Conditions That Diminish Myeloid-Derived Suppressor Cell Activities Stimulate Cross-Protective Immunity[▽]

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Immunity conferred by conventional vaccines is restricted to a narrow range of closely related strains, highlighting the unmet medical need for the development of vaccines that elicit protection against multiple pathogenic serotypes. Here we show that a *Salmonella* bivalent vaccine comprised of strains that lack and overproduce DNA adenine methylase (Dam) conferred cross-protective immunity to salmonella clinical isolates of human and animal origin. Protective immunity directly correlated with increased levels of cross-reactive opsonizing antibodies and memory T cells and a diminished expansion of myeloid-derived suppressor cells (MDSCs) that are responsible for the immune suppression linked to several conditions of host stress, including chronic microbial infections, traumatic insults, and many forms of cancer. Further, aged mice contained increased numbers of MDSCs and were more susceptible to *Salmonella* infection than young mice, suggesting a role for these cells in the immune declines associated with the natural aging process. These data suggest that interventions capable of reducing MDSC presence and activities may allow corresponding increases in B- and T-cell stimulation and benefit the ability of immunologically diverse populations to be effectively vaccinated as well as reducing the risk of susceptible individuals to infectious disease.

Salmonella enterica is a significant food-borne pathogen of humans that occurs via consumption of meat, animal products, and food products (e.g., fruits and vegetables) contaminated with animal waste (10, 34, 62). Clinical manifestations of human and animal salmonellosis range from self-limiting gastroenteritis to severe bacteremia and typhoid fever. More than 2,300 serovars of *S. enterica* have been identified, comprising six subspecies, with the vast majority of human and animal infections caused by strains belonging to subspecies I, which exhibits significant differences in virulence, host adaptation, and host specificity (59, 61).

Assessment of pathogenicity and risk to human and animal health depends on a number of variables, including the diversity of pathogenic salmonellae serotypes (10, 59, 61), the disparity among salmonella isolates from clinical versus surveillance submissions (5), management and environmental events that increase pathogen exposure and/or compromise host immunity (5, 17–19, 32, 33), and the emergence of strain variants that exhibit enhanced pathogenicity in humans and/or animals (27). Although vaccination is the best form of prophylaxis against disease caused by these infectious agents, the immunity conferred is generally limited to a narrow range of closely related strains. This presents a serious limitation under field conditions, wherein individuals can be exposed to multiple pathogenic serotypes.

The development of adaptive immune responses leading to cross-protective immunity can be compromised by the expan-

sion of myeloid-derived suppressor cell (MDSC) numbers and activities (13), which have been implicated in many conditions associated with immune suppression, including host stress from chronic microbial infection, severe trauma, and many forms of cancer (4, 9, 36). Such generalized immune suppression may also limit vaccination efficiency and increase susceptibility to infectious disease. For example, infection with live attenuated vaccines (i.e., *aroA* mutant) or wild-type *Salmonella* results in a transient state of generalized immune suppression (2–4, 14) attributed to the effects of nitric oxide (NO) produced by MDSCs. The *aroA*-associated immune suppression may restrict the nature of adaptive immunity induced, limiting protective efficiency to only closely related strains (25, 30, 31).

In contrast, a state of generalized immune suppression does not occur in animals infected with *dam* mutant vaccines (26). Additionally, *dam* mutant and Dam-overproducing (Dam^{OP}) salmonellae are known to constitutively express a unique set of proteins (and potential antigens) in vitro that are preferentially expressed by the wild-type strain only during infection (26, 28). Thus, immunization with a bivalent vaccine consisting of both *dam* mutant and Dam^{OP} strains might provide an expanded repertoire of antigens to immune-competent hosts not compromised by vaccine-associated MDSC activities. Herein we show that mice immunized with an *S. enterica* serovar Typhimurium bivalent *dam* mutant vaccine exhibited protection against the homologous strain, other serovar Typhimurium strains, and cross-protection against multiple other serotypes of pathogenic salmonellae. Cross-protective immunity in vivo directly correlated with the presence of *Salmonella*-specific cross-reactive opsonizing antibodies and memory T cells and diminished MDSC activities. Additionally, aged mice were shown to have elevated levels of MDSC activities and were more susceptible to infection than young mice. These data

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suggest that elevated levels of myeloid-derived suppressor cell activities limit vaccination efficiency and increase the risk of infectious disease for susceptible populations.

MATERIALS AND METHODS

Animals. Young (1.5 to 3 months) and aged (18 to 20 months) BALB/c and C57BL/6 mice were purchased from Charles River Laboratories or from the National Institute of Aging animal colony. DO11.10 T-cell receptor transgenic mice (ovalbumin 323-339 specific) on the BALB/c background were bred from animals originally purchased from Jackson Laboratories (Bar Harbor, ME). All mice were kept under specific-pathogen-free conditions at the Animal Resource Center, University of California, Santa Barbara, or the Center of Comparative Medicine Animal Facility, University of Utah. Use of laboratory animals for this study has been approved by the UC Santa Barbara and University of Utah Institutional Animal Care and Use Committees.

