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Recruited Macrophages Control Dissemination of Group A Streptococcus from Infected Soft Tissues

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Group A Streptococcus (GAS) causes diverse infections in humans, ranging from mild to life-threatening invasive diseases, such as necrotizing fasciitis (NF), a rapidly progressing deep tissue infection. Despite prompt treatments, NF remains a significant cause of morbidity and mortality, even in previously healthy individuals. The early recruitment of leukocytes is crucial to the outcome of NF; however, although the role of polymorphonuclear neutrophils (PMNs) in host defense against NF is well established, the role of recruited macrophages remains poorly defined. Using a cutaneous murine model mimicking human NF, we found that mice deficient in TNF-α were highly susceptible to s.c. infections with GAS, and a paucity of macrophages, but not PMNs, was demonstrated. To test whether the effects of TNF-α on the outcome of infection are mediated by macrophages/monocytes, we systemically depleted C57BL/6 mice of monocytes by pharmacological and genetic approaches. Systemic monocyte depletion substantially increased bacterial dissemination from soft tissues without affecting the number of recruited PMNs or altering the bacterial loads in soft tissues. Enhanced GAS dissemination could be reverted by either i.v. injection of monocytes or s.c. administration of peritoneal macrophages. These experiments demonstrated that recruited macrophages play a key role in defense against the extracellular pathogen GAS by limiting its spread from soft tissues. The Journal of Immunology, 2011, 187: 6022–6031.

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roup A Streptococcus (GAS) is a prominent Gram-positive human pathogen causing a wide variety of diseases, ranging from mild infections to life-threatening conditions, even in healthy children and adults. GAS is responsible for a minimally estimated 616 million cases of throat infections worldwide per year, as well as 111 million cases of skin infections (1). Although these infections are usually superficial and self-limiting, there has been an unexplained resurgence in several forms of severe invasive GAS diseases since the mid to late 1980s, including necrotizing fasciitis (NF), necrotizing pneumonia, and sepsis syndrome (2). NF is a rapidly progressive, highly destructive bacterial infection involving the skin, s.c. and deep soft tissues, and muscles that are referred to collectively in this article as soft tissues. Clinical studies demonstrated that a prompt surgical debridement of the infected soft tissues is critical for the patient’s better outcome (3). Yet, mortality from GAS NF remains high and, in some reports, exceeds 50% (4).

In skin infections, the primary line of local innate defense consists of antibiotic peptides and resident cells, including keratinocytes, dendritic cells, T cells, and macrophages (5). Upon breaching of these barriers, microorganisms are recognized by receptors of the innate immune system, thus triggering an inflammatory response aimed at eliminating the pathogens (6). One of the cardinal downstream proinflammatory cytokines generated is TNF-α, which, among other functions, controls leukocyte infiltration, including polymorphonuclear neutrophils (PMNs) and macrophages, to the site of bacterial invasion (7). PMNs, which are the most abundant among leukocytes, arrive promptly at the site of infection and are well equipped for clearing invading microorganisms. They kill invading bacteria by phagocytosis, degranulation, and the formation of neutrophil extracellular traps (8-10). Because PMNs fulfill a key role in preventing the transition of GAS soft tissue infections from local to rapidly disseminating life-threatening diseases, the interaction between GAS and PMNs has been extensively studied. It was found that GAS has evolved a plethora of virulence factors inhibiting main stages in the interaction of GAS with PMNs, including recruitment, phagocytosis, and intracellular and extracellular killing by PMNs and by extracellular traps (11-14).

The interaction of GAS with macrophages in soft tissues has received less attention. Although no definitive role for macrophages was described, recent work suggested the possibility that macrophages are important in GAS infections: using biopsies taken from patients with GAS NF, it was clearly demonstrated that macrophages are present in foci of soft tissue infections (15). Further-
more, in the context of i.v. challenge with GAS, systemic depletion of macrophages rendered mice highly susceptible to infection (16). To elucidate the role of macrophages in host resistance to GAS infection, we set out to study their effects in a murine model best simulating the conditions in human NF.

Monocytes are circulating blood leukocytes that play important roles in inflammation, homeostasis, and defense against invading microorganisms (17, 18). Recent studies showed that distinct subsets of human and mouse monocytes have different roles in the above-mentioned tasks. For example, Ly6C⁺ (Gr-1⁺) mouse monocytes are important players in defense against intracellular bacterial pathogens (18), whereas Ly6C⁻ (Gr-1⁻) monocytes constitutively patrol blood vessels and may play a role in scavenging lipids, dead cells, and potential pathogens (19, 20).

In this study, using a murine model resembling human NF, we showed that mice deficient in TNF-α are highly susceptible to GAS soft tissue infections as a result of impaired recruitment of macrophages but not PMNs. Furthermore, systemic depletion of monocytes substantially increased bacterial dissemination from soft tissues into blood and internal organs, which could be reverted by i.v. injection of monocytes or s.c. administration of peritoneal macrophages. These findings demonstrated that recruitment of macrophages into GAS-infected soft tissues plays a key role in containment of the subsequent spreading of GAS into blood and internal organs.

