laser for DAPI and were processed using the NIS-Elements software. Fluorescent intensities and/or number of immune cells infiltrated in the liver sections were quantified using ImageJ software. Neutrophil activity was quantitated in the liver sections using the Naphthol AS-D chloroacetate esterase enzyme activity kit from Sigma-Aldrich according to the manufacturer's recommendations. Images were acquired using a Zeiss brightfield microscope equipped with Zen Software. The purplish-red staining intensity was quantified using ImageJ software.

Serum ALT analysis

Sera samples from uninfected control mice and LM-infected mice were submitted to the University of Texas M.D. Anderson Cancer Center Veterinary Medicine and Surgery Department. Alanine aminotransferase (ALT) was quantitated using an automated COBAS Integra 400 Analyzer (Roche). Data are expressed as mean units per liter ± SEM.

Immunophenotyping

Leukocytes were harvested from spleens for FACS analysis from uninfected control mice and from infected mice on day 3 post-infection using the gentleMACS Dissociator. Prior to staining, erythrocytes were lysed using ACK lysing buffer (Lonza BioWhittaker). Total cells were counted using the Scepter 2.0 (Millipore). Non-specific antibody binding was blocked using mouse Fc block (BD Pharmingen). The following markers (Biolegend), with the clone listed in parentheses, were used to characterize the cell populations: CD4 (GK1.5), CD8a (53–6.7), NK1.1 (PK136), Ly-6G (1A8), F4/80 (BM8), and CD11c (N418). 7-AAD (BD Pharmingen) was added in the final 10 min of staining for dead cell exclusion. The cells were washed one time with PBS before analysis. A minimum of 20,000 events were collected on a FACSCalibur (BD Biosciences) flow cytometer, and the data was analyzed using Kaluza software (Beckman Coulter, Inc.). The gating strategy was as follows: the stained splenocytes were initially plotted based on SSC vs FSC, and a gate was drawn to eliminate cellular debris. This non-debris gate was then used to create another plot of 7-AAD vs FSC. A gate was drawn on the 7-AAD negative cells (live cells) to further analyze the live populations of each cell type, such as $CD4^+$, $CD8^+$, $NK1.1^+$, $Ly\text{-}6G^+$, $F4/80^+$, and $CD11c^+$.

Cytokine and chemokine analysis

Cytokines and chemokines were measured from sera, spleen, and liver taken from uninfected control mice as well as from infected mice on days 1 and 3 post-infection. Spleen and liver homogenates were centrifuged at 15,000 g for 15 min, and the supernatants were used for cytokine and chemokine analyses. The following ELISA kits were used: G-CSF (R&D Systems), IL-6, IFN-γ, and IL-12p70 (Invitrogen), MCP-1 and IFN-β (Biolegend). Data are expressed as mean pg/ml ± SEM.

In vitro splenocyte infection

Leukocytes were harvested from the spleens of naïve mice as described above in the "Immunophenotyping" section, except that all of the procedures were done in a biosafety cabinet to maintain sterility of the cells. The cells were cultured in RPMI-1640 (Lonza) containing 10% heat-inactivated FBS (Gibco). For the experiment, 1×10^6 cells were infected with 10^3 LM for 6 h or 20 h in a volume of 1 ml in 24 well plates (Costar). In some experiments, the cells were pre-treated in either diluted DMSO (vehicle control) or in 1 μM BAY11–7062 (Invivogen) for two hours before the LM infection. After 6 h or 20 h, the supernatants were collected, and the cells were lysed for RNA purification using the RNeasy Plus Mini Kit from Qiagen. IFN-γ and IL-12p70 protein was quantitated in the cell-free supernatants via ELISA. cDNA was synthesized from RNA using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. Quantitative PCR was performed using RT^2 qPCR Primers for mouse *Ifng*, *IL12a*, *Chuk* (*Ikka*), *Ikbkb* (*Ikkb*), *Ikbkg* (*Ikkg*), and *Gapdh* (Qiagen), iTaq Universal SYBR Green Supermix (Bio-Rad), and an ABI-7900 thermal cycler. Transcript abundance for the genes of interest was normalized to *Gapdh* for each sample.

Statistical analyses

All statistical analyses were done using GraphPad Prism software. Comparisons between C5aR2+/+ and C5aR2−/− mice were done with either the unpaired two-tailed t test or with one-way ANOVA with the Tukey post-test, with P values < 0.05 considered significant.

Results

Lack of C5aR2 leads to increased resistance to LM infection

To assess the overall impact of C5aR2 on the innate immune response to LM infection, C5aR2+/+ and C5aR2−/− mice were injected with LM, and the spleens and livers were harvested from the mice 1 or 3 d post-infection. As shown in Fig. 1A and 1B, the C5aR2−/− mice had 35% fewer bacteria in their spleens and 76% fewer bacteria in their livers on day 1 post-infection compared to the C5aR2+/+ mice, and they had 47% fewer bacteria in their spleens and 71% fewer bacteria in their livers on day 3 post-infection compared to the C5aR2+/+ mice. These data demonstrate that the C5aR2−/− are more resistant to LM infection and that the resistance phenotype observed in the C5aR2−/− mice was much more pronounced in the liver compared to the spleen on both days 1 and 3 post-infection, although the differences were statistically significant in both organs on both days. To determine if the decreased bacterial burden observed in the C5aR2−/− mice was due to their macrophages having an innate enhanced ability to kill LM, proteose-peptone elicited cells from C5aR2+/+ and C5aR2−/− mice were incubated with LM *in vitro*. As shown in Fig. 1C, the number of viable LM was similar in the C5aR2+/+ and C5aR2−/− cells at 2 h, 4 h, and 20 h post-infection, indicating that macrophages from C5aR2−/− mice do not have an enhanced innate killing capability compared to C5aR2+/+ mice, at least *in vitro*.

C5aR2−/− mice have less severe liver damage

Systemic LM infection leads to microabscess formation in the liver and subsequent liver damage. To evaluate the role of C5aR2 in this microabscess formation, the large lobe of the liver was collected on days 1 and 3 post-infection, and sections were stained with H&E. On day 1 post-infection, the abscess sizes looked similar in both the C5aR2+/+ and C5aR2−/− mice (Fig. 2A). However, by day 3 post-infection, the liver abscesses were significantly smaller in the C5aR2−/− mice compared to the C5aR2+/+ mice (Fig. 2A). The abscess area was quantitated using ImageJ software, and the abscess area was 40% less in the C5aR2−/− mice compared to the C5aR2+/+ mice ($P = 0.037$) (Fig. 2B). The C5aR2−/− mice also had lower C5aR1 expression, fewer $F4/80^+$ cells, and less neutrophil activity in their livers on day 3 post-infection compared to the C5aR2+/+ mice (data not shown). Serum ALT, which is a marker of liver damage, rose significantly in both groups of mice on day 3 post-infection compared to uninfected mice, but the serum ALT was 51% lower in the C5aR2−/− mice on day 3 compared to C5aR2+/+ mice (Fig. 2C). Overall, these data demonstrate that absence of C5aR2 protects the liver from damage caused by LM infection.

