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# Enhanced Macrophage M1 Polarization and Resistance to Apoptosis Enable Resistance to Plague

Emilia Pachulec,<sup>1</sup> Rym Ben Abdelwahed Bagga,<sup>1</sup> Lucie Chevallier,<sup>2</sup> Hope O'Donnell,<sup>1</sup> Chloé Guillas,<sup>1</sup> Jean Jaubert,<sup>2</sup> Xavier Montagutelli,<sup>2</sup> Elisabeth Carniel,<sup>1</sup> and Christian E. Demeure<sup>1</sup>

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**Background.** Susceptibility to infection is in part genetically driven, and C57BL/6 mice resist various pathogens through the proinflammatory response of their M1 macrophages (MPs). However, they are susceptible to plague. It has been reported elsewhere that *Mus spretus* SEG mice resist plague and develop an immune response characterized by a strong recruitment of MPs.

**Methods.** The responses of C57BL/6 and SEG MPs exposed to *Yersinia pestis* in vitro were examined.

**Results.** SEG MPs exhibit a stronger bactericidal activity with higher nitric oxide production, a more proinflammatory polarized cytokine response, and a higher resistance to *Y. pestis*-induced apoptosis. This response was not specific to *Y. pestis* and involved a reduced sensitivity to M2 polarization/signal transducer and activator of transcription 6 activation and inhibition of caspase 8. The enhanced M1 profile was inducible in C57BL/6 MPs in vitro, and when transferred to susceptible C57BL/6 mice, these MPs significantly increased survival of bubonic plague.

**Conclusions.** MPs can develop an enhanced functional profile beyond the prototypic M1, characterized by an even more potent proinflammatory response coordinated with resistance to killing. This programming plays a key role in the plague-resistance phenotype and may be similarly significant in other highly lethal infections, suggesting that orienting the MP response may represent a new therapeutic approach.

**Keywords.** *Yersinia pestis*; M1 macrophage polarization; SEG mice; apoptosis; inflammation.

The susceptibility of a patient to a given pathogen results from both environmental and genetic conditions. The innate immune response, first to develop after infection, directly depends on the host genetic background and is central to the interindividual heterogeneity observed during primary infection [1]. Faced with a fast-progressing and rapidly fatal infection, the robustness of the innate immune response is decisive in the outcome of the disease, because adaptive immunity mechanisms require days to develop and limit pathogen progression.

Macrophages (MPs) are among the key effectors of the innate immune response against pathogenic bacteria. Their functions include phagocytosis, killing of internalized organisms, release of antimicrobial factors, and recruitment of more effector cells by producing cytokines and chemokines. MPs are flexible cells with a highly dynamic phenotypic spectrum [2, 3]. The strongest antibacterial/proinflammatory profile is called M1, and at the opposite end, the “alternatively activated” MPs, designated

M2, drive tissue healing and turn off inflammation by producing anti-inflammatory cytokines. M1 and M2 MPs promote T-helper 1 and T-helper 2 lymphocyte adaptive responses, respectively.

A well-studied example of genetically driven resistance to infection is the C57BL/6 (B6) mouse strain, which is resistant to peritonitis and to infection by *Leishmania*, *Listeria*, and *Mycobacterium* [4–6]. This capacity was associated with the M1 MP orientation [7], as opposed to the susceptible BALB/c mouse strain, which displays an M2 response. However, the M1 genetic predisposition may not be sufficient to grant resistance to extremely potent pathogens. B6 mice, the prototypic M1 mice, are as susceptible to the plague agent *Yersinia pestis* [8, 9] as BALB/c mice [8].

It has been reported elsewhere that *Mus spretus* SEG/Pas (SEG) inbred mice are highly resistant to bubonic plague [8]. Paradoxically, *Y. pestis* disseminates faster and causes earlier clinical signs of infection in SEG mice, but the animals survive [9]. Bacterial foci in the SEG spleen 2 days after infection were well contained with limited histological lesions, contrasting with the major necrotic lesions noted in the B6 spleen. This implies that an innate immune mechanism is protective in SEG mice. A strong recruitment of MPs was also observed in SEG but not B6 spleens, pointing toward MPs as potential mediators of SEG resistance. This was however intriguing because MPs are among the first cell populations targeted by *Y. pestis* in vivo [10] and can serve as an intracellular niche for *Y. pestis* multiplication [11]. Thus, the aim of the current study was to analyze the MP response of plague-resistant SEG mice to *Y. pestis*, in

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order to identify cellular mechanisms that drive the capacity of a mammalian host to resist plague.

## MATERIALS AND METHODS

### Bacterial Growth Conditions

The virulent *Y. pestis* strain CO92 and its pYV-cured derivative CO92ΔpYV were cultured at 28°C on Luria-Bertani (LB) agar plates supplemented with 0.002% hemin (LBH) for 48 hours, followed by overnight culture in liquid LB broth at 37°C with shaking. Bacterial counts (measured in colony-forming units) were determined by plating serial dilutions on LBH. Experiments were performed in a biosafety level 3 laboratory.

### Mice

The Institut Pasteur animal facilities are accredited by the French Ministry of Agriculture (No. B75 15-01; 22 May 2008), in compliance with national and European regulations on care and protection of Laboratory Animals (French Law 2001-486; 6 June 2001). Protocols were approved by the institute's ethics committee (No. 2013-0054). The *M. spretus* SEG/Pas (SEG) inbred strain was described elsewhere [8]. Female SEG or C57BL/6J (B6) mice (Charles River), aged 7–9 weeks, were infected subcutaneously with *Y. pestis* (100 colony-forming units) in abdominal skin and followed up for 14 days.