Materials and Methods

Bacteria

The experiments were conducted using GAS strains JS95 of the M14 serotype (21) and 5448 of the M1T1 serotype (22). GAS strains were cultured in Todd–Hewitt medium supplemented with 0.2% yeast extract (Difco, BD Diagnostics) or on blood agar plates (Novamed, Jerusalem, Israel).

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of the Hebrew University School of Medicine Animal Research Committee (approval No. MD-10-12267-4). In all experiments, animals were euthanized by isoflurane prior to surgery. To minimize suffering, mice that either lost >20% of their body weight or showed impaired motility were euthanized.

Mice

All mice used in this study had a C57BL/6 genetic background. TNF-α-deficient (B6.129S-Tnfrsf1a−/−/J), TLR2-deficient (B6.129-Tlr2−/−/J), IL-1R-deficient (B6.129S7-Ilr1−/−/J), and Ccr2-deficient mice (B6.129S4-Ccr2−/−/J) were purchased from Jackson Laboratories (Bar Harbor, ME). Wild-type (WT) C57BL/6 control mice were purchased from Harlan Laboratories (Jerusalem, Israel). Mice were housed under specific pathogen-free conditions at the Hebrew University School of Medicine Animal Resource Center. Unless otherwise specified, the experiments were conducted using mice at 4–5 wk of age. CCR2 depleter mice were generated as previously described (23).

Mouse infection model

The murine model of human soft tissue infection was performed, as detailed previously (21, 24, 25). Briefly, GAS strains were cultured at 37°C in Todd–Hewitt medium supplemented with 0.2% yeast extract and grown to OD600 = 0.3–0.4. Bacteria were then washed with sterile PBS and brought to a concentration of 1 × 10⁸ CFU in 100 µl, which were injected s.c. into mice. Mice were monitored daily, and Kaplan–Meier survival curves were generated and analyzed for statistical significance using the log-rank test. For determination of CFU in the blood, spleen, and skin, mice were euthanized at indicated times after bacterial inoculation, and blood, spleen, and skin samples were removed from animals. Spleen and skin samples were homogenized in sterile PBS. Serial dilutions of organ homogenates and blood were plated on blood agar plates, and CFU was enumerated after overnight incubation at 37°C. CFU data were analyzed using the non-parametric Mann–Whitney U test.

Myeloperoxidase assay

Myeloperoxidase (MPO) levels were quantified using a commercial MPO assay kit (Cytostore, Calgary, AB, Canada), according to the manufacturer’s recommendations. The assays were performed on homogenized 6-mm punch biopsies (Acuderm, Fort Lauderdale, FL) of lesional skin specimens. Homogenates were obtained using Polytron homogenizer (Kinematica, Lucerne, Switzerland) for 30 s at 4°C. The MPO units of activity were normalized according to the protein content present in the corresponding samples, measured by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Data were analyzed using the Student t test.

Immunoperoxidase labeling

Sections were deparaffinized with xylene and rehydrated through gradient ethanol immersions (100% and 96%, respectively). Endogenous peroxidase activity was quenched with 3% H₂O₂ (DAKO, Glostrup, Denmark). Ag retrieval of Ly-6G was performed by microwaving the section for 20 min in citric acid buffer (pH 6) (Dako Cytomation, DAKO). Ag retrieval of F4/80 was performed using 1 mg/ml proteinase K (Sigma-Aldrich) for 10 min. The slides were washed twice in distilled water and treated again with 3% H₂O₂. The sections were washed with TBS auto wash buffer (Biocare Medical, Concord, CA) for 5 min and then incubated with the different Abs: a 1/100 dilution of anti-F4/80 (AbD Serotec, Oxford, U.K.) and a 1/25 dilution of anti-Ly-6G (Novus Biologicals, Littleton, CO). Sections were treated with anti-rat HRP (Nichirei Biosciences) for 30 min following three washes with TBS washing buffer; reaction product was visualized using the UltraVision Detection System (Thermo scientific) or the AEC staining kit (Sigma-Aldrich). Sections were counterstained with hematoxylin for 20 s and rinsed with tap water, immediately dehydrated by sequential immersion in gradient ethanol and xylene, and then mounted with mounting solution (Thermo scientific) on coverslips. Stained cells were counted under a light microscope. The data were analyzed using the nonparametric Mann–Whitney U test.

Determination of cytokines by ELISA

At indicated times after GAS infection, mice were sacrificed, and tissue sections surrounding the lesion were excised and minced with scissors. The chemokines were extracted from the minced tissues by incubation for 1 h at room temperature with lysis buffer containing 10 mM Tris-HCl (pH 7.8), supplemented with 1% octylphenoxypolyethoxyethanol (Nonidet P-40), 150 mM NaCl, 40 mM EDTA, and complete Minimix protease inhibitors (Roche). The extracts were centrifuged at 17,000 × g for 5 min at room temperature. Supernatants were stored at −80°C until all samples were collected. The amounts of the murine PMN-chemotaxtractant CXC chemokines KC and MIP-2; the monocyte chemotactic protein 1 CC chemokine MCP-1; TNF-α; the CCL5 chemokine RANTES; and the CX3CL1 chemokine Fractalkine were determined using Quantikine ELISA kits (R&D Systems), according to the manufacturer’s recommendations. The protein levels were normalized to the protein content of the corresponding samples, measured by Bradford protein assay (Bio-Rad Laboratories). Data were analyzed using the Student t test.