Reduced lymphocyte killing in C5aR2−/− mice

Systemic LM infection also leads to damage in the spleen, namely cellular necrosis and lymphocyte apoptosis. To determine if absence of C5aR2 protects the spleen from cellular damage in a similar fashion as the liver, spleens were collected from uninfected control mice as well as from day 3- infected mice. Total splenocytes were isolated, counted, and stained for cell surface markers. As shown in Fig. 3A, there was no difference in total cell numbers in uninfected C5aR2+/+ and C5aR2−/− mice. There was also no statistically significant difference in the percentages or total numbers of $CD4^+$, $CD8^+$, $NK1.1^+$, $LY\text{-}6G^+$, $F4/80^+$, or $CD11c^+$ cells in uninfected mice (Fig. 3B and 3C). On day 3 post-infection, there were no significant differences in the percentages or total numbers of $NK1.1^+$, $Ly\text{-}6G^+$, $F4/80^+$, or $CD11c^+$ cells in the spleens of C5aR2+/+ and C5aR2−/− infected mice (Fig. 3D and 3E). However, the infected C5aR2−/− mice had significantly higher percentages of $CD4^+$ and $CD8^+$ cells compared to infected C5aR2+/+ mice (Fig. 3D) ($P < 0.05$). In terms of total cell numbers in the spleen, the infected C5aR2−/− mice had 43% more $CD4^+$ cells, although this difference was not statistically significant, and 64% more $CD8^+$ cells ($P < 0.05$) (Fig. 3E). These data show that the absence of C5aR2 provides protection to $CD4^+$ and $CD8^+$ cells in the spleen from the destruction caused by LM. Production of type I interferon during LM infection sensitizes lymphocytes to apoptosis. To determine if absence of C5aR2 affects the production of type I interferons, we measured the level of IFN-β in the sera of control and day 1 and day 3-LM infected mice. On day 1 post-infection, the C5aR2−/− mice had 3-fold less IFN-β in their sera compared to the C5aR2+/+ mice ($P = 0.024$)(data not shown), but IFN-β was mostly undetectable in both groups of mice on day 3 post-infection (data not shown). Based on our day 1 results, it is possible that the lower level of IFN-β in the C5aR2−/− mice provided protection to the C5aR2−/− T cells against LM-induced apoptosis.

C5aR2−/− mice have lower cytokine and chemokine levels after infection

To determine if lack of C5aR2 alters the cytokine and chemokine responses to LM, the levels of G-CSF, IL-6, MCP-1, and IFN-γ were quantitated in the sera and spleen and liver

homogenates of C5aR2+/+ and C5aR2−/− mice on days 1 and 3 post-infection with LM. As shown in Fig. 4A, the serum of C5aR2−/− mice contained 65–68% less G-CSF, IL-6, and MCP-1 compared to C5aR2+/+ mice on day 1 post-infection ($P < 0.05$). However, IFN-γ was only reduced by 34% in the sera of C5aR2−/− mice compared to C5aR2+/+ mice on day 1 ($P < 0.05$). Next, we examined the levels of these cytokines and chemokines in the cell-free spleen homogenates (Fig. 4B). In the C5aR2−/− spleens compared to the C5aR2+/+ spleens on day 1 post-infection, G-CSF was reduced 59%; IL-6 was reduced 51%; and MCP-1 was reduced 31% ($P < 0.05$ for all three). There was no difference in spleen IFN-γ levels between the two groups of mice on day 1 post-infection (Fig. 4B). Finally, we quantitated the levels in the cell-free liver homogenates (Fig. 4C). In the C5aR2−/− livers compared to the C5aR2+/+ livers on day 1 post-infection, G-CSF was reduced 52%; IL-6 was reduced 51%; MCP-1 was reduced 32%; and IFN-γ was reduced 29% compared to the C5aR2+/+ livers ($P < 0.05$ for all four) (Fig. 4C). The differences between groups on day 3 did not reach significance for any cytokine or chemokine in the serum, spleen, or liver. Overall, the data demonstrate that the decreases in cytokine and chemokine production in the infected C5aR2−/− mice compared to the infected C5aR2+/+ mice were most prominent on day 1 post-infection in the serum, spleen, and liver with the differences between groups much less pronounced on day 3 post-infection.

C5aR2−/− splenocytes produce more IFN-γ and IL-12p70 in vitro

The decreased levels of the inflammatory cytokines and chemokines in the infected C5aR2−/− mice reported in Fig. 4 was most likely due to their having fewer remaining bacteria (and thus less inflammation) compared to the infected C5aR2+/+ mice (Fig. 1). Despite these overall decreases in cytokines and chemokines, IFN-γ on day 1 was not reduced as much in the serum of C5aR2−/− mice as compared to G-CSF, IL-6, and MCP-1 (34% reduction for IFN-γ versus 65–68% for G-CSF, IL-6, and MCP-1) (see Fig. 4A). In addition, despite there being 35% fewer bacteria on day 1 in the C5aR2−/− spleens (see Fig. 1A) compared to the C5aR2+/+ spleens, the C5aR2−/− mice still produced similar amounts of IFN-γ in their spleens as the C5aR2+/+ mice on day 1 (see Fig. 4B). These differences in the IFN-γ levels compared to the other cytokines and chemokines tested suggested that the IFN-γ response was not as dependent as the others on the levels of remaining bacteria. Therefore, we hypothesized that C5aR2 directly modulates IFN-γ production in the early response to LM infection (especially in the spleen). To examine this possibility, splenocytes from untreated C5aR2+/+ and C5aR2−/− mice were incubated with either media alone or LM for 6 h or 20 h *in vitro*. In data not shown, the amount of LM was very similar in the C5aR2+/+ and C5aR2−/− splenocytes after 20 h of infection *in vitro*. Since IL-12 is an important inducer of IFN-γ expression, IL-12p70 as well as IFN-γ proteins were quantitated in the cell-free supernatants by ELISA. At 6 h, there was no detectable IL-12p70 or IFN-γ protein (data not shown), but at 20 h the infected C5aR2−/− splenocytes produced significantly more IL-12p70 and IFN-γ protein compared to the infected C5aR2+/+ splenocytes (Fig. 5A) ($P < 0.05$). Even though IL-12p70 and IFN-γ protein were not detectable at 6 h, message for these cytokines was detectable at 6 h and was quantitated by Q-PCR. The infected C5aR2−/− splenocytes produced significantly more *IL12a* (which is the p35 subunit of IL-12p70) and *Ifng* message at 6 h compared to the infected C5aR2+/+ splenocytes (Fig. 5B) ($P \leq 0.018$). To determine if LM induces IL-12p70 and IFN-γ production through the NF-kB pathway,