Bacterial strains. *Salmonella* human and animal clinical isolates were recovered from different outbreaks or individual cases submitted to diagnostic laboratories; animal nonclinical isolates were derived from on-farm surveillance studies of healthy animals (27). *Salmonella* reference pathogenic strain *S. enterica* serovar Typhimurium ATCC 14028 (CDC 6516-60) was used in all studies.

Vaccine efficacy assay. *aroA*, *dam*, or *dam*/Dam^{OP} serovar Typhimurium vaccine strains were grown overnight in LB medium with aeration at 37°C. Overnight-grown bacterial cells were used to orally infect BALB/c mice (6 to 8 weeks) at a dose of 10⁹ CFU via gavage (28). Eleven weeks postvaccination, immunized mice were orally challenged with virulent salmonellae at a dose of 10⁷ to 7 × 10⁸ CFU which, for the challenge strains tested, is approximately 100 times the lethal dose required to kill 50% (LD₅₀) of naïve animals. The oral LD₅₀ for reference wild-type serovar Typhimurium strain 14028 is 10⁵ organisms (28).

Antigen-specific cross-reactive immunoglobulin G (IgG) antibody assay. Splenocytes isolated from T-cell receptor transgenic (ovalbumin 323-339-specific) DO11.10 mice (2 × 10⁵ cells/well) were cultured with purified Gr1⁺ CD11b⁺ MDSCs at various ratios in the presence or absence of 100 µg/ml of ovalbumin. MDSCs were isolated from the spleens of naïve BALB/c mice or from age-matched mice that were intraperitoneally (i.p.) infected with *aroA* or *dam* mutant serovar Typhimurium (10⁴ CFU) at times of peak of Gr1⁺ CD11b⁺ cell number postinfection (days 7 and 14, respectively). Cocultures were maintained in the presence of 100 IU/ml penicillin and 100 µg/ml streptomycin, because MDSCs were isolated from infected mice at time points prior to splenic clearance. After 4 days, cell cultures were analyzed for T-cell proliferation by the incorporation of [³H]thymidine. Proliferation of DO11.10 cells in all experimental groups was related to that observed with the same ratio of Gr1⁺ CD11b⁺ cells from naïve donors.

Opsonizing antibody assay. BALB/c mice were orally infected with *aroA*, *dam*, or *dam*/Dam^{OP} serovar Typhimurium (14028). Blood was collected 11 weeks postvaccination (*n* = 5 to 10 mice per serum pool). Heat-inactivated pooled serum was diluted 1:50 and incubated with an equal volume of bacterial suspension for 1 h at 4°C. RAW 264.7 cultured macrophages were infected with the opsonized bacteria at a multiplicity of infection (MOI) of 20:1 (bacteria:phagocytic cells). After 1 h of incubation at 37°C, cells were washed and incubated with gentamicin (100 µg/ml) for 1 h prior to cell lysis with 0.1% Triton X-100. Internalized bacteria were enumerated by direct colony counting.

HeLa cell infection assay. Heat-inactivated pooled serum derived from animals 11 weeks postimmunization was added at a 1:40 dilution to cultured HeLa cells. Immediately thereafter, salmonellae were added at an MOI of 50:1 (bacteria:HeLa cells) for 1 h at 37°C. After washing, the cells were incubated with gentamicin (100 µg/ml) for 1 h prior to cell lysis with 0.1% Triton X-100. Internalized bacteria were enumerated by direct colony counting.

Detection of memory CD4⁺ and CD8⁺ T cells producing IFN-γ. Eleven weeks postvaccination with *aroA*, *dam*, or *dam*/Dam^{OP} serovar Typhimurium (14028), BALB/c mice were intravenously (i.v.) challenged with virulent strains of salmonellae (10⁶ CFU/challenge). Age-matched naïve mice were used as a control. Three hours later, spleens were harvested, and splenocytes were stained with monoclonal antibodies directed against mouse CD4 or CD8 and CD11a (BD Pharmingen, San Diego, CA) and intracellular gamma interferon (IFN-γ; Miltenyi Biotec, Auburn, CA). Activated memory CD4⁺ or CD8⁺ T cells were identified as CD4⁺ CD11a⁺ IFN-γ⁺ or CD8⁺ CD11a⁺ IFN-γ⁺ by fluorescence-activated cell sorter (FACS) analysis using CellQuest software.

Analysis of myeloid-derived suppressor cells. BALB/c mice (8 to 10 weeks) were i.p. infected with log-phase *aroA* or *dam* strains of serovar Typhimurium 14028 (10⁴ CFU/mouse). Age-matched naïve mice were used as a control. At various times postinfection, animals were sacrificed and the number of viable

bacteria (CFU) present in the spleen was enumerated by direct colony count. Splenocytes were stained with monoclonal antibodies directed against mouse Gr1 and CD11b (BD Pharmingen), and the presence of MDSCs of the Gr1⁺ CD11b⁺ phenotype was determined by FACS analysis. Splenocytes were also cultured for 48 h and resultant supernatants were evaluated for the production of NO by Griess assay (Sigma, St. Louis, MO).