Isolation of peritoneal macrophages

Mouse macrophages were isolated according to the procedure of Kumagai et al. (26). Briefly, mouse peritoneal exudate cells were harvested from C57BL/6 mice 5 d after i.p. injection of heat-killed (20 min at 80°C) JS95 GAS. Cells were washed and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 10,000 U/ml penicillin, 10 mg/ml streptomycin, 1250 U/ml nystatin, 4 mM l-glutamine, and 25 mM HEPES (Biological Industries, Beit Haemek, Israel). Peritoneal cells were incubated at 37°C in a CO₂ (5%) cell culture incubator for 3 h in plastic petri dishes. The nonadherent cells were removed by washing with PBS, and adherent cells were cultured overnight under the same conditions. Cells were detached by incubation with PBS containing 0.2% EDTA and adherent cells were cultured overnight under the same conditions. Cells were detached by incubation with PBS containing 0.2% EDTA and 5% PBS for 30 min at 4°C and resuspended in PBS or RPMI 1640. Based on flow cytometry analysis using an anti-F4/80 mAb, >95% of the cells were macrophages.

Systemic depletion of monocytes

Systemic monocyte depletion was achieved by two successive i.p. injections of clodronate liposomes (Clo-lipo; 0.4 ml/mouse/injection) 72 and 24 h before s.c. inoculation with GAS. Clo-lipo and PBS liposomes (PBS-lipo) were purchased from Clodronate Liposome Organization (http://www.
clodronateliposomes.org). Control mice were injected with PBS-lipo at the same time. A typical flow cytometry analysis obtained for monocyte-depleted mice is shown in Supplemental Fig. 2A.

**Monocytes purification from bone marrow**

Bone marrow (BM) was harvested from femurs and tibias of C57BL/6 mice under sterile conditions and suspended in PBS. RBCs were lysed using lysis buffer containing 0.15 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA, and monocytes were purified using the Easy-Sep mouse monocyte enrichment negative selection kit (STEMCELL Technologies), according to the manufacturer’s protocol.

**Systemic complementation of monocytes**

BM-derived monocytes (BMDMs) were isolated, as detailed above, and injected (1–2 × 10⁶ cells suspended in 0.1 ml PBS) into the lateral tail vein of monocyte-depleted mice (see above). Four hours later, mice were inoculated s.c. with GAS, and bacterial dissemination into sterile tissues was examined 24 h postinfection.

**Monocyte depletion in CCR2 depleter mice**

Systemic monocyte depletion in CCR2 depleter mice was achieved by two successive i.p. injections with 10 mg/g body weight of diphtheria toxin, 24 h prior to, and just before, s.c. inoculation with GAS. Control mice were injected with PBS at the same times. Flow cytometry analysis performed on blood cells isolated from monocyte-depleted CCR2 depleter mice is shown in Supplemental Fig. 4.

**Subcutaneous transfer of macrophages**

Peritoneal macrophages were isolated, as described above, and injected (1–2 × 10⁶ cells suspended in 0.1 ml PBS) s.c. into monocyte-depleted mice (see above). Four hours later, mice were inoculated s.c. with GAS, and GAS dissemination into sterile tissues was examined 24 h postinfection.

**Phagocytosis assay**

Bacteria were grown to exponential phase, opsonized with 10% mouse serum and 20% heat-inactivated FBS for 30 min at room temperature, and then resuspended in RPMI 1640 medium. Peritoneal macrophages that were isolated, as described above, were seeded in a six-well plate (2 × 10⁶ cells/well) and infected with 0.1 ml bacterial suspension (2 × 10⁸ bacteria) for 1 h at 37°C in a CO₂ (5%) cell incubator. Cells were then washed three times with PBS and incubated with gentamicin (500 μg/ml) for 1.5 h to allow killing of remaining extracellular bacteria. The medium containing gentamicin (500 μg/ml) was removed, and fresh medium containing gentamicin (100 μg/ml) was added. Cells were further incubated for different time periods, after which cells were washed with PBS to remove the antibiotics and lysed by the addition of distilled water supplemented with 2% saponin (Sigma-Aldrich). Dilutions were plated on blood agar plates, and CFU were enumerated after overnight incubation at 37°C. Data were analyzed using the Student t test.

**Whole-blood survival assay**

The ability of GAS to survive in mouse blood was tested by the direct bacterial test of Lancefield, as we described previously (21).

**Flow cytometry**

Blood, single-cell suspension of spleen, and peritoneal lavage were suspended in flow cytometry buffer (PBS supplemented with 2% FCS and 0.01% sodium azide) and stained with FITC-conjugated anti–Mac-1 (CD11b), allophycocyanin-conjugated anti-F4/80, allophycocyanin-conjugated anti–Gr-1(Ly6C/G), PE-conjugated anti-CD115, or matched isotype controls (eBioscience, San Diego, CA). Immunostained cells were analyzed with CyAnADP (Dako, Carpinteria, CA) using Summit v4.3 software.