C5aR2+/+ and C5aR2−/− splenocytes were pre-treated for 2 h before LM infection with either BAY11–7082, an inhibitor of the NF-kB pathway, or diluted DMSO as a vehicle control. As shown in Fig. 6A, C5aR2−/− splenocytes that were pre-treated with DMSO vehicle produced significantly more IL-12-p70 and IFN-γ than vehicle-treated C5aR2+/+ splenocytes, and pre-treatment of the cells with the BAY11–7082 inhibitor completely abolished IL-12-p70 and IFN-γ production in both C5aR2−/− and C5aR2+/+ splenocytes (Fig. 6A), demonstrating a vital role for the NF-kB pathway in LM-induced IL-12-p70 and IFN-γ production. Finally, we wanted to determine how absence of C5aR2 enhances NF-kB activation in response to LM infection. The central regulator of the NF-kB pathway is the IkB kinase [IKK] complex (39), and these molecules are directly upstream of the BAY11–7082 inhibitor that we used. The IKK complex consists of IKKα and IKKβ, which are kinases, and IKKγ, which is a regulatory subunit. We looked at expression of these *Ikk* genes by QPCR in our C5aR2+/+ and C5aR2−/− splenocytes that had been infected with LM for 6 h or 20 h. As shown in Fig. 6B, at 6 h the C5aR2−/− splenocytes had 25% higher gene expression of *Ikka* (formal gene name is *Chuk*) and 50% higher gene expression of *Ikkg* (formal gene name is *Ikbkg*) ($P < 0.05$) compared to infected C5aR2+/+ splenocytes (which were set to a value of 1.0). There was no difference in *Ikkb* (formal gene name is *Ikbkb*) at 6 h (Fig. 6B). At 20 h post-infection, the differences were even more substantial. The C5aR2−/− splenocytes had 3-fold higher gene expression of *Ikka* and *Ikkg* ($P \leq 0.028$) and 60% higher gene expression of *Ikkb* ($P = 0.021$) compared to infected C5aR2+/+ splenocytes (Fig. 6C). These data demonstrate that absence of C5aR2 led to early enhanced activation of the NF-kB pathway during LM infection via upregulation of the *Ikk* genes, which resulted in greater production of the protective cytokines IL-12p70 and IFN-γ compared to C5aR2+/+ cells.

Discussion

Since its discovery in 2000, a significant amount of research by several different laboratories has been devoted to delineating the biological function of C5aR2. Despite this overall effort, C5aR2 remains largely an enigmatic receptor with no clear unifying function. C5aR2 was initially characterized as a decoy or anti-inflammatory receptor by diverting C5a from binding and signaling through C5aR1 as well as by forming a complex with C5aR1 and β-arrestin, thereby blocking C5aR1-mediated MAPK activation in inflammatory cells. Its role as a decoy receptor was initially accepted as its primary, if not sole function, since it is devoid of Gα protein coupling and down-stream signaling. Opposing results in C5aR1−/− and C5aR2−/− mice subjected to mouse models of allergic contact dermatitis (34), LPS-induced sepsis (22, 38), and immune complex-induced lung injury (37) support C5aR2 as a decoy or anti-inflammatory receptor. In contrast, in other disease models such as cecal ligation and puncture (29), colitis (31, 40), atherosclerosis (41, 42), and renal ischemia-reperfusion injury (32, 33, 43), C5aR1 and C5aR2 exhibited similar inflammatory pathological roles. The mechanism(s) by which C5aR2 in the absence of direct downstream signaling can produce inflammatory responses similar to that of C5aR1 remain unclear. Some investigations have indicated that C5aR2 may function via dimerization with C5aR1 or through intracellular interactions with internalized C5aR1 (28). However, these

mechanisms have not been proven as responsible for the C5aR2 inflammatory responses observed in mouse models of disease.

In our previous investigations, we found that C5a via C5aR1 signaling was very important for host protection against LM systemic infection. In the absence of C5aR1, mice were extremely susceptible to LM systemic infection (increased mortality and elevated bacterial burden) due to massive IFN-β-mediated apoptosis of major leukocyte populations in the spleen, including critical $CD4^+$ and $CD8^+$ T-cells (19). Further research by our laboratory discovered that C5a via direct C5aR1 signaling is critical for suppressing IFN-β expression and LM-mediated cellular apoptosis by inhibition of the cyclic dinucleotide-activated cytosolic surveillance pathway (20). In our current study, we show that unlike C5aR1 deficiency, absence of C5aR2 provided protection to mice systemically infected with LM (reduced bacterial burden in spleens and livers). On day 1 post infection, the C5aR2−/− infected mice exhibited a transient decrease of IFN-β in their circulation. This decrease of IFN-β may have provided some protection to the LM-infected C5aR2−/− mice since their spleens contained significantly higher percentages of $CD4^+$ and $CD8^+$ T-cells compared to LM- infected C5aR2+/+ mice on day 3 post infection. C5a suppresses IFN-β production in C5aR2+/+ and C5aR2−/− BMDCs to the same extent (S. L. Mueller-Ortiz and R. A. Wetsel, unpublished observations), demonstrating that absence of C5aR2 does not affect LM-induced IFN-β production directly. Instead these findings collectively support C5aR2 functioning as a decoy receptor during LM infection, since in the absence of C5aR2 more C5a will be available to suppress IFN-β expression via C5aR1 signaling.

Despite the decreased IFN-β expression in the LM-infected C5aR2−/− mice, it is unlikely that is the major reason that the C5aR2−/− mice exhibited host protection against systemic LM infection. For example, in the C5aR1−/− LM-infected mice there was massive IFN-β mediated apoptosis of all leukocyte populations in the spleen (19). In contrast, only the $CD4^+$ and $CD8^+$ T-cells were affected in the infected C5aR2−/− mice; moreover, the impact on the survival of $CD4^+$ and $CD8^+$ T-cells in the C5aR2−/− mice was not as dramatic as was their cell death in the C5aR1−/− infected mice. This led us to examine other possible mechanisms that could explain why C5aR2−/− mice are protected from LM systemic infection.