Morphological characterization of immature myeloid-derived suppressor cells. MDSCs of the Gr1⁺ CD11b⁺ phenotype were isolated from the spleens of BALB/c mice i.p. infected with *aroA* or *dam* mutant serovar Typhimurium (10⁴ CFU) at the times of peak Gr1⁺ CD11b⁺ cell number postinfection (days 7 and 14, respectively). Gr1⁺ CD11b⁺ cells were stained with Wright-Giemsa, and the percentages of mature polymorphonuclear leukocytes (PMN), immature PMN ring-shaped nucleus cells (PMN rings), mature mononuclear cells (MNC), and immature MNC ring-shaped nucleus cells (MNC rings) were identified based on cytoplasmic and nuclear morphology. MDSCs with the greatest immunosuppressive activities are designated by the immature MNC ring phenotype (12).

Antigen-specific CD4⁺ T-cell proliferation inhibition assay. Splenocytes isolated from T-cell receptor transgenic (ovalbumin 323-339-specific) DO11.10 mice (2 × 10⁵ cells/well) were cultured with purified Gr1⁺ CD11b⁺ MDSCs at the ratios indicated in the presence or absence of 100 µg/ml ovalbumin. MDSCs were isolated from the spleens of naïve BALB/c mice or from age-matched mice that were i.p. infected with *aroA* or *dam* mutant serovar Typhimurium (10⁴ CFU) at peak times of Gr1⁺ CD11b⁺ cell number postinfection (days 7 and 14, respectively). Cocultures were maintained in the presence of 100 IU/ml penicillin and 100 µg/ml streptomycin, because MDSCs were isolated from infected mice at time points prior to splenic clearance. After 4 days, cell cultures were analyzed for proliferation of ovalbumin-specific CD4⁺ T cells by the incorporation of [³H]thymidine. Proliferation of DO11.10 cells in all experimental groups was related to that observed with the same ratio of Gr1⁺ CD11b⁺ cells from naïve donors.

Statistical analysis. Statistical significance for proportions was calculated with a chi-square test. For all statistical analyses, a significance level (*P*) of less than 0.05 was used to reject the null hypothesis.

RESULTS

Immunization with *dam* mutant *Salmonella* conferred cross-protective immunity to multiple pathogenic isolates of human and animal origin. Previous reports have shown that live vaccines (e.g., *aroA*) confer transient, nonspecific cross-protective immune responses that decline immediately after the vaccine strain is cleared from the animals (25, 30, 31). Here we examined whether a bivalent vaccine consisting of both *dam* mutant and Dam^{OP} serovar Typhimurium strains could elicit cross-protective immunity, since each individual vaccine strain has the capacity to express a unique subset of antigens with an enhanced potential for immune stimulation in the absence of the immune-suppressive activities that have now been ascribed to *Salmonella* vaccine-associated MDSCs (26, 37). Immunization of BALB/c mice with the bivalent vaccine conferred robust cross-protective immunity against challenge with a number of clinical isolates of salmonellae derived from human and animal origin, as well as to field isolates from surveillance submissions of on-farm healthy animals (Table 1) (*P* < 0.05 for vaccinates relative to nonvaccinates). Such cross-protective immune responses were not attributed to the presence of the vaccine strain, as immunized animals were challenged more than 4 weeks after vaccine clearance.

Cross-reactive antibodies with opsonizing and anti-infective activities are increased in animals immunized with *dam* mutant vaccines. Conceptually, cross-protection could be facilitated by cross-reactive antibodies capable of opsonizing salmonellae for efficient killing by phagocytic cells and/or neutralizing salmonellae antigens that are vital for host-pathogen interactions essential to infection. To test this hypothesis, mice immunized with *aroA*, *dam*, or *dam*/Dam^{OP}

TABLE 1. *dam* mutant vaccines elicit cross-protective immunity to pathogenic isolates of human and animal origin

<i>Salmonella</i> source and strain	Serovar	Serogroup	Immunization results (no. of survivors/total immunized) ^a	
			<i>dam</i>	<i>dam</i> /Dam ^{OP}
Gastrointestinal and extraintestinal animal isolates				
174	Bovismorbificans	C2-C3	14/22	19/22
225	Bovismorbificans	C2-C3	13/18	18/18
χ3246	Choleraesuis	C1	5/20	6/21
(05)-8895	Dublin	D1	14/21	19/25
(02)-1226	Dublin	D1	5/22	14/22
(98)-9534	Enteritidis	D1	7/19	13/23
4973	Enteritidis	D1	7/19	11/23
(05)-6192	Typhimurium	B	17/20	16/19
(97)-1332	Typhimurium	B	12/24	18/22
131	Typhimurium	B	17/18	20/22
12	Typhimurium var. 5 [−]	B	14/22	17/22
(04)-8511	Typhimurium var. 5 [−]	B	16/18	21/22
14028	Typhimurium	B	20/20	20/20
Isolates from surveillance submissions of on-farm healthy animals				
BL7W2FL	Enteritidis	D1	5/22	18/18
EPIMD142	Typhimurium var. 5 [−]	B	13/18	14/20
Fecal isolates from human gastroenteritis patients				
F9	Typhimurium	B	10/20	11/19
5h	Typhimurium	B	7/15	10/18
7h	Typhimurium var. 5 [−]	B	ND	7/18
Blood isolates from human bacteremic patients				
Lane	Dublin	D1	17/22	18/21
B1	Enteritidis	D1	10/20	11/19
B3	Enteritidis	D1	7/15	10/18
B5	Enteritidis	D1	ND	7/18
B4	Typhimurium	B	10/18	13/21
B9	Typhimurium	B	9/15	13/20