**Statistical analysis**

Because the absolute values of the three experiments described in Fig. 9B varied, we converted the results to Z-scores (the difference between each CFU result and that experiment’s average, divided by the experiment’s SD) and assessed the significance of the difference among the three groups of mice by the Mann–Whitney U test.

**Results**

**TNF-α-deficient mice are highly susceptible to GAS soft tissue infection**

TLR2, IL-1R, and TNF-α have been implicated in the innate detection and response to skin and soft tissue infections caused by the Gram-positive pathogen *Staphylococcus aureus* (27, 28). Therefore, we decided to test their involvement in GAS-mediated soft tissue infections. To this end, we used transgenic mice deficient (−/− = knockout [KO]) in these components and a murine soft tissue infection model that mimics human NF (13, 24, 29, 30). WT (C57BL/6) and mutant mice were inoculated s.c. with 10⁸ CFU of the GAS strain JS95, which was isolated from a patient with NF (24). TNF-α KO mice showed a rapidly progressing infection and an enhanced death rate within 2–7 d after challenge. In contrast, only half of the WT mice challenged with GAS strain JS95 succumbed to the infection after 14 d (Fig. 1A). Mice inoculated s.c. with GAS die as a result of bacterial dissemination from soft tissues into blood and internal organs (29). Therefore, we compared the amount of bacteria present in the blood of WT
and TNF-α KO mice and found that, at both 48 and 72 h after GAS inoculation, TNF-α KO mice had a significantly higher bacterial load in the blood than did WT mice (Fig. 1B, 1C).

In contrast to TNF-α KO mice, there were no significant differences in survival rates of WT versus IL-1R KO or TLR2 KO mice (Fig. 2). These data suggested that TNF-α, but not IL-1R or TLR2, is involved in host defense against GAS soft tissue infection. Interestingly, these findings are in accordance with reports that anti–TNF-α therapy may increase the risk for GAS NF in rheumatoid arthritis and dermatomyositis patients (31, 32).

**TNF-α–deficient mice are impaired in macrophage, but not in PMN, recruitment following s.c. inoculation with GAS.**

We examined the level of TNF-α production in lesional soft tissues from infected WT and TNF-α KO mice using ELISA. In WT mice, the level of TNF-α was already elevated 6 h postinfection and remained high after 48 h; as expected, no TNF-α was detected in the TNF-α KO mice (Fig. 3A). Histological examination of lesional soft tissues from WT and TNF-α KO mice revealed muscle inflammation with extensive necrosis of fascia, dermis, and epidermis, along with very high numbers of GAS present in the fascia. In contrast to the low level of PMN infiltration observed in young BALB/C mice (24, 25), in both WT and TNF-α KO C57BL/6 mice, we noted a significant influx of PMNs that concentrated mainly at the boundaries of the necrotic fascia (Fig. 3B). It has been reported that the mouse innate immune response to GAS challenge differs among different inbred mouse strains and is age dependent (33, 34). The above-mentioned finding implied that PMN recruitment into the infected GAS soft tissues is independent of TNF-α. To verify this notion, we also assessed the amount of recruited PMNs by measuring MPO activity (Fig. 3C), as well as by enumerating anti–Ly-6G–labeled cells in tissue sections of mice at 48 h postinfection (Fig. 3D). Both assays showed that the number of PMNs recruited to soft tissues infected with GAS was similar in WT and TNF-α KO mice. Furthermore, the levels of MPO activities in soft tissue sections derived from WT and TNF-α KO mice 24 and 72 h after GAS challenge were similar (data not shown), thus corroborating that the increased susceptibility of TNF-α–deficient mice to s.c. GAS challenge is not caused by altered PMN recruitment.

It was reported that TNF-α is essential for the adequate recruitment of systemic leukocytes to the focus of infection (35). Because PMN recruitment was unaffected by TNF-α deficiency (Fig. 3), we tested the recruitment of macrophages. To this end, we stained the soft tissue sections with a mAb against F4/80, a murine macrophage marker. Comparison of the stained sections from WT versus TNF-α KO mice (Fig. 4A) and enumeration of macrophages (Fig. 4B) revealed that the deficiency in TNF-α signifi-
significantly diminished macrophage influx to the infected soft tissues. We also used flow cytometry to measure the flux of PMNs and macrophages into the peritoneum of WT and TNF-α KO mice challenged i.p. with heat-killed GAS. Although the number of PMNs trafficking into the peritoneum of WT and TNF-α KO mice was similar, the number of macrophages entering the peritoneum of TNF-α KO mice was significantly lower than that entering the peritoneum of WT mice, both 48 and 72 h after bacterial challenge (Supplemental Fig. 1A, 1B). Taken together, these results suggested that TNF-α deficiency affects the ability of macrophages to traffic into the site of GAS infection.