Several cytokines and chemokines are protective during LM infection, including G-CSF, IL-6, MCP-1, and IFN-γ. The LM-infected C5aR2−/− mice had a controlled cytokine and chemokine response, producing lower levels of G-CSF, IL-6, MCP-1, and IFN-γ in their sera and livers as early as day 1 post-infection (see Fig. 4A and 4C) versus the C5aR2+/+ mice. The decreased cytokine levels would not be expected to protect the C5aR2−/− mice from infection. More likely the decrease in cytokine levels are a consequence of decreased inflammation due to a reduction in bacterial burden but are not the reason that the number of bacteria are reduced. However, we did notice that the C5aR2−/− mice produced a similar level of IFN-γ in their spleens as C5aR2+/+ mice on day 1 post-infection despite having significantly fewer bacteria than the C5aR2+/+ mice (see Fig. 4B). Therefore, we hypothesized that the host protection to LM infection observed in the C5aR2−/− mice may be due to very early increased expression of IFN-γ. To examine this possibility, purified splenocytes from C5aR2−/− and C5aR2+/+ mice were infected with LM and examined for

IFN-γ expression 20 h post-infection. In support of our hypothesis, C5aR2−/− infected splenocytes were found to produce significantly more IFN-γ protein *in vitro* at 20 h post-infection compared to infected C5aR2+/+ splenocytes (See Fig. 5A). This upregulation of IFN-γ in the C5aR2−/− splenocytes could be seen as early as 6 h post-infection at the message level (Fig. 5B). We also discovered that LM-infected splenocytes from C5aR1−/− mice also produced elevated levels of IFN-γ compared to infected C5aR1+/+ splenocytes (Supplemental Figure 1), indicating that during early stages of LM infection both C5aR1 and C5aR2 modulate IFN-γ expression similarly. Given the interrelationship between IL-12 and IFN-γ, we also looked at IL-12p70 production in the *in vitro*-infected splenocytes. Just like IFN-γ, the infected C5aR2−/− splenocytes produced more IL-12p70 protein at 20 h and more *IL12a* message at 6 h compared to the infected C5aR2+/+ splenocytes (Fig. 5). IL-12 is rapidly induced during infection and is produced by macrophages, dendritic cells, and B cells (44, 45). IL-12 stimulates the production of IFN-γ by NK cells and T cells, which then causes more IL-12 production in a positive feedback loop. In studies done by Tripp *et al.* (46), neutralization of IL-12 prior to LM infection resulted in greater mortality, decreased macrophage activation, and increased bacterial burden in the spleen compared to isotype-treated control mice. Concomitant administration of recombinant IFN-γ with the neutralizing IL-12 antibody reversed the phenotype (46), demonstrating a critical role for IL-12 in stimulating IFN-γ production and subsequent macrophage activation during LM infection. In these same studies, mice that were given purified IFN-γ (but no antibody) at the time of LM infection had fewer LM in their spleens 3 days post-infection (46), showing the protective effect of IFN-γ in controlling LM *in vivo*. To our knowledge, our data is the first demonstration that C5aR2 modulates both IFN-γ and IL-12p70 in a bacterial infection model and does so very rapidly – within six hours of infection.

Our further experiments demonstrated that C5aR2 regulates IFN-γ and IL-12p70 through the NF-κB pathway during LM infection. Pre-treatment of splenocytes with a NF-κB pathway inhibitor (BAY11–7082) was able to shut down production of IL-12p70 and IFN-γ in LM-infected splenocytes from both C5aR2+/+ and C5aR2−/− mice (see Fig. 6A), and using QPCR, we showed that C5aR2−/− splenocytes infected with LM had significantly higher expression of *Ikk* genes as early as 6 h post-infection compared to infected C5aR2+/+ splenocytes (see Fig. 6B). IKKα and IKKβ are both kinases, and IKKγ is a regulatory subunit (39). Together, they are involved in phosphorylating IκB, which leads to activation of NF-kB. As far as we know, we are the first to demonstrate that absence of C5aR2 increases *Ikk* gene expression.

In summary, our findings show that in a systemic model of LM infection, C5aR2 is detrimental to the host. During early infection, C5aR2 appears to act as a decoy receptor by impairing the suppression of IFN-β by C5aR1 signaling. However, the impact of C5aR2 on C5aR1- mediated IFN-β expression is short-lived and likely is not the major reason that C5aR2 impairs the host response to LM infection. Absence of C5aR2 led to higher expression of the *Ikk* genes and subsequent increased activation of the NF-κB pathway in splenocytes early during LM infection (as early as 6 h post-infection), which led to greater production of the protective cytokines IL-12p70 and IFN-γ. These two cytokines are important for macrophage activation and early killing of LM, and their higher early expression may largely explain the reduced bacterial burden in the C5aR2−/− mice. This

increased killing of LM was evident in the C5aR2−/− mice as early as day 1 post-infection in both the liver and spleen. Of note, absence of C5aR1 also led to increased production of IFN-γ during very early stages of LM infection, indicating that in addition to acting as a decoy receptor, C5aR2 also responds similarly to C5aR1 in suppressing IFN-γ during early LM infection. The suppression of IFN-γ by C5aR1 and C5aR2 during the early stage of LM infection does not appear to impact the overall and sustained host protection provided by C5aR1 suppression of IFN-β mediated apoptosis of LM-infected leukocytes as documented in C5aR1−/− LM-infected mice (19). A model depicting C5aR1 and C5aR2 regulation of the host response to LM infection is shown in Figure 7.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ALT	alanine aminotransferase
BHI	brain heart infusion
C5aR2	second receptor for C5a
LM	*Listeria monocytogenes*
MOI	multiplicity of infection
BMDCs	bone marrow-derived dendritic cells
IKK	IκB kinase

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Key Points

C5aR2 is detrimental to the host immune response to early *L. monocytogenes* infection.

C5aR2 suppresses NFκ-B-mediated expression of IFN-γ.

C5aR2 acts as a decoy receptor for C5aR1-mediated inhibition of IFN-β expression.