### Cell Culture and In Vitro Stimulation

Peritoneal MPs collected from euthanized mice by lavage were plated in 48 wells plates for adherence, and adherent cells were further cultured in Roswell Park Memorial Institute 1640 medium supplemented with 5% fetal bovine serum. Bone marrow-derived MPs (BM-MPs) were obtained by culturing femoral bone marrow cells 6 days in medium containing 15% L929-conditioned medium. SEG and B6 MPs did not differ in size or shape, and MPs were homogeneously F4/80+ CD11b+ by flow cytometry. They were also CD80<sup>low</sup> and Toll-like receptor (TLR) 4+, indicating that they were not preactivated [12]. MPs were stimulated with 10 *Y. pestis* per MP and briefly centrifuged to

favor bacterial contact (800G at 5 minutes). Lactate dehydrogenase and cytokines were tested after 5 hour, or 24 hours for nitric oxide (NO). To obtain splenic MPs, an erythrocyte-free suspension of splenocytes ( $3 \times 10^6$ /mL) in Roswell Park Memorial Institute 1640 medium was cultured for 2 hours to allow adherence, before washing with phosphate-buffered saline. To polarize MPs toward M1, BM-MPs were precultured with recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (50 ng/mL; Immunotools) for 16 hours [13].

### Adoptive Transfer of MPs

B6 mice received autologous BM-MPs ( $10^6$  cells in 100  $\mu$ L) subcutaneously in the abdominal skin, followed 5 hours later by a second dose into the tail vein ( $2 \times 10^6$  cells in 200  $\mu$ L), before subcutaneous infection with *Y. pestis*.

### Statistical Analyses

The unpaired Student *t* test was used to compare groups. The Fisher exact test and the Mantel-Cox (log-rank) test were used to analyze survival (outcome and kinetics, respectively).

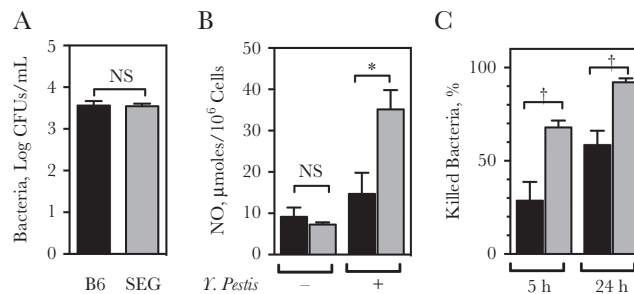
## RESULTS

### SEG and B6 MPs' Capacity to Kill Internalized *Y. pestis*

Peritoneal MPs from SEG and B6 mice exposed in vitro to *Y. pestis* CO92 (multiplicity of infection, 10) phagocytosed the bacterium with the same efficiency (Figure 1A). However, SEG MPs produced higher levels of NO (Figure 1B). Because NO is a potent antimicrobial agent, this suggested that SEG MPs killed internalized bacteria more efficiently. Indeed, the percentage of killed bacteria was significantly higher in SEG than in B6 MPs, and most bacteria internalized by SEG MPs were dead by 24 hours (Figure 1C). Therefore, SEG MPs exhibit a higher bactericidal activity against *Y. pestis* than B6 MPs.

### SEG MP's Inflammatory Response

SEG MPs infected with *Y. pestis* produced higher levels of proinflammatory cytokines (tumor necrosis factor [TNF]