TNF-α could trigger macrophage recruitment through the induction of various chemokines and by controlling the expression of adhesion molecules on endothelial cells (36–39). Thus, we used ELISA to measure the levels of MCP-1 (CCL2), CX3CL1, MIP-2 (CXCL2), KC (CXCL1), and RANTES (CCL5) in GAS-infected soft tissues from WT and TNF-α KO mice (Figs. 4C, 5) and stained soft tissues with Ab directed against the adhesion molecule ICAM-1 (data not shown). We found that only RANTES levels were significantly elevated in WT mice compared with TNF-α KO mice 48 h after challenge (Fig. 4C), suggesting that TNF-α may increase the influx of macrophages by augmenting the level of RANTES in GAS-infected soft tissues.

To test whether the increased susceptibility of TNF-α KO mice could be attributed to inefficient phagocytosis and killing of GAS by TNF-α–deficient macrophages, we compared the ability of the corresponding types of macrophages to kill GAS ex vivo. To this end, peritoneal macrophages were isolated from WT and TNF-α–deficient mice after challenge with heat-killed GAS strain JS95. The two types of macrophages were infected in vitro with live GAS strain JS95, and surviving bacteria were enumerated. Both types of macrophages killed GAS with the same efficiency.
In addition to altering macrophage influx to GAS-infected soft tissues infected with GAS but not to an altered ability to eliminate the bacteria. We tested whether the migration of macrophages into GAS-infected fascia plays a role in defense against GAS infection (Fig. 6B–D, Supplemental Fig. 3). Taken together, these data showed that, in the absence of systemic monocytes, GAS dissemination from soft tissues is substantially increased, despite the fact that the load of bacteria in soft tissues are unaffected (Fig. 6B–D, Supplemental Fig. 3).

Immunostaining of soft tissue sections harvested 12 h postinfection showed a number of macrophages that were recruited into the infected fascia in PBS-lipo–treated mice, whereas in Clo-lipo–treated mice, the number was significantly smaller (Fig. 7A, 7B). In addition, ex vivo proliferation of GAS in whole blood isolated from untreated mice, which had been treated with Clo-lipo, was unexpectedly lower than in blood withdrawn from control mice treated with PBS-lipo (41, 42). The Clo-lipo treatment depleted monocytes in blood and spleen without affecting the number of PMNs (Supplemental Fig. 2A). Furthermore, this treatment did not affect recruitment of PMNs into GAS-infected soft tissues compared with mice treated with PBS-lipo at 12, 24, and 48 h postinfection (Supplemental Fig. 2B). Notably, despite the normal PMN recruitment, treatment of mice with Clo-lipo rendered them more sensitive to s.c. inoculation with GAS compared with mice treated with PBS-lipo or mice injected with PBS alone, as reflected by an increased mouse mortality (Fig. 6A).

Survival curves of monocyte-depleted (Clo-lipo; n = 28) and control (PBS [n = 28] and PBS-lipo [n = 14]) mice after inoculation with GAS. The Kaplan–Meier analysis is based on results from four separate experiments. p < 0.0001, log rank (Mantel–Cox) test. Horizontal lines represent medians. *p < 0.001, nonparametric Mann–Whitney U test. (Supplemental Fig. 1C). This suggested that the increased susceptibility of TNF-α–deficient mice to s.c. inoculation with GAS can be mainly attributed to the altered influx of macrophages to soft tissues infected with GAS but not to an altered ability to eliminate the bacteria.

**Systemic depletion of monocytes by Clo-lipo renders mice sensitive to s.c. inoculation with GAS**

In addition to altering macrophage influx to GAS-infected soft tissues, TNF-α may modulate innate immunity in several interdependent ways (7). Therefore, we wished to isolate the effect of macrophages on mice susceptibility to s.c. GAS infection. Because it was suggested that resident macrophages efficiently take up and kill GAS during in vivo infection (16), we attempted to deplete this subset of macrophages by s.c. injection of Clo-lipo, according to a protocol that has been used to eliminate skin macrophages in mice (40). We found that Clo-lipo–treated mice were as sensitive to s.c. challenge with GAS as were untreated mice or mice treated with PBS-lipo (data not shown). Therefore, we tested whether the migration of macrophages into GAS-infected soft tissues plays a role in defense against GAS infection. To examine this notion, we systemically depleted monocytes by injecting mice i.p. with Clo-lipo: a method that has been widely used to address monocyte/macrophage functions in mice (41, 42). The Clo-lipo treatment depleted monocytes in blood and spleen without affecting the number of PMNs (Supplemental Fig. 2A). Furthermore, this treatment did not affect recruitment of PMNs into GAS-infected soft tissues compared with mice treated with PBS-lipo at 12, 24, and 48 h postinfection (Supplemental Fig. 2B). Notably, despite the normal PMN recruitment, treatment of mice with Clo-lipo rendered them more sensitive to s.c. inoculation with GAS compared with mice treated with PBS-lipo or mice injected with PBS alone, as reflected by an increased mouse mortality (Fig. 6A). Moreover, a sharp increase in bacterial load was detected in spleen and blood of Clo-lipo–treated mice compared with PBS-lipo–treated mice at 3, 6, 12, and 24 h postinfection (Fig. 6B–D, Supplemental Fig. 3). Taken together, these data showed that, in the absence of systemic monocytes, GAS dissemination from soft tissues is substantially increased, despite the fact that the load of bacteria in soft tissues are unaffected (Fig. 6B–D, Supplemental Fig. 3).