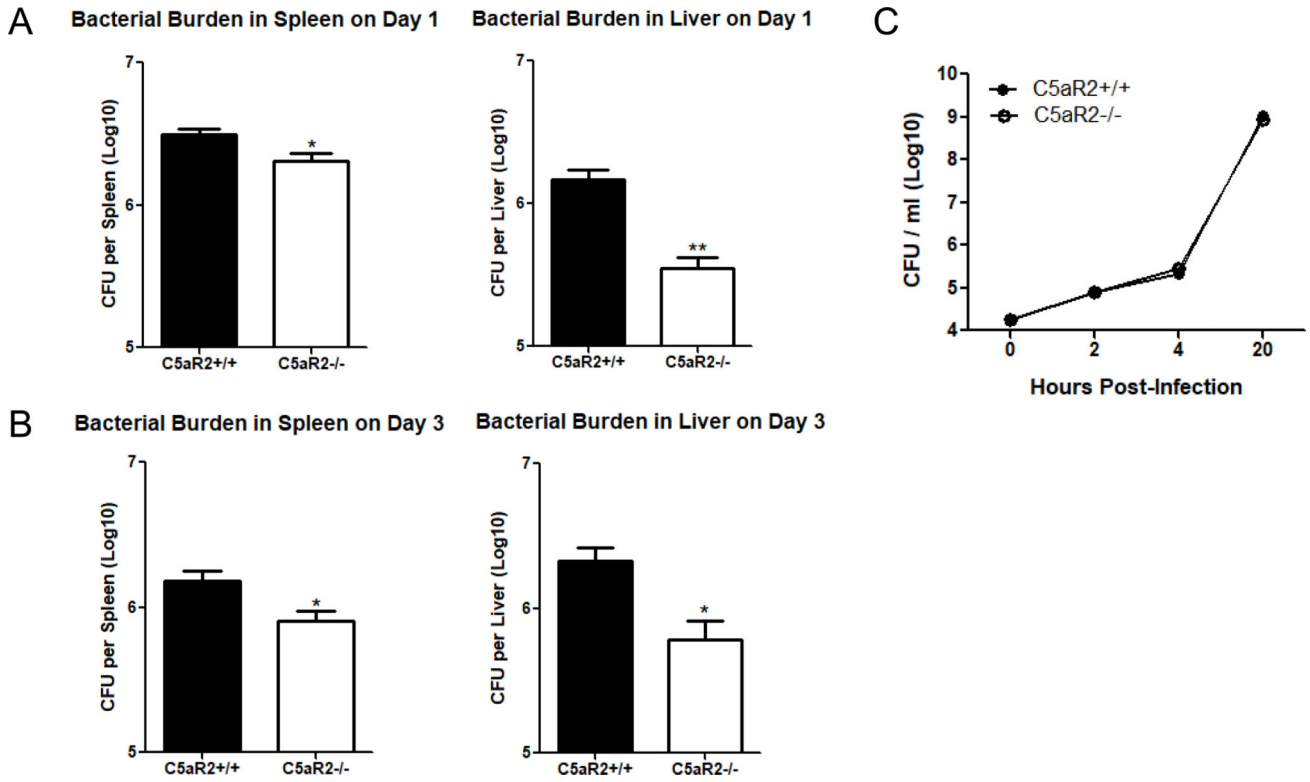

Figure 1. C5aR2−/− mice have decreased bacterial burdens in the spleen and liver following LM infection.

C5aR2+/+ and C5aR2−/− mice were infected i.v. with 1×10^5 LM, and 1 d (A) and 3 d (B) post-infection, the spleens and livers were harvested and homogenized. The data for day 1 are pooled from two experiments (n = 8–12 mice / group), and the data for day 3 are pooled from four experiments (n = 15–19 mice / group) and are presented as mean CFU/organ (log10) ± SEM. * $P \leq 0.029$, ** $P = 0.003$, by t test. (C) Proteose peptone-elicited exudate cells were harvested and infected with LM. At 2 h, 4 h, or 20 h post-infection the number of live LM was determined, and the data are presented as CFU/ml (log10) ± SEM. Data are combined from two experiments performed in triplicate.

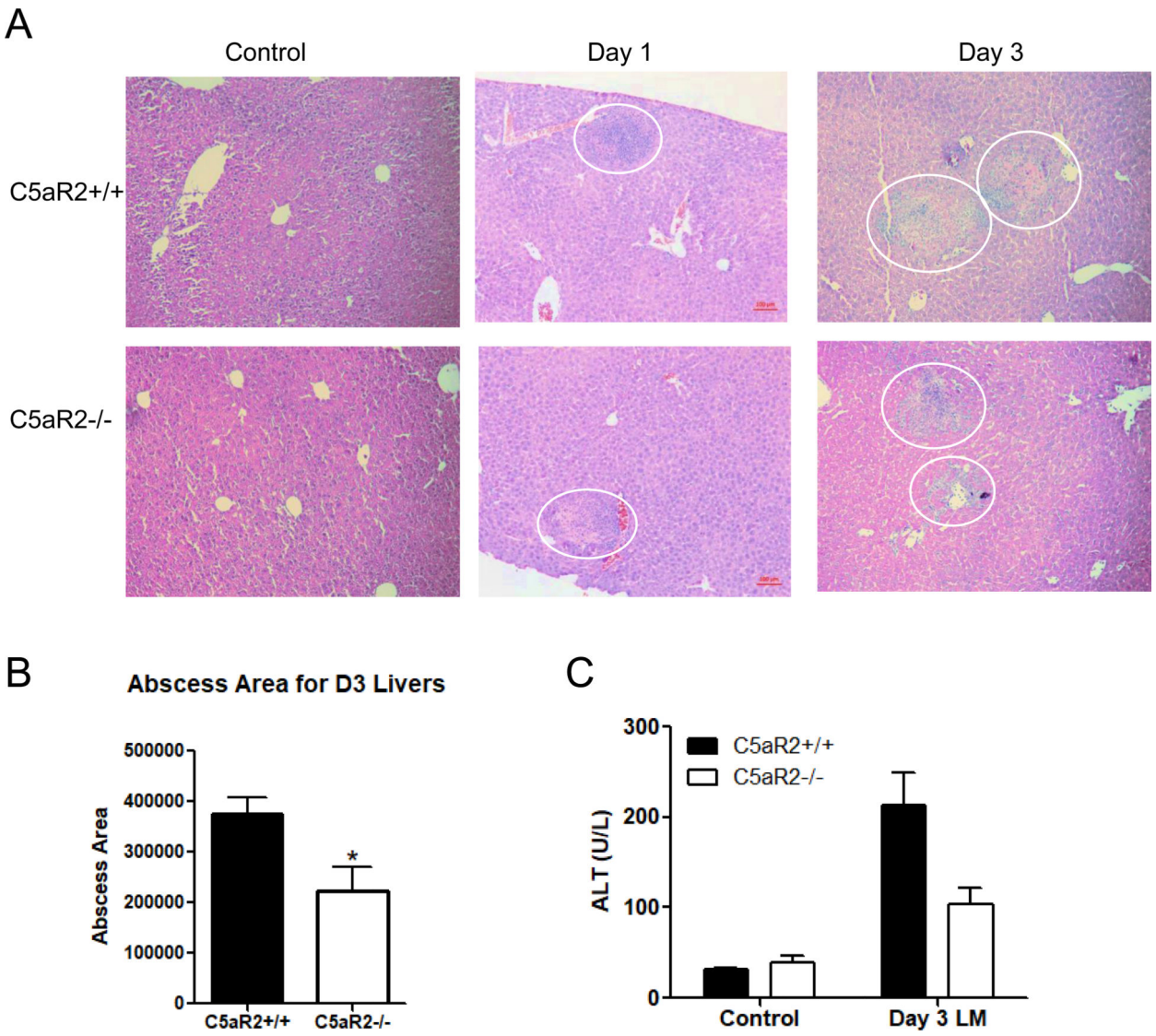

Figure 2. C5aR2−/− mice have less severe liver damage following LM infection.

C5aR2+/+ and C5aR2−/− mice were infected i.v. with 1×10^5 LM, and 1 day and 3 days post-infection, (A) the large lobe of the liver was removed and embedded in paraffin. Sections were stained with hematoxylin and eosin. The abscesses are outlined in white, and the sections are representative of at least 3 mice per group. Magnification is 100x final. (B) The liver abscess area was quantitated from H&E images taken at 100x as described in Materials and Methods and is presented as mean liver abscess area ± SEM. The data was quantitated from 3–5 mice per group on day 3. * P = 0.037, by t test. (C) Sera levels of ALT from uninfected (n = 3) and day 3-infected (n = 15–19) mice were measured and are presented as mean Units/L ± SEM.