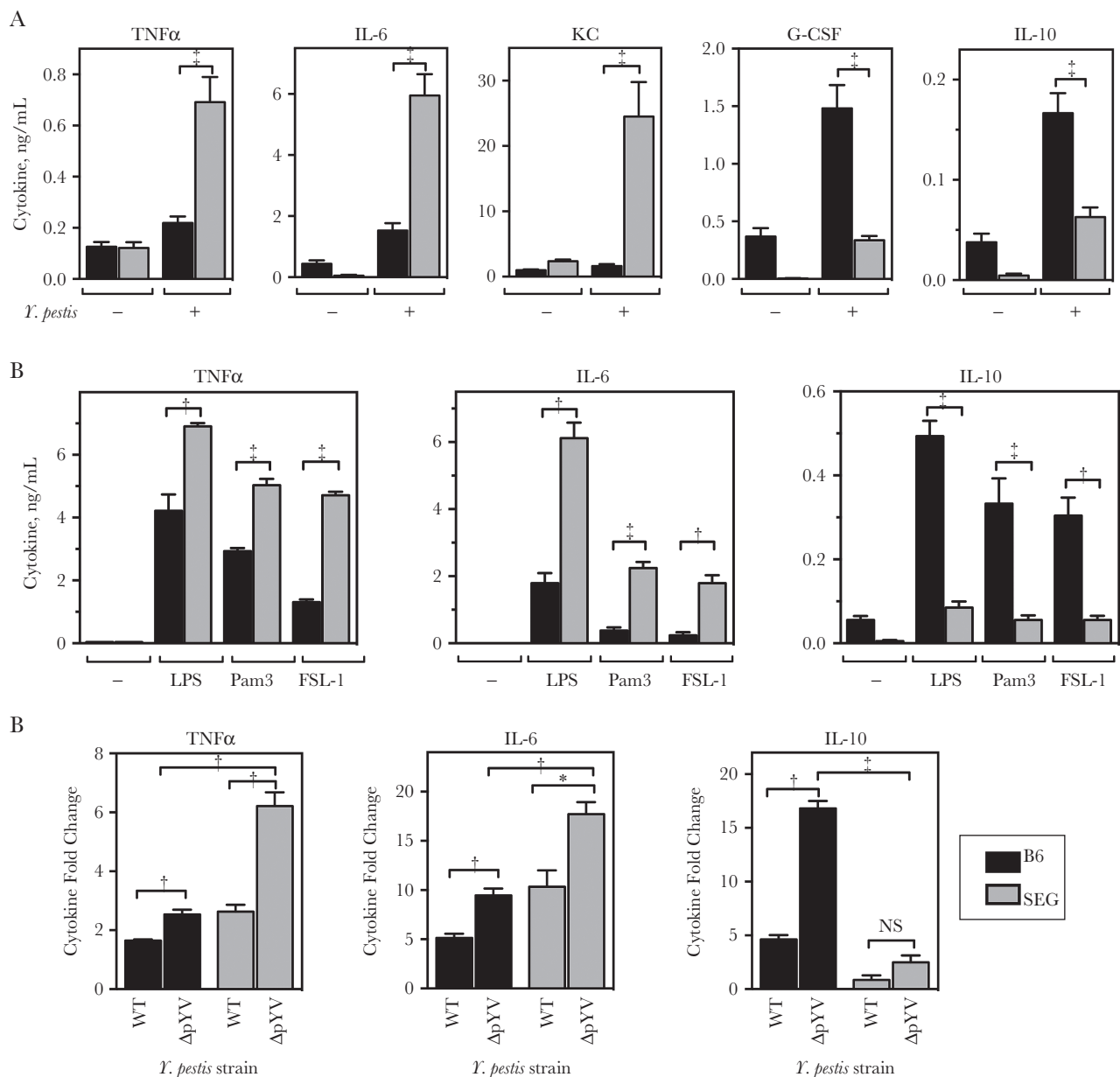


**Figure 1.** Phagocytosis and bactericidal activity against *Yersinia pestis* of SEG and B6 macrophages (MPs). Peritoneal MPs collected from SEG (gray bars) and B6 mice (black bars) were exposed ex vivo to *Y. pestis* CO92 (multiplicity of infection, 10) for 30 minutes and then treated with gentamicin (40  $\mu$ g/mL) to eliminate extracellular bacteria. A, Quantification of intracellular bacteria 2 hours after infection. B, Nitric oxide (NO) production 24 hours after infection. C, Percentage of bacterial killing 5 and 24 hours after infection. Data are shown as means with standard errors of the mean from individual samples obtained from 6 mice, representative of 2 experiments. Abbreviations: CFUs, colony-forming units; NS, not significant. \* $P < .05$ ; † $P < .01$ .

$\alpha$ , interleukin 6 [IL-6], and keratinocyte chemoattractant/CXCL1), and less anti-inflammatory cytokines (granulocyte colony-stimulating factor and interleukin 10 [IL-10]) than B6 MPs (Figure 2A). The lower proinflammatory cytokines of B6 MPs was not caused by an IL-10 retroinhibition mechanism, because blocking IL-10 with antibodies had little or no effect on either cytokine profile (Supplementary Figure S1). Compared

with B6 MPs, the prototypic proinflammatory M1 [7], the SEG profile was an enhanced M1.

Different bacterial products preferentially trigger specific sets of cytokines [14]. To determine whether SEG MPs could preferentially respond to certain bacterial products, MPs were stimulated with well-defined TLR ligands: *Escherichia coli* lipopolysaccharide (TLR4 agonist), Pam3CSK (TLR1/2 agonist)



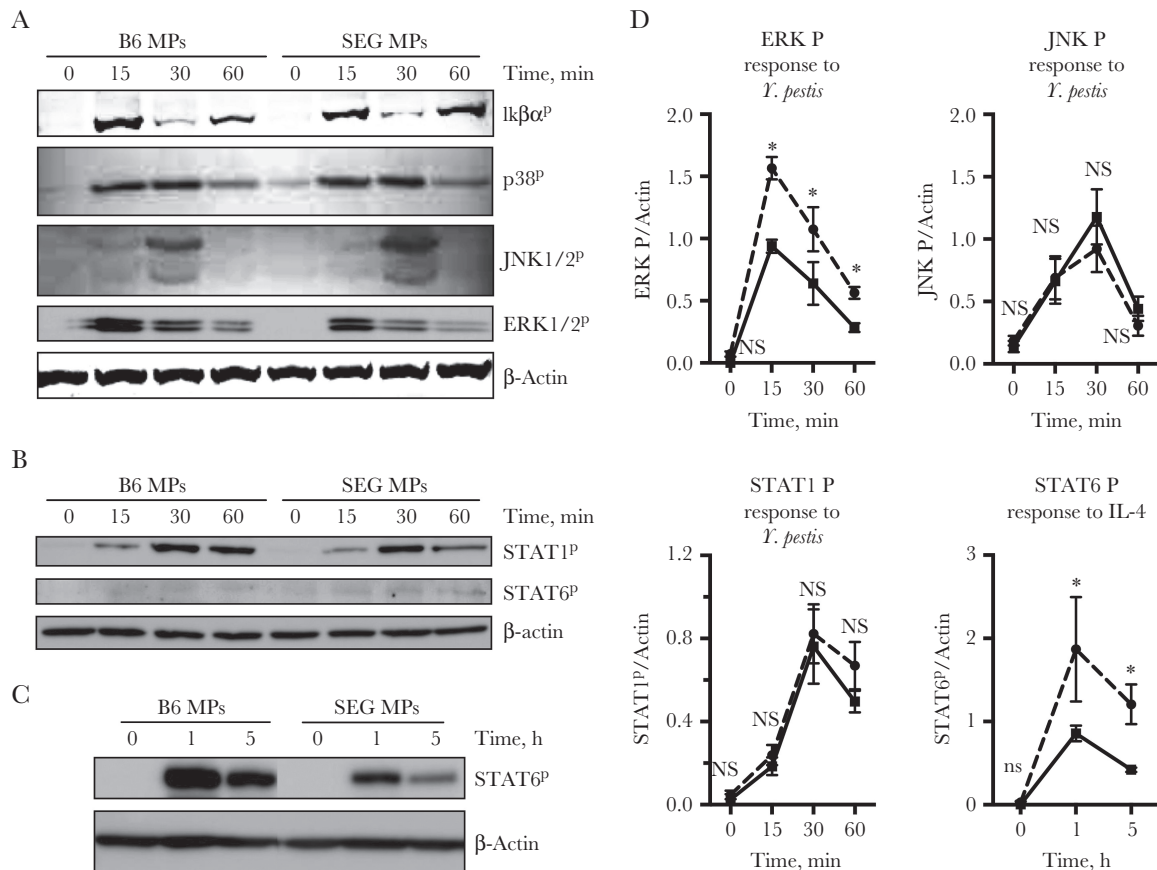
**Figure 2.** Cytokine production by SEG and B6 macrophages (MPs) exposed to *Yersinia pestis*. **A**, B6 (black bars) and SEG (gray bars) peritoneal MPs were exposed (+) or not (–) to *Y. pestis* (multiplicity of infection, 10) for 5 hours to measure tumor necrosis factor (TNF)  $\alpha$ , interleukin 6 (IL-6), keratinocyte chemoattractant (KC), granulocyte colony-stimulating factor (G-CSF) and interleukin 10 (IL-10) production. Data are shown as means with standard errors of the mean [SEMs] for individual cell samples from 12 mice pooled from 2 independent experiments. **B**, Bone marrow–derived MPs (BM-MPs) from B6 and SEG mice were untreated (–) or stimulated for 5 hours with 100 ng/mL of *Escherichia coli* lipopolysaccharide (LPS) (Toll-like receptor [TLR] 4 agonist), Pam3CSK (TLR1/2 agonist), or FSL-1 (TLR2/6 agonist) before measurement of TNF- $\alpha$ , IL-6 and IL-10 in supernatants. Data represent means with SEMs from triplicate cultures, representative of 2 independent experiments. **C**, B6 and SEG peritoneal MPs were infected with *Y. pestis* pYV+ (wild type [WT]) or the isogenic strain cured of pYV ( $\Delta pYV$ ) for 5 hours before measurement of TNF- $\alpha$ , IL-6, and IL-10 production. Data represent the ratios of cytokines from stimulated MPs to those from unstimulated MPs, from 6 mice (means with SEMs), and are representative of 2 independent experiments. Abbreviation: NS, not significant. \* $P < .05$ ; † $P < .01$ ; ‡ $P < .001$ .