Immunostaining of soft tissue sections harvested 12 h postinfection showed a number of macrophages that were recruited into the infected fascia in PBS-lipo–treated mice, whereas in Clo-lipo–treated mice, the number was significantly smaller (Fig. 7A, 7B). In addition, ex vivo proliferation of GAS in whole blood isolated from untreated mice, which had been treated with Clo-lipo, was unexpectedly lower than in blood withdrawn from control mice.

**FIGURE 6.** Systemic depletion of monocytes renders mice susceptible to s.c. inoculation with GAS. Mice were injected i.p. with control (PBS or PBS-lipo) or Clo-lipo on days 1 and 3. On day 4, mice were inoculated s.c. with GAS. A, Survival curves of monocyte-depleted (Clo-lipo; n = 28) and control (PBS [n = 28] and PBS-lipo [n = 14]) mice after inoculation with GAS. The Kaplan–Meier analysis is based on results from four separate experiments. *p < 0.0001, log rank (Mantel–Cox) test. Horizontal lines represent medians. B, C, D, Bacterial counts (CFU) in skin (soft tissues), spleen, and blood of monocyte-depleted (Clo-lipo) and control (PBS-lipo) mice 12 h after GAS inoculation. *p < 0.001, nonparametric Mann–Whitney U test. Horizontal lines represent medians.

**FIGURE 7.** The effect of systemic depletion of monocytes on macrophage presence in infected soft tissues and on GAS survival in whole blood. A, Representative photomicrographs of sections labeled with anti-F4/80 mAb (immunoperoxidase method) 12 h after inoculation with GAS. B, Macrophage counts in skin biopsies taken 12 h after inoculation. Each symbol represents the number of macrophages in one high-power field (HPF; ×1000), as determined by F4/80 staining. *p < 0.0001, nonparametric Mann–Whitney U test. Horizontal lines represent medians. C, Proliferation of GAS in whole blood. GAS strain JS95 was added to blood obtained from control (PBS and PBS-lipo) and Clo-lipo–treated mice. CFU after 3 h of growth at 37°C was determined. The multiplicity factor was calculated by dividing the CFU present in blood after 3 h by the original CFU added. The mean ± SEM was calculated from the results of at least two separate experiments using blood from different donors.
mice that did not receive BMDMs, although the probability that spleen was decreased compared with the group of Clo-lipo–treated group of mice, GAS dissemination from the soft tissues into the blood and internal organs, suggesting that monocyte-mediated control of GAS spreading represents a general trait independent of GAS serotype.

In an attempt to provide final support for monocyte-mediated control of GAS dissemination from infected soft tissues, we performed the following complementation experiment. Mice that had been depleted of monocytes by Clo-lipo treatment subsequently received a single i.v. injection of 1–2 × 10^6 BMDMs from untreated WT mice, prior to the s.c. challenge with GAS. In this group of mice, GAS dissemination from the soft tissues into the spleen was decreased compared with the group of Clo-lipo–treated mice that did not receive BMDMs, although the probability that the two groups are different was not sufficiently high (p = 0.072; Fig. 9A), probably as a result of residual clodronate killing of the grafted monocytes. In two additional experiments, a partial decrease in GAS dissemination was again observed in the groups of mice that received BMDMs. To improve the significance, we wished to combine results from all three experiments, but because the CFU ranges varied between them, we converted the results to Z-scores (the difference between each CFU result and that experiment’s average, divided by the experiment’s SD). Mann–Whitney U analysis of the combined data’s Z-scores showed a statistically significant difference between the group of mice treated with Clo-lipo and the group of Clo-lipo–treated mice that subsequently received BMDMs (Fig. 9B), demonstrating that monocytes are directly involved in the control of GAS spreading from infected soft tissues.

Depletion of monocytes in CCR2 depleter mice increases dissemination of GAS from soft tissues

The Ly6C+ (Gr1+) subset of mouse monocytes is considered to act as inflammatory monocytes, mainly responsible for dealing with bacterial infections. These monocytes express the CCR2 chemokine receptor that promotes their emigration from BM to the circulation (17, 45). Thus, we tested CCR2 KO mice and, to our surprise, found that they are not more susceptible to s.c. inoculation with GAS than parental WT C57BL/6 mice (n = 34 mice); correspondingly, the loads of GAS detected in soft tissues, blood, and spleen 24 h postinfection were similar in both types of mice (data not shown). Nonetheless, it was reported that the absence of CCR2 only reduced circulating monocytes by ~80% during homeostatic conditions, and CCR2-independent emigration of Ly6Ch+ subtype monocytes from the BM may confound the interpretation of experiments using CCR2-deficient mice (23, 45). To examine this possibility and to confirm the findings obtained using Clo-lipo (Fig. 6), we used a CCR2 depleter mouse strain, in which administration of diphtheria toxin (DT) obliterates >99% of the BM Ly6Ch+ monocyte subtype, along with Ly6Ch+ monocytes (23). Treatment of CCR2 depleter mice with DT significantly increased the presence of GAS in spleen compared with PBS-treated and control mice, without affecting GAS load in soft tissues (Fig. 10). The difference between GAS counts in the blood of WT mice and the group of CCR2 depleter mice with DT was significant, but the difference in CFU between CCR2 depleter mice with DT and CCR2 depleter mice with PBS was not (Fig. 10). Nonetheless, taken together, these results add genetic evidence that monocytes fulfill a central role in the control of GAS dissemination from infected soft tissues.