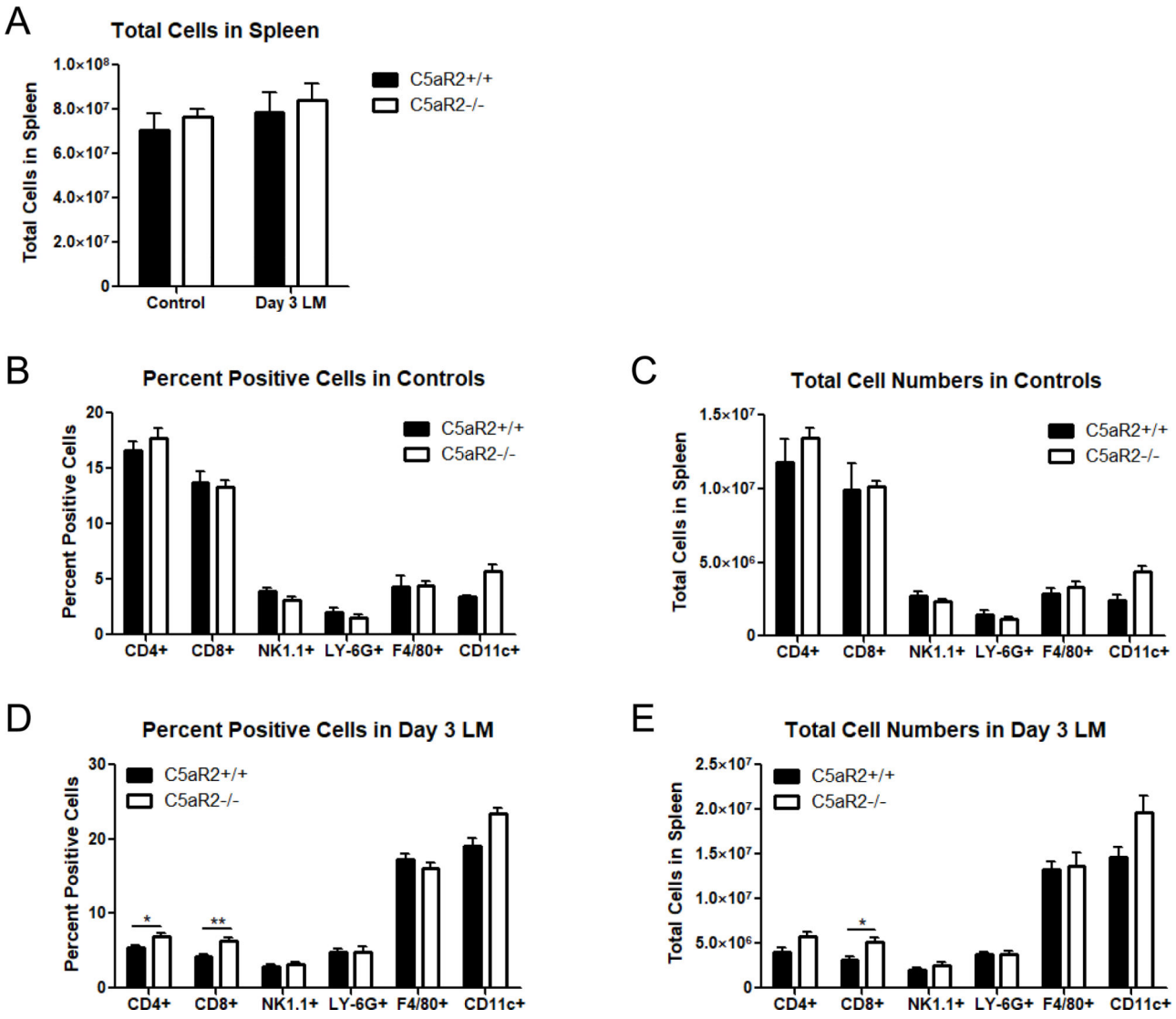

Figure 3. Leukocyte populations in spleens following LM infection.

C5aR2+/+ and C5aR2−/− mice were infected i.v. with 1×10^5 LM, and 3 days post-infection, the spleens were removed and total cells were counted (A). Spleens from uninfected mice were also harvested as controls. Splenocytes from uninfected (B and C) and day 3 (D and E) LM-infected mice were stained with various cell surface markers to determine cell populations. The data are presented as mean percent positive cells ± SEM (B and D) and mean cells per spleen ± SEM (A, C and E) and are pooled from two independent experiments. $n = 5$ for the uninfected mice, $n = 6–8$ for the infected mice. *, ** $P < 0.05$ by one-way ANOVA with Tukey post-test.