mimicking bacterial lipopeptides) and FSL-1, an agonist of TLR2/6 that favors IL-10 production on recognition of *Y. pestis* LcrV [14]. Whatever the TLR triggered, SEG MPs produced more proinflammatory cytokines (TNF- $\alpha$  and IL-6) and less IL-10 than B6 MPs (Figure 2B), indicating that the enhanced M1 profile was not ligand specific.

The *Y. pestis* virulence plasmid pYV (pCD1) encodes *Yersinia* outer protein (Yop) cytotoxins that are injected into the host cell cytosol [15] and block intracellular signaling pathways critical for cytokine production [15]. We hypothesized that the enhanced M1 response could result from a lower capacity of Yops to block these signaling pathways in SEG MPs. When MPs were infected with a pYV-cured *Y. pestis* derivative ( $\Delta$ pYV), production of IL-6, TNF- $\alpha$ , and IL-10 was increased, as expected, compared with that of the wild-type strain (Figure 2C). However, even without the pYV, the levels of proinflammatory cytokines remained higher and the levels of anti-inflammatory cytokines lower in SEG than in B6 MPs (Figure 2C), indicating that Yops did not shape the response.

### Activation of Intracellular Signaling Pathways in SEG and B6 MPs

The MP cytokine response triggered by bacteria results from the activation of various intracellular pathways. To determine whether these pathways could be activated differently in SEG MPs, BM-MPs from SEG and B6 mice were exposed to *Y. pestis*, and the activation levels of these different pathways were compared. The NF- $\kappa$ B pathway, central to cytokine production, was not differently activated (Figure 3A), as shown by comparable levels of I $\kappa$ B $\alpha$  phosphorylation, degradation, and resynthesis [16]. Production of TNF- $\alpha$  and other proinflammatory cytokines requires c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase pathways [17, 18]. JNK and p38 mitogen-activated protein kinase were not different in SEG MPs (Figure 3A and 3D). The extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase pathway favors production of IL-10 [19]. ERK was more phosphorylated in B6 MPs (Figure 3A and 3D), potentially accounting for their higher IL-10 production.



**Figure 3.** Intracellular signaling pathways activated in SEG and B6 macrophages (MPs). Bone marrow–derived MPs (BM-MPs) were incubated with *Yersinia pestis* (10 bacteria per MP) (A, B, D), or interleukin 4 (IL-4) (10 ng/mL) (C) for the indicated times. Phosphorylation of indicated factors were determined using Western blot analysis; superscript *p* denotes phosphorylated protein. D, Quantification of Western blots shown in A–C. Solid lines represent SEG MPs; dashed lines, B6 MPs.  $\beta$ -Actin is shown as a control and was used as loading control. Data represent factor-to-actin ratios, means with standard errors of the mean from 4 independent experiments performed with distinct MP cultures. Quantification and statistics were performed only when the visual aspect of blots suggested differences. Abbreviations: ERK, extracellular signal-regulated kinase; I $\kappa$ B $\alpha$ , endogenous cytoplasmic inhibitor of NF- $\kappa$ B; JNK, c-Jun N-terminal kinase; NS, not significant; p38, p38 mitogen-activated protein kinase; STAT, signal transducer and activator of transcription. \**P* < .05 (paired Student *t* test).

SEG MPs could have an enhanced or decreased sensitivity to polarizing signals, leading to activation of transcription factors signal transducer and activator of transcription (STAT) 1 and STAT6 controlling M1 (“classic activation”) and M2 (“alternative activation”) signature genes, respectively. We observed (Figure 3B, 3C, and 3D) that SEG MPs do not have an increased capacity to activate the STAT1 pathway in response to *Y. pestis*, but they show a markedly reduced ability to activate the STAT6 pathway in response to interleukin 4. Unphosphorylated factors were not different in the 2 species (not shown). That no factor phosphorylation was observed before infection confirmed that MPs were not preactivated.