^a BALB/c mice orally immunized with *dam* or *dam*/Dam^{OP} serovar Typhimurium (14028) were orally challenged with a dose of 100 LD₅₀ of virulent salmonellae. Naïve (nonvaccinated) control mice (18 to 22/group) all died by day 21 postinfection. ND, not determined. *P* < 0.05 for vaccinated versus nonvaccinated animals.

serovar Typhimurium were evaluated for the presence of antibodies that exhibited cross-reactivity, opsonization, and/or neutralization activities. Although *aroA*-derived serum antibodies (anti-*Salmonella* IgG) reacted strongly with antigens present on several serovar Typhimurium strains, they exhibited far less cross-reactivity to the non-Typhimurium strains tested (Table 2). Additionally, they were poor at opsonizing salmonellae for uptake into phagocytic cells (RAW 264.7 macrophages) (Fig. 1A) regardless of the corresponding IgG titers and were poor at blocking invasion of nonhomologous salmonellae into nonphagocytic (HeLa) cells (Fig. 1B). In contrast, serum antibodies derived from *dam* mutant-immunized mice exhibited high levels of cross-reactivity, opsonization, and blocking activities to all salmonellae strains tested. These correlations were further improved when utilizing serum derived from *dam*/Dam^{OP}-vaccinated animals.

Taken together, these findings suggest that cross-reactive antibodies with opsonizing and anti-infective activities are more effectively induced by vaccination with *dam* mutant strains than with the *aroA* strain. The presence of these serum antibody activities directly correlates with the efficiency of cross-protective immunity, possibly through enhanced targeting of infecting bacteria for efficient killing by phagocytic immune cells (29, 58, 60) and reduced invasion

TABLE 2. *dam* mutant immunization results in elevated levels of serum antibodies with cross-reactivity to multiple salmonellae serotypes

Strain	Serovar	IgG (ng/ml) after immunization with ^a :		
		<i>aroA</i>	<i>dam</i>	<i>dam</i> /Dam ^{OP}
174	Bovismorbificans	387	2,855	3,203
225	Bovismorbificans	520	3,087	3,425
χ3246	Choleraesuis	262	505	940
(05)-8895	Dublin	692	1,833	1,580
(02)-1226	Dublin	352	1,565	3,258
Lane	Dublin	290	1,526	3,548
BL7W2FL	Enteritidis	378	666	3,425
(98)-9534	Enteritidis	456	1,358	1,315
4973	Enteritidis	397	1,340	2,321
(05)-6192	Typhimurium	7,038	5,079	12,143
(97)-1332	Typhimurium	7,800	7,022	9,376
131	Typhimurium	4,027	5,726	6,423
12	Typhimurium var. 5 ⁻	542	1,618	3,553
EPIMD142	Typhimurium var. 5 ⁻	2,343	6,129	13,430
(04)-8511	Typhimurium	9,025	8,058	7,645
14028	Typhimurium	13,178	14,327	12,904

^a Detection of *Salmonella*-specific cross-reactive IgG antibodies in pooled serum (*n* = 5 to 10 mice per serum pool) isolated 11 weeks postvaccination from BALB/c mice immunized with *aroA*, *dam*, or *dam*/Dam^{OP} serovar Typhimurium (14028) was performed by enzyme-linked immunosorbent assay (26). Naïve mouse serum had no detectable levels of *Salmonella*-specific IgG. Values given are averages of triplicates of pooled serum samples; standard deviations were <15% of the means.

FIG. 1. *dam* mutant immunization results in increased levels of cross-reactive opsonizing antibodies, blocking antibodies capable of inhibiting the infection of nonphagocytic cells, and cross-reactive memory CD4⁺ and CD8⁺ cells. BALB/c mice were orally immunized with *aroA*, *dam*, or *dam/Dam^{OP}* serovar Typhimurium (14028). After 11 weeks, pooled serum samples collected from immunized mice (heat inactivated, 1:50 dilution) were incubated with salmonellae (10⁹ CFU) for 1 h at 4°C. (A) Opsonized salmonellae were cocultured with RAW 264.7 macrophages (MOI, 20:1) for 1 h at 37°C. (B) Immune serum (1:40) was added directly to HeLa cell cultures followed by the addition of bacteria (MOI, 50:1). After 1 h at 37°C, the numbers of internalized bacteria were determined by direct colony counting after gentamicin treatment (1 h at 37°C) prior to cell lysis with Triton X-100. Values represent enhancement of phagocytosis and percent inhibition of invasion into nonphagocytic cells, respectively, relative to naive serum. (C and D) Immunized and naive mice were i.v. challenged with salmonellae (10⁶ CFU); 3 h later, splenocytes were isolated and stained with monoclonal antibodies to quantitate CD4⁺ CD11a⁺ IFN- γ ⁺ or CD8⁺ CD11a⁺ IFN- γ ⁺ cells by FACS. Values represent the number of memory CD4⁺ or CD8⁺ T cells producing IFN- γ per 10³ splenocytes. Memory CD4⁺ or CD8⁺ T cells from naive challenged mice producing IFN- γ were less than 1 per 10³ (data not shown). SB, serovar Bovismorbificans; SD, serovar Dublin; SE, serovar Enteritidis; ST, serovar Typhimurium. Data represent the means of triplicates $\pm$ the standard deviations. *, $P < 0.05$; **, $P < 0.005$.