Subcutaneous transfer of macrophages prevents GAS dissemination

To test whether it would be possible to decrease GAS dissemination from infected soft tissues by complementing them locally with macrophages, we performed the experiment described below. Mice that were treated with Clo-lipo were injected s.c. with peritoneal macrophages derived from WT mice. Four hours later, the mice (designated in this article as complemented) were inoculated s.c. with GAS strain JS95. After an additional 3 h, we enumerated bacterial loads in the soft tissues, spleen, and blood of three groups of mice: PBS-lipo treated, Clo-lipo treated, and complemented. The results shown in Figure 11 clearly demonstrated that mice from the group regained their ability to control bacterial spreading at a level comparable to the group of mice treated with PBS-lipo (control) (Fig. 11), demonstrating that the presence of recruited macrophages in soft tissues is required to control GAS dissemination.
Discussion

Although GAS is a human-specific pathogen, GAS NF can be partly reproduced in mice upon either s.c. or i.m. inoculation with serotypes causing invasive GAS diseases in human (13, 21, 24, 25, 29, 30, 43, 44). Studies based on the above-mentioned murine models demonstrated that PMNs constitute an essential defense component preventing the transition of GAS from local to disseminated infection and, hence, GAS produces a plethora of virulence factors that hamper PMN responses (11–14). The role of recruited monocytes/macrophages in protecting the host against GAS cutaneous infection remains undefined. On one hand, it was reported that GAS survives inside macrophages present in necrotic soft tissues debrided from NF patients previously treated with antibiotics. This led the investigators to propose that macrophages may act as “Trojan horses” facilitating GAS spreading (15). On the other hand, based on an i.v. route of mouse infection, it was concluded that resident macrophages can efficiently phagocytose and kill GAS in vivo, and it was suggested that this killing may play an important role in cutaneous host resistance (16). Although this study was not conducted in a murine model resembling human soft tissue infections, and the number of PMNs was altered by the depletion procedure both in the blood and the spleen (16), it suggested that macrophages may play a major role in host resistance against GAS infections.

Our initial indication that recruited macrophages may be involved in the protection of the host against invasive GAS infection was provided by the fact that mice deficient in TNF-α are substantially more sensitive to invasive GAS infection in the cutaneous model mimicking human NF. However, TNF-α modulates innate immunity by affecting a complex network of cytokines and chemokines, and it participates in the orchestration of the inflammatory response by regulating the migration, activation, and biological properties of various immune effector cells, particularly those of PMNs and monocytes/macrophages (38, 46, 47). We
found that PMN responses in TNF-α–proficient and -deficient mice, s.c. challenged with GAS, were similar as assessed by immunostaining of PMNs, determination of MIP-2 and KC levels in soft tissues, and assays of MPO activity in soft tissues. In contrast, we found that TNF-α–deficient mice have a reduced ability to recruit macrophages and this, in part, could account for an increased ability of GAS to spread and cause a systemic lethal disease.

To corroborate this notion, we undertook two independent approaches. The first was to systemically deplete monocytes using liposome-encapsulated bisphosphonate (clodronate), a method widely used to address monocyte/macrophage functions in mice (41, 42). The second approach is based on the use of a transgenic CCR2 depleter mouse strain in which >99% of the Ly6C+ and 90% of Ly6C– of BM monocyte subtypes are temporarily ablated upon administration of DT (23). These two approaches lead to the same phenotype: enhanced GAS spreading without affecting either bacterial load or PMN content in GAS-infected soft tissues.

Subcutaneous injection of Clo-lipo was shown to eliminate resident skin macrophages (40). Although we did not see a change in the ability of GAS to spread after such a treatment, one cannot completely rule out the possibility that some macrophage depletion from soft tissues occurs in our systemic-depletion methodologies and contributes to the enhanced GAS dissemination. However, our ability to reverse GAS dissemination by i.v. transfer of BMDMs from WT mice into mice systemically depleted of monocytes argues against this possibility.

It is possible that local proliferation of GAS in blood and internal organs could be, in part, accountable for the increased load of bacteria seen in monocyte-depleted mice inoculated s.c. with GAS. Nevertheless, there are several lines of experimental evidence arguing against this scenario. First, the ability of GAS to grow in whole blood derived from monocyte-depleted mice is significantly lower than its ability to grow in blood from control mice, suggesting that the number of disseminated GAS bacteria may, in fact, be higher. The reason for the decreased rate of GAS proliferation in blood in the absence of monocytes is unknown. It may be linked to the fact that GAS temporarily survive within monocytes and macrophages (15), and, in their absence, the efficiency of killing by PMNs in whole blood might increase. Second, although the bacterial loads in soft tissues from monocyte-depleted and control mice were similar at 3, 6, 12, and 24 h after s.c. GAS inoculation, the bacterial loads in blood and internal organs in the former group of mice were significantly higher at all of the indicated times, even at 3 h after GAS inoculation. Lastly, our ability to decrease the rate of the systemic appearance of GAS by s.c. injection of peritoneal macrophages constitutes a strong argument against the option of proliferation in the blood and spleen.