#### SEG and B6 MPs Resistance to *Y. pestis*-Induced Apoptosis

*Y. pestis* can kill MPs by injecting Yop cytotoxins into their cytosol [15], leading to death by apoptosis. The resistance of SEG mice to plague could result from a higher capacity of their MPs to resist this cytotoxic effect. SEG peritoneal MPs, but also spleen MPs and BM-MPs, were indeed able to better resist *Y. pestis*-induced killing than B6 MPs (Figure 4A). Furthermore, the resistance of in vitro-grown SEG BM-MPs demonstrated that their ability to

resist *Y. pestis* cytotoxicity was not driven by signals produced in vivo and was therefore genetically programmed.

That Yop injection is required to kill MPs was confirmed here, because the pYV-cured *Y. pestis* failed to kill (Figure 4B). SEG MP survival could also result from a lower Yop injection into cell's cytosol. To test this, Yop injection was quantified using a *Y. pestis* producing a YopE-Cya reporter fusion protein [20]. The cyclic adenosine monophosphate level (produced by adenylate-cyclase Cya) was higher in SEG than in B6 MPs (Figure 4C), clearly excluding the possibility that a low Yop injection explained SEG MP survival.

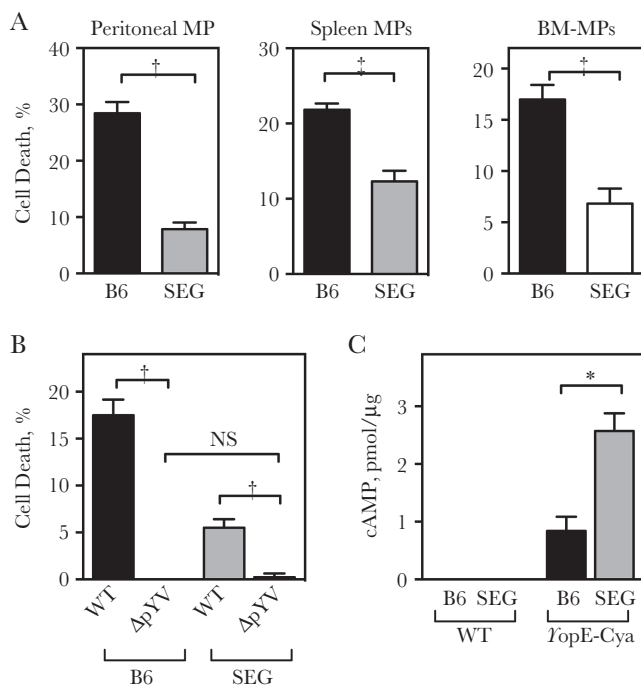
*Y. pestis* can activate different death programs: a noninflammatory apoptosis [15], but also an inflammatory death by pyroptosis [21] or a more extensive death by necrosis [22]. A different type of death program could explain the difference in cell death intensity observed in SEG and B6 MPs. *Y. pestis*-induced death of B6 and SEG MPs was inhibited by glycine, which acts as cytoprotectant by inhibiting nonspecific ions fluxes (Figure 5A), indicating that apoptosis in both MP types ends with necrosis features [23, 24]. Interleukin 1 $\beta$  production accompanies death by pyroptosis in activated MPs [21]. Interleukin 1 $\beta$  levels were low and comparable in SEG and B6 MPs (Figure 5B), excluding pyroptosis as a cause of lower SEG MP death. Caspases are the enzymes mediating apoptosis, and form 2 signaling cascades triggered by external (eg, Fas) or internal causes (eg, stress). Both pathways end by activation of effector caspase 3. Caspase 3 was activated in both MPs but was much higher in B6 than in SEG MPs infected with *Y. pestis* (Figure 5C). SEG MPs were as susceptible as B6 MPs to the apoptosis inducer staurosporin (Supplementary Figure S2), indicating that SEG MP resistance to killing by *Y. pestis* was not due to an intrinsic defect of their apoptosis machinery. Therefore, the higher cytotoxic effect of *Y. pestis* on B6 MPs did not result from the triggering of different death programs in the 2 mouse strains but from a lower susceptibility of SEG MPs to *Y. pestis*-induced death.

#### Differential Activation of Apoptotic Pathways in SEG and B6 MPs

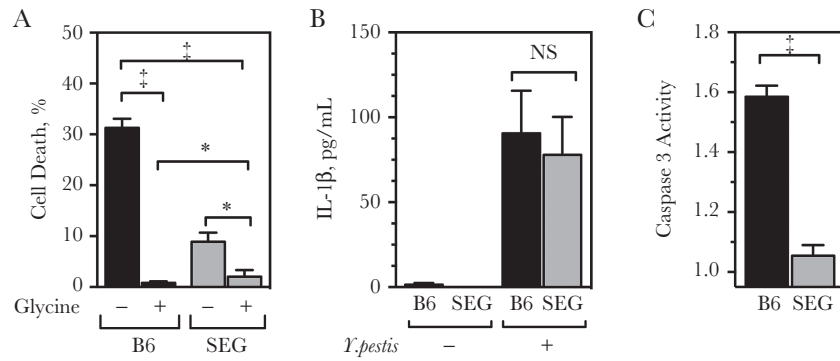
Like external death factors, *Y. pestis* is known to induce the “extrinsic” pathway of apoptosis, which signature is caspase 8 activation [25]. Caspase 8 was activated in B6 MPs exposed to *Y. pestis*, but, remarkably, caspase 8 activity was almost undetectable in SEG MPs (Figure 6A), indicating that a much lower activation of the extrinsic apoptosis pathway contributes to their resistance to *Y. pestis*-induced killing.

By activating the transcription of anti-apoptotic factors, the NF- $\kappa$ B pathway is a major switch for death processes [26]. We examined its contribution to SEG MP survival. The NF- $\kappa$ B inhibitor BMS-345541 considerably increased *Y. pestis*-induced death of B6 MPs, but not SEG MPs (Figure 6B).