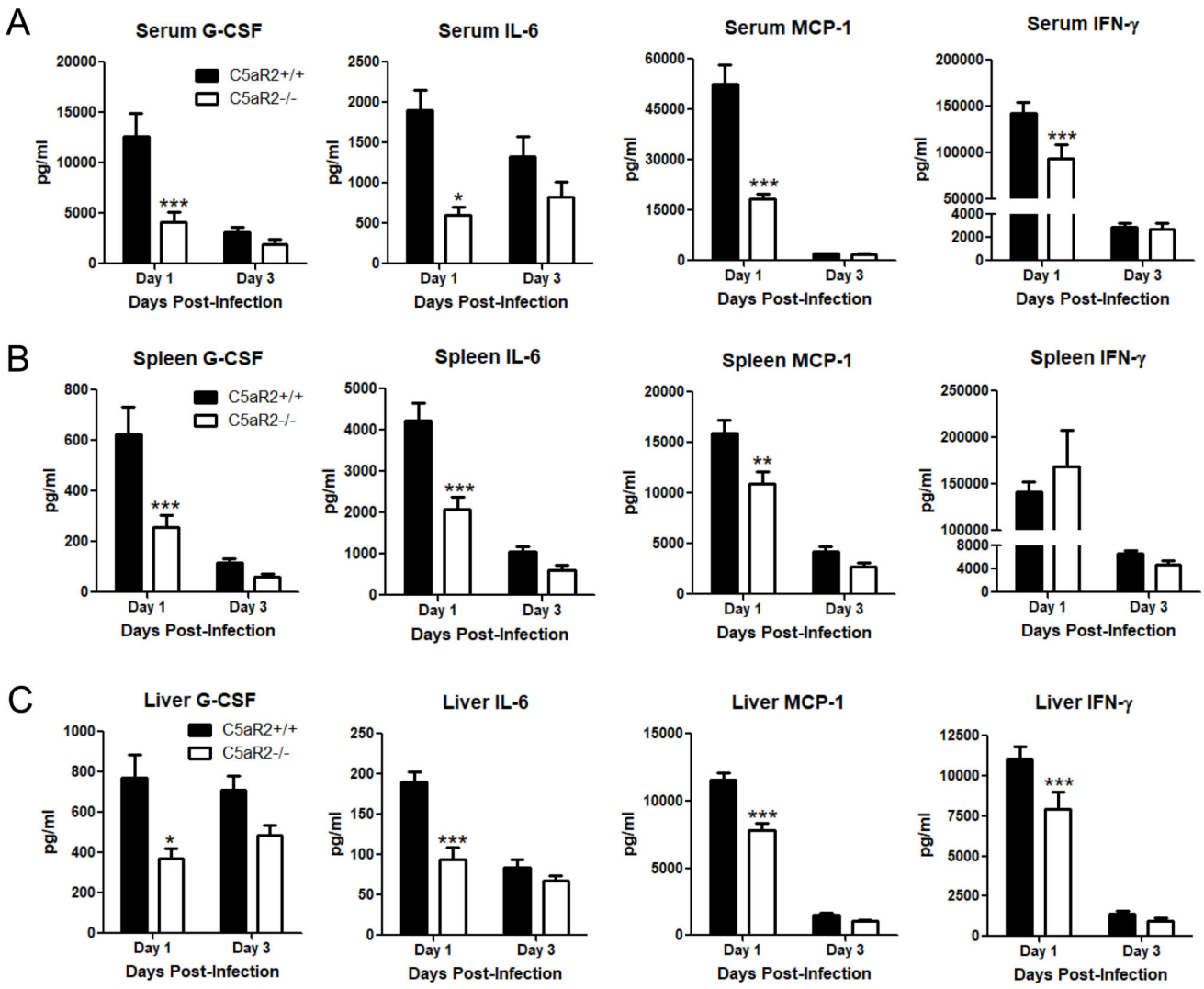

Figure 4. Cytokine and chemokine production following LM infection.

C5aR2+/+ and C5aR2−/− mice were infected i.v. with 1×10^5 LM, and blood, spleen, and liver were harvested from the mice 1 day and 3 days post-infection. G-CSF, IL-6, MCP-1, and IFN-γ levels were measured from (A) serum (B) spleen and (C) liver. Uninfected mice (n = 3 per group) had no detectable levels of these cytokines and chemokines at these tested dilutions (data not shown). The data for the LM-infected mice is presented as mean pg/ml ± SEM, with n = 8–12 mice / group for day 1 and n = 15–19 mice / group for day 3. *, **, *** $P < 0.05$ by one-way ANOVA with Tukey post-test.

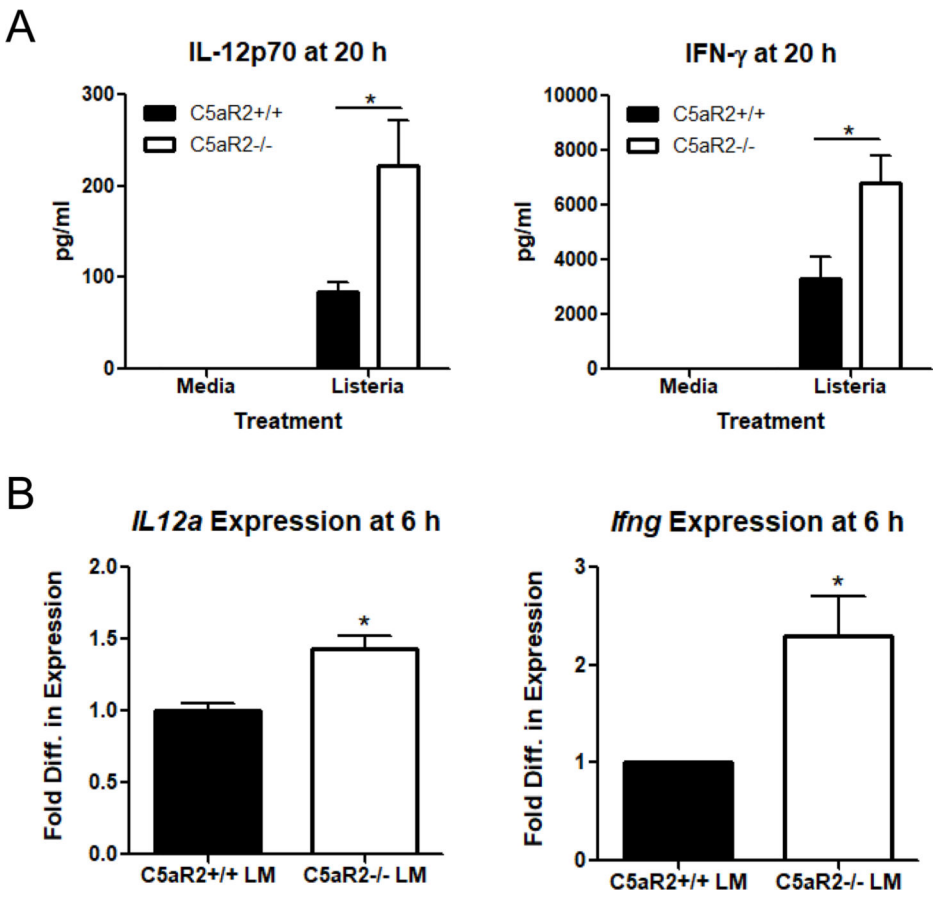

Figure 5. C5aR2−/− splenocytes produce more IL-12p70 and IFN-γ *in vitro*.

Splenocytes were purified from C5aR2+/+ and C5aR2−/− mice and incubated with either media or infected with 10^3 LM for either 6 h or 20 h. (A) IL-12p70 and IFN-γ were quantitated via ELISA from the cell-free culture supernatants at 20 h. Data are presented as mean pg/ml ± SEM and are combined from three independent experiments. * $P < 0.05$ by one-way ANOVA with Tukey post-test. (B) RNA was harvested from whole cell lysates at 6 h, and transcript abundance of *IL12a* and *Ifng* were quantitated from cDNA by QPCR. Message for *IL12a* and *Ifng* were normalized to *Gapdh* for each sample, and the infected C5aR2+/+ splenocytes were set as a value of 1.0 for statistical purposes. Data are presented as mean fold-difference in expression ± SEM and are combined from three independent experiments. * $P < 0.05$, by t test