of infecting bacteria into weakly bactericidal nonphagocytic cells (43, 44). The reduced titers of these activities in serum derived from *aroA*-immunized animals are consistent with the ineffective cross-protective immunity reported for *aroA* vaccines (25, 30, 31).

***dam* mutant immunization elicits memory CD4⁺ and CD8⁺ T cells that can be reactivated to produce IFN- γ by challenge with nonhomologous salmonellae.** To further investigate the immunologic basis for efficient cross-protection, the presence of IFN- γ -producing memory CD4⁺ and CD8⁺ T cells (45, 56) was evaluated in mice immunized, 80 days previously, with *aroA*, *dam*, or *dam/Dam^{OP}* *Salmonella*. Three hours following i.v. challenge with nonhomologous salmonellae, spleens of *dam* mutant-immunized mice showed increased numbers of activated IFN- γ -producing memory CD4⁺ and CD8⁺ T cells relative to those observed in *aroA*-immunized mice (Fig. 1C and D). Such memory T-cell reactivation was further improved in nearly every case in *dam/Dam^{OP}*-vaccinated animals. These findings indicate that cross-reactive CD4⁺ and CD8⁺ memory cells producing IFN- γ , in addition to cross-reactive antibodies with opsonizing and anti-infective activities, are induced efficiently in response to *dam* mutant vaccination and may be collectively responsible for the cross-protective immunity observed.

***dam* mutant vaccines failed to induce a state of immune suppression caused by elevated levels or activities of myeloid-derived suppressor cells.** Infection with *aroA* mutant or wild-type *Salmonella* results in a profound state of generalized immune suppression (2–4, 14) attributed to the effects of NO produced by myeloid-derived suppressor cells that curtail the induction of adaptive immunity (13). In contrast, a state of immune suppression is not observed in *dam* mutant-immunized animals (26). Since increased numbers of MDSCs of the Gr1⁺ CD11b⁺ phenotype have been linked to NO-mediated immune suppression (6, 8, 9, 12, 24, 38), we examined whether the lack of immune suppression observed in *dam* mutant vaccines correlated with reduced numbers of these cells.

Infection of mice with *aroA* serovar Typhimurium caused significant increases in the percentages and absolute numbers of Gr1⁺ CD11b⁺ cells in the spleen relative to those of uninfected mice, peaking at day 7 postvaccination (Fig. 2A and data not shown). These kinetics were paralleled by increased levels of NO production (Fig. 2B) that directly correlated with peak CFU present in the spleen (Fig. 2C). Alternatively, following *dam* mutant immunization, smaller increases in Gr1⁺ CD11b⁺ cell number were observed, with the maximum at a later time (day 14 instead of day 7). This was accompanied by lower levels of NO and lower CFU in the spleen. These data suggest that

FIG. 2. *dam* mutant immunization does not result in a significant expansion of myeloid-derived suppressor cells. BALB/c mice were i.p. infected (10^4 CFU) with *aroA* or *dam* mutant serovar Typhimurium (14028). Spleens were collected from animals at days 3, 7, 10, 14, 21, 28, and 35 postinfection and were evaluated for number of Gr1⁺ CD11b⁺ MDSCs per spleen analyzed by FACS analysis (A), NO production in 48-h culture supernatants, quantitated by Griess assay (B), or CFU of infecting bacteria, determined by direct colony counting (C). Data represent the means of triplicates $\pm$ the standard deviations. Differences between the numbers of Gr1⁺ CD11b⁺ MDSCs, the levels of NO produced by Gr1⁺ CD11b⁺ MDSCs, and the CFU numbers in spleens of mice infected with *aroA* or *dam* mutant serovar Typhimurium were statistically significant ($P < 0.004$) on days 3, 5, and 7.

the rapid increase in the numbers of MDSCs in the spleen and resultant NO produced following *aroA* mutant infection might contribute to the restricted state of protective immunity induced by this vaccine strain. In contrast, the muted upregulation of MDSCs and lower NO levels observed in *dam* mutant-vaccinated animals correlate strongly with the lack of immune suppression and the robust cross-reactive adaptive immunity generated in response to immunization.