The JNK factor is known to play a role in cell survival [27]. When JNK inhibitor SP600125 was added during MP infection, deaths of both B6 and SEG MPs were increased, reaching a level



**Figure 4.** Resistance of SEG and B6 macrophages (MPs) to *Yersinia pestis* cytotoxicity. *A*, Death of B6 (black bars) and SEG (gray bars) peritoneal, bone marrow-derived MPs (BM-MPs), and spleen MPs after 5-hour incubation with *Y. pestis* was quantified based on lactate dehydrogenase (LDH) release. *B*, B6 and SEG peritoneal MPs were infected with wild-type (WT) *Y. pestis* or *Y. pestis* cured of the pYV ( $\Delta$ pYV), and LDH release after 5 hours was used to quantify cell death. *C*, Peritoneal MPs were exposed for 2 hours to WT *Y. pestis* or *Y. pestis* carrying the pYopE-Cya reporter plasmid (carrying the YopE-adenylate cyclase fusion protein gene). Intracellular accumulation of cyclic adenosine monophosphate (cAMP) was measured, and values were normalized to the sample protein content. Data represent means with standard errors of the mean from 2 independent experiments. Abbreviation: NS, not significant. \* $P < .01$ ; † $P < .001$ .



**Figure 5.** Type of cell death induced by *Yersinia pestis* in SEG and B6 macrophages (MPs). B6 (black bars) and SEG (gray bars) MPs were exposed to *Y. pestis* for 5 hours (multiplicity of infection, 10). *A*, Cell death measured using lactate dehydrogenase release by peritoneal MPs preincubated (+) or not (–) for 1 hour with glycine before infection. Data were obtained from 2 independent experiments with 6 mice each. *B*, Interleukin 1 $\beta$  (IL-1 $\beta$ ) release was measured by enzyme-linked immunosorbent assay after 5 hours of exposure of peritoneal MPs to *Y. pestis*. Data represent means with standard errors of the mean (SEMs) for 12 mice pooled from 2 experiments. *C*, Caspase 3 activity in *Y. pestis*-infected bone marrow–derived MPs (BM-MPs), expressed as the infected-noninfected ratio. Results are shown as means (with SEMs) from triplicate cultures, representative of 2 independent experiments. Abbreviations: NS, not significant. \* $P < .05$ ; † $P < .001$ .

comparable in the 2 types of MPs (Figure 6C). Therefore, the JNK pathway is necessary to the resistance of MPs to *Y. pestis* but not sufficient. Together, these data suggest that the higher resistance of SEG MPs to *Y. pestis*-induced cytotoxicity results from the combination of a lower caspase 8 activation and the capacity to resist apoptosis independently of the NF- $\kappa$ B signaling pathway.

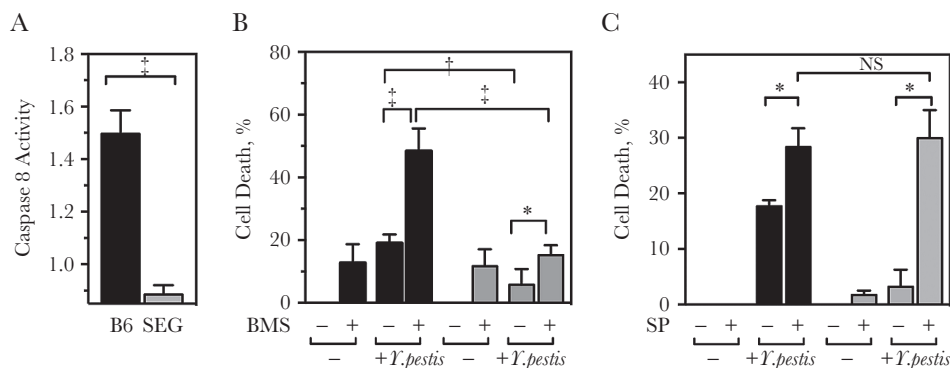
#### Reprogramming of B6 MPs to Display an Enhanced M1 Response to *Y. pestis*

It has been shown that GM-CSF increases the proinflammatory cytokine response of B6 MPs [13]. We first determined whether GM-CSF could induce the enhanced M1 response to *Y. pestis*. Indeed, IL-6 and NO levels of GM-CSF-treated B6 MPs increased significantly, to become higher than those of untreated SEG MPs (Figure 7A and 7B). In contrast, IL-10 production was decreased in GM-CSF-treated B6 MPs to levels comparable to those of SEG and GM-CSF-treated SEG MPs (Figure 7A).