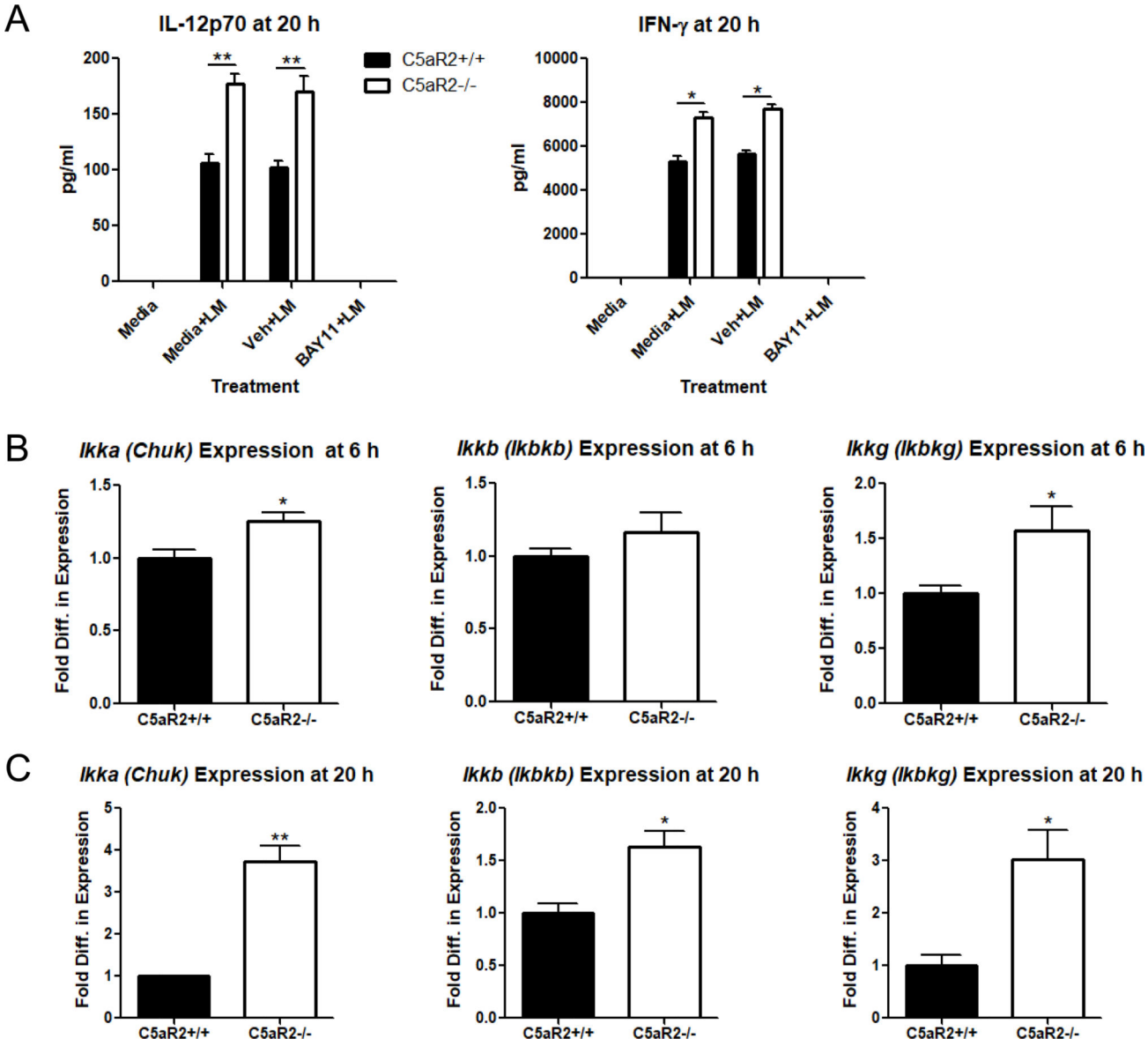

Figure 6. C5aR2 modulates the NF-kB pathway during *in vitro* splenocyte infection.

Splenocytes were purified from C5aR2+/+ and C5aR2−/− mice and were pre-treated with either media, diluted DMSO vehicle, or 1 μM BAY11–7082 for 2 h prior to infection with 10^3 LM for 20 h. (A) IL-12p70 and IFN-γ were quantitated via ELISA from the cell-free culture supernatants at 20 h. Data are presented as mean pg/ml ± SEM and are combined from three independent experiments. *, ** $P < 0.05$ by one-way ANOVA with Tukey post-test. (B) and (C) Purified splenocytes from C5aR2+/+ and C5aR2−/− mice were infected with 10^3 LM for either 6 h (B) or 20 h (C). RNA was harvested from whole cell lysates, and transcript abundance of *Ikka (Chuk)*, *Ikkb (Ikbkb)*, *Ikkg (Ikbkg)*, and *Gapdh* were quantitated from cDNA by QPCR. Message for each *Ikk* gene was normalized to *Gapdh* for each sample, and the infected C5aR2+/+ splenocytes were set to a value of 1.0 for statistical purposes. Data are presented as mean fold-difference in expression ± SEM and are combined from three independent experiments. * $P < 0.05$, ** $P = 0.002$, by t test

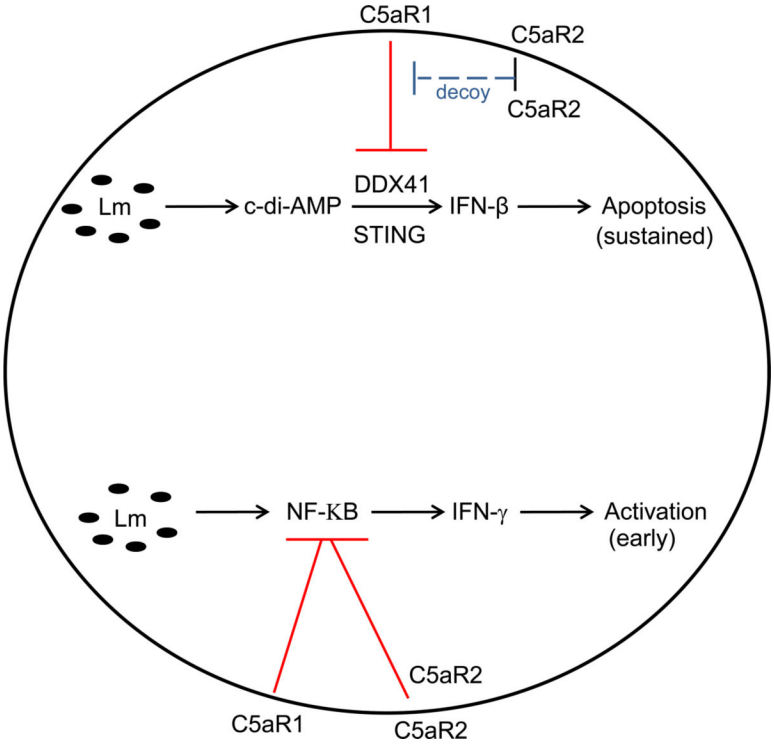

Figure 7. Model depicting the regulation of the host cellular response to systemic LM infection by C5aR1 and C5aR2.

The top of the figure illustrates how C5aR1 signaling protects host cells against LM-mediated apoptosis by suppressing IFN-β expression via early as well as sustained inhibition of the cyclic dinucleotide-activated surveillance pathway, which involves DDX41, STING, as well as other intracellular protein mediators (see reference 20 for more details). Also shown in the top of the figure is how C5aR2 acts as a decoy receptor during LM infection by impairing to some degree C5aR1 suppression of IFN-β production. In the bottom part of the figure, C5aR1 and C5aR2 are depicted suppressing NF-KB-mediated expression of IFN-γ during the early stage of LM infection. Importantly, neither the C5aR2 actions as a decoy receptor nor the C5aR1/C5aR2 suppression of IFN-γ during the early stage of infection are able to override the overall and sustained host protection provided by C5aR1 impairment of IFN-β expression in this systemic model of LM.