Antigen-specific CD4⁺ T-cell proliferation was inhibited by myeloid-derived suppressor cells derived from *aroA* mutant-but not *dam* mutant-infected mice. To further understand the mechanism of vaccine-induced immunosuppression, we examined whether the proliferation of CD4⁺ T cells was inhibited to a greater extent by MDSCs derived from BALB/c mice immunized with *aroA* versus *dam* mutant *Salmonella*. The cocultivation of splenocytes from naïve DO11.10 mice (T-cell receptor transgenic, ovalbumin specific) with purified MDSCs of the Gr1⁺ CD11b⁺ phenotype from *aroA*-immunized mice resulted in a significant inhibition of CD4⁺ T-cell proliferation in response to ovalbumin addition (Fig. 3). In contrast, the proliferation of ovalbumin-specific CD4⁺ T cells was largely unaffected by the addition of Gr1⁺ CD11b⁺ cells from *dam* mutant-immunized mice compared to naïve control mice. Taken together, these data indicate that a diminished immune suppression associated with *dam* mutant vaccination directly correlates with a reduced suppressive activity of the Gr1⁺ CD11b⁺ cell fraction that adversely affects the development of adaptive immune responses.

The immune-suppressive subpopulation of immature myeloid-derived suppressor cells was increased in *aroA* mutant-but not *dam* mutant-infected mice. MDSCs bearing the Gr1⁺ CD11b⁺ phenotype are morphologically heterogeneous and differ with regard to their immune-suppressive properties. They can be divided into four morphological subgroups: immature and mature polymorphonuclear leukocytes (PMNs) as well as immature and mature monocytes, with the greatest immune-suppressive properties residing in the immature ringed monocyte (MNC ring) subpopulation (12). We therefore examined the subset distribution of MDSCs in the spleens of animals vaccinated with *aroA* or *dam* mutant *Salmonella* at

FIG. 3. Ovalbumin-specific CD4⁺ T-cell proliferation was inhibited by myeloid-derived suppressor cells isolated from *aroA* *Salmonella*-infected mice. Purified MDSCs of the Gr1⁺ CD11b⁺ phenotype isolated from the spleens of BALB/c mice i.p. infected with *aroA* or *dam* mutant serovar Typhimurium (10^4 CFU) at the times of peak Gr1⁺ CD11b⁺ cell numbers postinfection (days 7 and 14, respectively) were cocultured with splenocytes from T-cell receptor transgenic (ovalbumin 323-339-specific) DO11.10 mice at different ratios (1:8, 1:16, and 1:32) in the presence of ovalbumin (100 μ g/ml). After 4 days of coculture, CD4⁺ T-cell proliferation was assessed by the ability of proliferating cells to incorporate [³H]thymidine. Values given reflect the percent inhibition of CD4⁺ T-cell proliferation compared to cell cultures containing similar ratios of Gr1⁺ CD11b⁺ cells derived from uninfected mice. Data represent the means of triplicates $\pm$ standard deviations. **, $P < 0.002$.

the peak times of Gr1⁺ CD11b⁺ cell number postinfection (days 7 and 14, respectively). A higher percentage of immature ringed monocytes populated spleens of animals vaccinated with *aroA* versus *dam* mutant *Salmonella* (Table 3). These data provide an explanation as to why immunization of mice with *aroA* mutant *Salmonella* is more immune suppressive than *dam* mutant immunization.

Aged mice had increased numbers of myeloid-derived suppressor cells and were more susceptible to *Salmonella* infection than young mice. Since generalized immune deficits are known to accompany the natural aging process (11, 15, 21–23), we evaluated whether spleens of aged mice had increased numbers of myeloid-derived suppressor cells and whether aged mice were more susceptible to infection, relative to young mice. In this analysis, two different strains of susceptible mice were examined. While the absolute number of splenocytes in the two age groups of BALB/c and C57BL/6 mice was similar,

TABLE 3. Immature myeloid-derived suppressor cells were largely unaffected by *dam* mutant immunization

Morphology	Spleen Gr1 ⁺ CD11b ⁺ cells (%) from mice immunized with mice ^a :		
	Naïve	<i>aroA</i>	<i>dam</i>
PMN ring	17.8	11.5	12.0
PMN	19.8	26.8	26.0
MNC ring	6.4	17.8*	8.3
MNC	56.0	43.9	53.7

^a MDSCs of the Gr1⁺ CD11b⁺ phenotype were isolated from the spleens of 8- to 10-week-old BALB/c mice ($n = 3$) i.p. infected with *aroA* or *dam* mutant serovar Typhimurium (10^4 CFU) at the peak times of Gr1⁺ CD11b⁺ cell number postinfection (days 7 and 14, respectively). The purified Gr1⁺ CD11b⁺ cells were stained with Wright-Giemsa. A total of 1,000 cells were counted for each sample. The percentages of mature PMN, PMN ring, MNC, and MNC ring cells were identified based on cytoplasmic and nuclear morphologies. Values given are averages of percentages counted in individual samples; standard deviations were $<15\%$ of the means. *, $P < 0.004$ for *aroA*-infected mice versus naïve or *dam* mutant-infected mice.