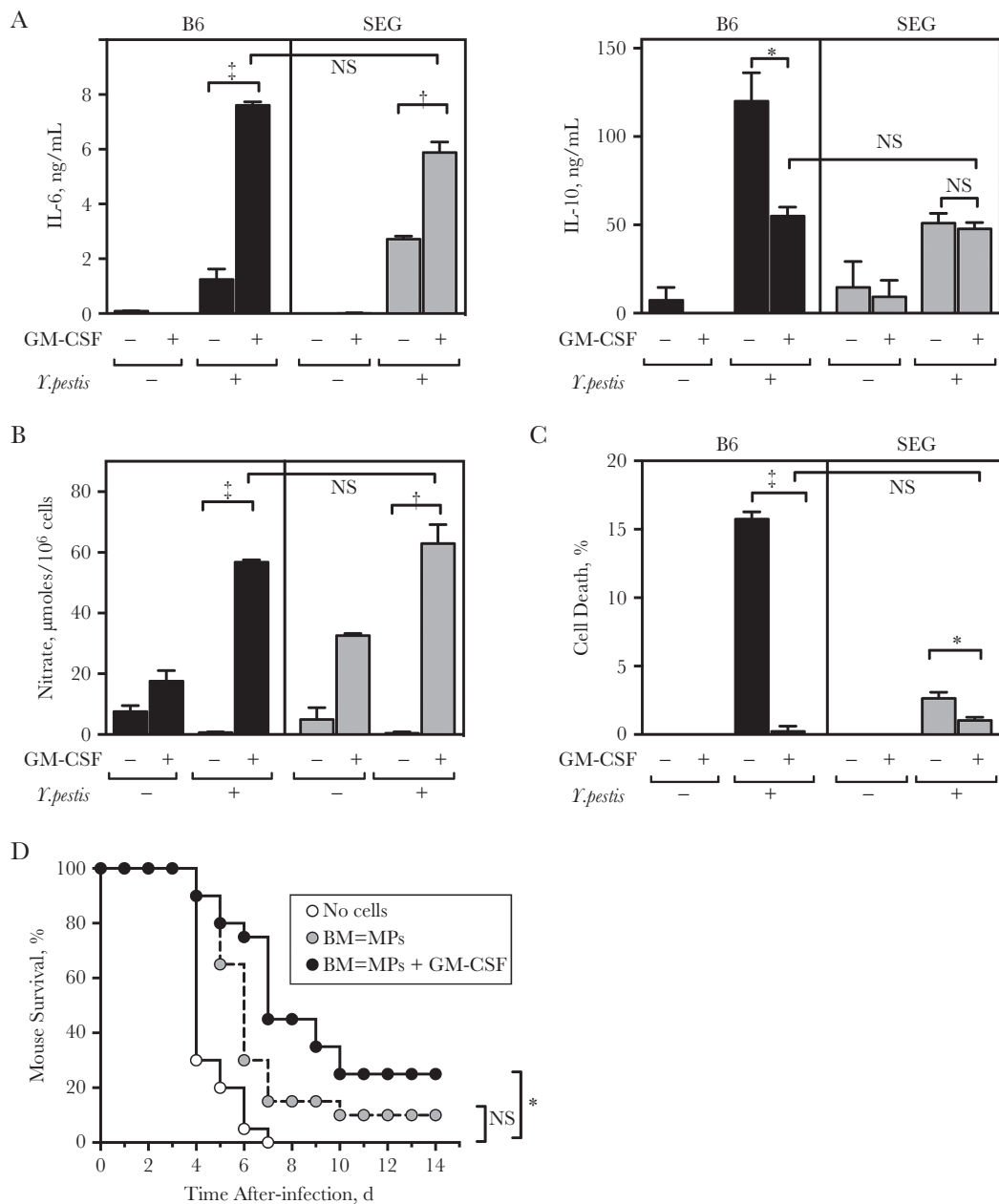
We then examined whether the enhanced M1 reprogramming could confer resistance to *Y. pestis*-induced apoptosis. Pretreated B6 MPs became even more resistant to *Y. pestis* cytotoxicity than SEG MPs and survived as well as GM-CSF-treated SEG MPs (Figure 7C). Therefore, resistance to apoptosis and the high M1 cytokine response are functionally linked. Together, our results demonstrate that B6 MPs do have the capacity to display a higher M1/anti-apoptotic response to *Y. pestis*, and therefore the difference between B6 and SEG MPs is due to different functional programs, controlled by an unknown genetic element.

#### Contribution of an enhanced M1/Anti-Apoptotic Response to Resistance to Bubonic Plague

We examined whether grafting MPs with the enhanced M1 profile to susceptible mice conferred resistance. SEG and B6 mice are not histocompatible, and grafting SEG MPs to F1 (B6  $\times$  SEG)



**Figure 6.** Apoptosis signaling pathways activated in SEG and B6 macrophages (MPs) exposed to *Yersinia pestis*. B6 (black bars) and SEG (gray bars) bone marrow–derived MPs (BM-MPs) were exposed to *Y. pestis* for 5 hours (multiplicity of infection, 10). *A*, Caspase 8 activity in *Y. pestis*-infected MPs expressed as the infected-noninfected ratio. *B*, *Y. pestis*-induced cell death of MPs pretreated 1 hour before infection with the NF- $\kappa$ B inhibitor BMS-345541 (BMS). *C*, *Y. pestis*-induced cell death of MPs pretreated (+) or not (–) with the JNK inhibitor SP600125 (SP), 1 hour before infection. Data are shown as means and standard errors of the mean for of triplicate BM-MP cultures, representative of 2 independent experiments. Abbreviation: NS, not significant. \* $P < .05$ ; † $P < .01$ ; ‡ $P < .001$ .



**Figure 7.** Bone marrow–derived macrophages (BM-MPs) from B6 mice reprogrammed for enhanced M1 enable survival of bubonic plague when grafted to autologous susceptible mice. SEG and B6 BM-MPs were incubated with 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (+) or not (–) for 16 hours before exposure (+) or not (–) to *Yersinia pestis* for 5 hours (multiplicity of infection, 10). A–C, Cytokines (A), nitric oxide (NO) (B), and cell death (C) were then measured. Data represent means with standard errors of the mean for MPs from 6 mice (1 representative experiment of 2). IL-6, interleukin 6; IL-10, interleukin 10; NS, not significant ( $P \geq .05$ ). \* $P < .05$ ; † $P < .01$ ; ‡ $P < .001$ . D, B6 BM-MPs preincubated with or without GM-CSF were injected intravenously and subcutaneously into B6 mice just before subcutaneous infection of the animals with 100 colony-forming units of *Y. pestis*. Nongrafted mice were also infected as control (no cells). Survival was followed for 14 days, and survival curves represent 20 mice, pooled from 2 independent experiments. Abbreviation: NS, not significant. ( $P \geq .05$ ). \* $P < .05$  (Fisher exact test).

mice would not be informative because F1 mice are already 75% resistant to plague [8]. We therefore injected normal or GM-CSF–treated B6 BM-MPs into B6 mice, before a subcutaneous challenge with *Y. pestis*. Mice having received polarized B6 MPs had a longer time to death (mean [standard error of the mean], 6.9 [0.5] days; Figure 7D) than nongrafted B6 mice (4.5 [0.2] days) or B6 mice that had received untreated B6 MPs (5.8 [0.3] days). Furthermore, none

of the nongrafted mice survived. Grafting-enhanced M1-polarized MPs provided 25% survival ( $P < .05$ ), whereas untreated B6 MPs had no significant effect (10% survival). GM-CSF–treated MPs also had a strong favorable effect on mouse survival kinetics (log-rank test:  $P < .001$ ). Taken together, these data indicate that MPs displaying a high M1/antiapoptotic response contribute significantly to the capacity of a host to resist plague.