It is important to note that although the initial inflammatory responses that are aimed at restricting the dissemination of cutaneous pathogens, such as GAS and S. aureus, are absolutely dependent on MyD88 (28, 48), the downstream responses appear to be fundamentally different. Although the control of S. aureus infections is independent of TNF-α, marginally dependent on TLR2, and primarily dependent on IL-1R (28), the control of GAS dissemination primarily relies on TNF-α but is independent of either IL-1R or TLR2.

The histological analyses showed that the number of recruited monocyte/macrophages to the site of infection is much lower than the number of recruited PMNs. Although PMNs surround the focus of infection, monocytes/macrophages are recruited directly to the center of the infection and can be detected in the necrotic fascia, which is filled with GAS (Fig. 7). Thus, it is possible that the roles of recruited monocytes/macrophages are to phagocytose and kill GAS at specific loci from where dissemination of GAS occurs, whereas PMNs are unable to reach these sites. The discovery that recruited monocytes/macrophage control GAS spreading from infected fascia paves the way for the design of novel treatments against this life-threatening NF disease.

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Disclosures

The authors have no financial conflicts of interest.

References

Supporting information

**FIGURE S1.** The influx of macrophages and PMNs into the peritoneum of WT and TNF-α KO mice. Peritoneal cells from WT and TNF-α KO mice were harvested (A) 48 and (B) 72 hr after i.p. inoculation with heat-killed GAS, and analyzed by flow cytometry. PMNs: SSC**high**Gr-1**high** CD11b+. Macrophages: F4/80+CD11b+. Black bar-WT, white bar-TNF-α KO mice. Each bar represents the mean determination of 4 mice. Error bars represent SEM (*P<0.05 for 48 hr; *P<0.025 for 72 hr; student's T test). (C) GAS survival in peritoneal macrophages derived from WT and TNF-α KO mice. Peritoneal macrophages were infected with GAS strain JS95 at a multiplicity of infection (MOI) of 1 and were incubated for 1 hr, after which gentamicin was added in order to kill extracellular bacteria. Macrophages were lysed at the indicated time points and CFU of intracellular GAS were enumerated. Black bar-WT, white bar-TNF-α KO mice. Results represent mean ± SEM. Experiments were repeated 4 times yielding similar results.

**FIGURE S2.** Intraperitoneal treatment of mice with clodronate liposomes systemically depletes monocytes but not PMNs. (A) Flow cytometry analysis of spleen and blood cells isolated from mice treated with either PBS-lipo (control) or Clo-lipo. mAbs against F4/80, CD115 and Gr-1 were used for staining. PMNs: SSC**high**Gr-1**high**. Macrophages: F4/80**+**CD115**+**. The results shown represent a typical experiment which was repeated 3 times yielding similar results. The numbers in the upper panels stand for the percentage of gated cells. (B) Influx of PMNs into the soft tissues is not affected by systemic depletion. Mean myeloperoxidase (MPO) activity (pg/mg protein) ± SD in skin biopsies taken 12, 24 and 48 hr after inoculation of control (PBS-lipo) and monocyte-depleted (Clo-lipo)
mice with GAS. Each bar represents the mean determination in 4 mice performed in duplicates (P>0.05 student's t test).

**FIGURE S3.** Systemic depletion of monocytes increases dissemination of GAS from soft tissues into blood and spleen. Bacterial counts (CFU) in blood, spleen, and soft tissues of monocyte-depleted (Clo-lipo) and control (PBS-lipo) mice, at (A) 3, (B) 6 and (C) 24 hr after inoculation. Statistical analyses were performed using non-parametric Mann-Whitney test (*P< 0.001 for 3 and 24 hr.* *P< 0.05 for 6 hr). Horizontal lines represent medians.

**Figure S4.** Treatment of CCR2 depleter mice with diphtheria toxin (DT) systemically depletes monocytes but not PMNs. Flow cytometry analysis of blood cells isolated from CCR2 depleter mice 1 day after i.p. administration of either PBS or 10 ng/gr body weight of DT. For staining we used mAbs against CD115 and Gr-1. PMNs: SSC\textsuperscript{high}Gr-1\textsuperscript{high}. Monocytes: CD115\textsuperscript{+}. Upper right dot blot is gated on CD115\textsuperscript{+} cells. R2, R3 and R4 refer to Gr1\textsuperscript{+} monocytes, Gr1\textsuperscript{-} monocytes and neutrophils respectively. The numbers stand for the percentage of gated cells. Depletion of monocytes (A), and survival of PMNs (B) are demonstrated.