FIG. 4. Aged mice contained an increased percentage of myeloid-derived suppressor cells and were more susceptible to *Salmonella* infection than young mice. (A and B) Splenocytes isolated from uninfected young (3-month-old) and aged (18- to 20-month-old) BALB/c or C57BL/6 mice were analyzed by FACS for the presence and percentage of MDSCs of the Gr1⁺ CD11b⁺ phenotype. (C) Young and aged mice were i.p. infected with *aroA* or *dam* (10^3 CFU) or wild-type (10^2 CFU) serovar Typhimurium. Three days postinfection, spleens of infected mice were evaluated for bacterial load by direct colony count. Data represent the means of triplicates $\pm$ standard deviations. **, $P < 0.001$.

the spleens of aged mice (18 to 20 months) of both strains were found to be populated with increased numbers of MDSCs bearing the Gr1⁺ CD11b⁺ phenotype relative to that observed in young (3-month-old) mice (Fig. 4A and B). In both mouse strains, this increase in Gr1⁺ CD11b⁺ cells was compounded by a marked increase in the percentage of immature ringed monocytes populating the spleen (Table 4 and data not shown), which have been associated with the greatest immune-suppressive properties (12).

Additionally, aged C57BL/6 mice i.p. infected with wild-type serovar Typhimurium (10^2 cells) displayed a 10-fold increase in the number of bacteria in the spleen at day 3 postinfection and succumbed to the infection sooner, relative to young mice (Fig. 4C and data not shown). Although *aroA* and *dam* mutant vaccine strains were significantly attenuated relative to the wild type at a 10-fold-higher i.p. dose (10^3 cells), aged mice exhibited a ~ 50 -fold increase in vaccine CFU in the spleen relative to that of young mice. Similar increases in disease susceptibility following challenge with wild-type or *dam* mutant serovar Typhimurium were observed when aged versus young BALB/c mice were compared (data not shown). Thus, two strains of genetically identical animals differing only in age showed marked differences in basal numbers of MDSCs and susceptibility to bacterial infection with wild-type or attenuated mutant strains. Taken together, these findings suggest that protocols capable of reducing MDSC numbers or activities in the

elderly may facilitate their ability to better develop protective immune responses to vaccination.

DISCUSSION

The development of cross-protective vaccines against salmonellosis is a high priority for human and animal health due to the large number of pathogenic serotypes, the emergence of multidrug-resistant strains, the difficulties associated with preventing *Salmonella* contamination of food and water supplies, and the potential for the emergence of strain variants that exhibit enhanced pathogenicity in humans and/or animals. Here we show that *Salmonella dam* mutant vaccines confer robust cross-protective immune responses to multiple pathogenic salmonellae isolates. Effective cross-protection appears to be dependent upon the generation of cross-reactive antibodies and antigen-specific memory T cells, as well as immune responses that could be due to the diminished expansion of MDSCs that negatively impact the development of adaptive immune responses. Additionally, the spleens of naïve aged mice contained a marked increase in MDSC numbers and a concomitant increased susceptibility to bacterial infection relative to young mice. Collectively, these data suggest that vaccine protocols designed to control MDSC presence or activities may augment B- and T-cell stimulation, enabling immunologically diverse populations to be effectively vaccinated as well as reducing the risk of susceptible individuals to infectious disease.

Conventional vaccines typically confer highly specific immunity that does not encompass multiple pathogenic serotypes. Subsequent exposure to more than one strain, therefore, may override the immunity elicited by a single vaccine, and pathogenic strains can develop in vaccinated individuals that render current vaccines ineffective. These concerns are exacerbated by the potential emergence of virulent strain variants that differ with regard to host range and degree of host adaptation (51). Epidemiological studies of livestock indicate that the persistence of serovar Typhimurium is characterized by a succession of relatively small epidemics in which one dominant clone is successively replaced by another dominant clone (52). However, some strain variants, e.g., serovar Typhimurium DT104 (57), circulate in a variety of livestock and humans (16, 57) and have the capacity for pandemic spread (49). Further, some

TABLE 4. Aged mice contain an increased percentage of immature myeloid-derived suppressor cells

Morphology	Spleen Gr1 ⁺ CD11b ⁺ cells (%) ^a	
	Young mice	Aged mice
PMN ring	8.5	6.0
PMN	26.0	9.5*
MNC ring	2.0	11.0*
MNC	63.5	73.5

^a MDSCs of the Gr1⁺ CD11b⁺ phenotype were isolated from the spleens of naïve young (3 months old; 3 mice/group) and aged (18 to 20 months old; 3 mice/group) C57BL/6 mice. The purified Gr1⁺ CD11b⁺ cells were stained with Wright-Giemsa. A total of 1,000 cells were counted for each sample. The percentages of PMN, PMN ring, MNC, and MNC ring cells were identified based on cytoplasmic and nuclear morphologies. Values given are averages of percentages counted in individual samples; standard deviations were $<10\%$ of the means. *, $P < 0.005$ for aged versus young mice.

Salmonella strains obtained from human salmonellosis patients are distinct from those of animal origin (27). This finding suggests that either a minor fraction of the strains present in animals are responsible for disease in humans, the epidemiological path of bacterial transmission from animals to humans enables the selection of human-specific strains, and/or bacterial strain variants are selected in humans that have an increased capacity to cause human disease. Consequently, a sustainable vaccine approach must be one that affords protection to multiple pathogenic serotypes.