## DISCUSSION

MPs play a crucial role in the capacity of B6 mice to resist diverse bacterial diseases, a capacity associated with the programming of their MPs to display an M1 response [7]. However, this skewing is not sufficient to protect B6 mice against plague. We had previously reported that SEG mice resist bubonic plague, with a response characterized by an active recruitment of monocytes/MPs in infected organs [9]. The current study further determined and characterized the role MPs play in SEG capacity to survive plague, by comparing SEG and B6 MPs exposed in vitro to *Y. pestis*. We report that 3 components of the MP response that may actively participate in the control and killing of this bacterium were more effective in SEG than in B6 MPs.

The first was the higher production of NO by SEG MPs on infection with *Y. pestis*. The plague bacillus has to subvert NO production to survive and multiply intracellularly [11], so the capacity of SEG MPs to produce higher amounts of this toxic compound may explain their higher bactericidal activity against *Y. pestis*. Second, SEG MPs resisted *Y. pestis*-induced apoptosis. The plague bacillus, like other pathogenic bacteria, induces programmed cell death by injecting toxins into host cells [28]. By resisting this apoptosis, more SEG MPs could thus exert their bactericidal activity. Finally, the cytokine response of SEG MPs was even more strongly proinflammatory. In laboratory mice, inflammation is lacking in early plague [29], creating a local permissive environment enabling *Y. pestis* growth [30]. In SEG mice, this preinflammatory phase is much shorter [9], and this could be explained by the stronger proinflammatory action of their MPs. All together, the combination of a strong bactericidal activity, abundant production of proinflammatory cytokines, and the capacity to better resist *Y. pestis*-induced apoptosis strongly argued for a MP role in the resistance of SEG mice to bubonic plague. Transferring MPs exhibiting a SEG-like response significantly enhanced the survival of susceptible B6 mice to plague, confirming that MPs play a key role in host genetic resistance to plague.

The M1 response was initially characterized by a cytokine-mediated proinflammatory response and a potent bactericidal activity [7]. Our analysis shows that the resistance of SEG MPs is not limited to their proinflammatory and bactericidal response but also involves the resistance to bacteria-induced apoptosis. These properties have been observed independently but have not been associated until now. Linking the 2 properties is JNK, a known regulator of cell death [27, 31], which also induces the production of inflammatory cytokines [32] and is required for SEG MPs to survive apoptosis. Similarly, caspase 8, which is involved in apoptosis via the extrinsic pathway [25, 33] also inhibits M1 polarization of MPs [34] and is not activated in SEG MPs. Therefore, this suggests that the proinflammatory response and resistance to apoptosis are coordinated as part of a unique functional program conferring an effective response to pathogens.

It has been reported elsewhere that SEG resistance to plague is genetically controlled by at least 4 distinct genetic loci in the genome [8, 35]. However, these loci encompass more than a thousand genes, including multiple genes coding for actors of inflammation, and further reduction of the loci will be needed. Interestingly, B6 MPs can display the enhanced M1 response after receiving an external polarization signal demonstrates that the necessary genetic determinants are functional. This indicates that the response of B6 and SEG MPs is under the control of upstream signals (enhancers, regulators, transcription factors) that differentially activate or inhibit the same signaling pathways. In agreement, SEG MPs have a decreased responsiveness via STAT6, a transcription factor central to M2/alternative activation that counteracts the M1 response. Therefore, SEG MPs may have an enhanced M1 profile due to resistance to M2 polarization.

We evaluated the possibility of injecting GM-CSF directly into mice at the time of infection, following conditions that protected rats against *Staphylococcus aureus* [36]; however, GM-CSF did not improve mouse survival of plague (data not shown). Although GM-CSF is one of the most studied immunostimulants in human sepsis ([37, 38]), there has been a consistent lack of survival benefit in these studies, with failure to confirm promising in vitro results. GM-CSF is not produced spontaneously by SEG MPs and is not produced by SEG MPs not more than by B6 MPs on infection (not detailed), and another factor probably exerts the observed effects.

There is hope that the present findings may be translated to human disease if safe treatments increasing the M1/apoptosis resistance profile are found. The SEG mice suggest that having more active MPs is not pathogenic. In spite of the enhanced response of their MPs, they are not hypersensitive to lipopolysaccharide [39] and no autoimmune disease, chronic inflammation disease, or severe inflammatory tissue damage was observed during infection by various pathogens ([40] and our unpublished observations). In humans, increasing inflammation must be used with care to avoid potential adverse effects [41]. Transient enhancement of MP functions would be preferable, and an adequate treatment dosage must be found. However, functional polarization is not permanent activation, and an activating signal is required to cytokines and NO release. Enhanced proinflammatory MPs could be induced by means of cytokines, possibly from the GM-CSF family, but also immunostimulants such as CpG oligodeoxynucleotides [42]. There is increasing recognition that nonspecific MP memory exists, also in humans, called "trained immunity", which results from long-lasting epigenetic modifications by factors such as  $\beta$ -glucan or bacille Calmette-Guérin (BCG). Those could be used to enhance innate immunity [43]. Such a mechanism provides hope that treatments fostering innate immunity could be beneficial against plague and other infectious diseases and might be

proposed in plague-endemic regions as a prophylactic measure to limit the risk of contagion.

In conclusion, our study unraveled the important role played by MPs in the innate resistance of mice to bubonic plague. We show that a classic M1 response is not sufficient to survive plague and that a more proinflammatory MP response is required. Such a response is driven by a preprogrammed capacity to activate signaling pathways that elicit a potent proinflammatory response and resistance to *Y. pestis*-induced apoptosis. This response could also take place and confer resistance to other highly lethal diseases, such as viral hemorrhagic fevers, which skew MP functions to their benefit [44–46]. Skewing MPs toward an enhanced M1 phenotype could represent a strategy to combat otherwise fatal infectious diseases.

### Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

### Notes

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