Our findings show that the protection conferred by the *dam*/*Dam*^{OP} serovar Typhimurium vaccine spanned multiple *Salmonella* clinical isolates derived from human and animal sources, as well as from surveillance submissions of on-farm healthy animals. Further, immunity to some nonhomologous strains exceeded that of many serovar Typhimurium isolates tested. These data indicate that the Typhimurium serotype is comprised of a spectrum of strain variants (27, 51) that differ more from each other immunologically rather than serotypically, a classification principally determined by the presence of distinct lipopolysaccharide and flagellar antigens (47, 53). Thus, in an outbreak scenario, although knowledge of the strain serotype is useful epidemiologically, it has limited predictive value as to whether protection will be conferred by a vaccine strain derived from a homologous serotype. Accordingly, careful consideration must be given to improvements in vaccine design that result in cross-protective efficacy against multiple pathogenic serotypes as well as multiple variants within a given serotype.

The molecular basis of attenuation and protection conferred by immunization with the *dam* mutant may be the result of a number of changes in the physiology of *dam* mutant bacteria, including changes in the levels of bacterium-associated and secreted proteins, a reduction in the ability of the bacterium to invade nonphagocytic cell types, and a minimal cytotoxicity to M cells of the Peyer's patches (20, 26, 50). Consequently, pathways involved in antigen processing and presentation by antigen-presenting cells, including dendritic cells, and the ability to effectively initiate antigen-specific B- and T-cell responses within the Peyer's patches, may be uniquely stimulated in animals infected with *dam* mutant *Salmonella* (54, 55). Such immune responses may facilitate cross-protection due to the stimulation of the following: (i) cross-reactive opsonizing antibodies that enhance bactericidal activities of macrophages and dendritic cells by targeting infecting bacteria for efficient lysosomal degradation (29, 58, 60); (ii) cross-reactive blocking antibodies capable of inhibiting the infection of nonphagocytic cells which are inherently deficient in bactericidal activities; and (iii) memory CD4⁺ and CD8⁺ cells that confer cell-mediated activities that facilitate immune defenses or are directly cytotoxic to infected cells (45, 56). While it has been reported that both antigen-specific IgG antibodies and memory T cells are required for protection against disease following challenge with homologous *Salmonella* (39–42, 45, 46), these data suggest that the efficiency of cross-protective immunity following *dam* mutant immunization may have a similar set of requirements.

Efficient cross-protection conferred by *dam* mutant immunization correlated with the diminished induction of myeloid-derived suppressor cells and activities (13) and the resultant

lack of generalized immune suppression that may adversely affect adaptive immune responses via lymphocyte inactivation (14). This is in contrast to infection with *aroA* mutant or wild-type *Salmonella* that results in a transient state of generalized immune suppression (2–4, 14), which is credited to the effects of NO produced by MDSCs. The *aroA*-associated immune suppression may constrain the adaptive immune responses elicited in vaccinated hosts to a limited set of closely related strains (25, 30, 31). Vaccine-induced immunosuppression is particularly undesirable from the standpoint of public health, military, and veterinary immunization programs, as multiple vaccines are often delivered simultaneously, which may delay or preclude a response to a subsequent antigen exposure.

The increased percentage and numbers of myeloid-derived suppressor cells in aged mice suggests that they may also contribute significantly to the decline in immune competence associated with natural aging. Thus, whether directly induced by a given vaccine itself, as seen with *aroA* mutant *Salmonella* vaccines, or representing a preexisting condition, as demonstrated with aging or observed with a variety of stressful conditions (e.g., chronic microbial infection, traumatic insult, and many forms of cancer), activities by MDSCs appear to compromise the efficient generation of adaptive immunity (4, 9, 36). Additionally, MDSCs may also be responsible for the generalized immune suppression observed under conditions of severe psychological stress, limiting vaccination efficiency and increasing the susceptibility of a stressed host to infectious disease (7). Thus, conditions that diminish MDSC activities may have benefit to otherwise-healthy individuals subjected to severe stress, e.g., personnel involved in military training and combat.

Protocols designed to inhibit the expansion of MDSCs and negate their immune-suppressive activities are currently being investigated in tumor-bearing patients and animal models (1, 35). Treatment of tumor-bearing hosts with antioxidant therapies, such as all-*trans* retinoic acid, an active form of vitamin A, is able to promote the terminal differentiation of MDSCs, eliminating their immune-suppressive activities. Mechanistically, all-*trans* retinoic acid was found to promote terminal differentiation via neutralization of elevated reactive oxygen species, a functional attribute of MDSCs (48). Other antioxidants may be equally effective, as treatment of aged mice with supplemental vitamin E decreases the number of MDSCs and significantly restores immune competence (unpublished data) (11, 15). It is possible that interventions capable of reducing MDSC numbers and activities warrant special consideration, as they may promote the development of safe and effective cross-protective vaccines while reducing the public health risk of susceptible populations to microbial infection.

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