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Immunization with *Staphylococcus aureus* iron regulated surface determinant B (IsdB) confers protection via Th17/IL17 pathway in a murine sepsis model

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Abbreviations: amorphous aluminum hydroxyphosphate sulfate adjuvant, AAHSA; opsonophagocytosis, OP; severe combined immunodeficiency, SCID; bovine serum albumin, BSA; intracellular cytokine staining, ICS

We have previously shown that IsdB, a conserved protein expressed by *Staphylococcus aureus,* induces a robust antibody response which correlates with protection in a murine challenge model. Here we investigate the role of cellular immunity in IsdB mediated protection using lymphocyte deficient SCID mice. As opposed to WT CB-17 mice the CB-17 SCID mice were not protected against a lethal challenge of *S. aureus* after active and passive immunizations with IsdB. Adoptive transfer of in vitro isolated lymphocyte subsets revealed that reconstituting mice with IsdB specific CD3+ or CD4+ T-cells conferred antigen specific protection while $CD8^+$ T-cells, $CD19^+$ B-cells and plasma cells ($CD138^{high}B220^{int}CD19^{lo}$) alone were not protective. A combination of $CD3^+$ T-cells plus $CD19^+$ B-cells conferred protection in CB-17 SCID mice, whereas bovine serum albumin (BSA) immune lymphocytes did not confer protection. Active immunization experiments indicated that IsdB immunized Jh mice (B-cell deficient) were protected against lethal challenge, while nude (T-cell deficient) mice were not. In vitro assays indicated that isolated IsdB specific splenocytes from immunized mice produced abundant IL-17A, much less IFN-γ and no detectable IL-4. IL-23 deficient mice were not protected from a lethal challenge by IsdB vaccination, pointing to a critical role for $CD4^+$ Th17 in IsdB-mediated vaccination. Neutralizing IL-17A, but not IL-22 in vivo significantly increased mortality in IsdB immunized mice; whereas, neutralizing IFN-γ did not alter IsdB-mediated protection. These findings suggest that IL-17A producing Th17 cells play an essential role in IsdB vaccine-mediated defense against invasive *S. aureus* infection in mice.

Introduction

The need for a vaccine to prevent invasive disease caused by *S. aureus* has become an important public health concern, increasing in urgency over the last decades.[1-3] Development of a vaccine often depends on gaining an understanding of the immune response to an organism, which can then be enhanced through rational vaccine design. Although extensively investigated, natural protective immunity to *S. aureus* is still poorly understood. Acute infection with *S. aureus* does not prevent re-infection.[1] Preclinical and clinical data indicate that immunization with intact whole bacteria induces high immune titers to staphylococcus, but does not confer protection from *S. aureus* disease.[1,4] Clearance of *S. aureus* is historically thought to be dependent upon antibody (Ab) and complement mediated uptake and killing by phagocytes,[5-10] with neutrophils being essential to resolution of disease.[11,12] However it has been demonstrated that *S. aureus* can in fact survive within neutrophils, leading to exacerbated disease.[11,13] Additionally, the *S. aureus* stimulated humoral immune response may not play a meaningful role in bacterial clearance in some models.[14-17] While antibodies undoubtedly play some role in protection, they may not be determinative for vaccine protective efficacy because animals and humans already have sufficient baseline opsonins to allow for phagocytic uptake by neutrophils.

Recently, the importance of T-cells and T-cell cytokines in innate immunity to *S. aureus* has been the object of investigation.[14,18-20] T cells were observed to play a pivotal role in the prevention of *S. aureus* infection, as demonstrated in models of disseminated as well as local infection. These studies established a connection between T cells and neutrophils wherein IL-17A is important for recruiting neutrophils, enhancing chemotaxis, acting synergistically with TLR2 to enhance killing (non-antibody, i.e., pattern recognition), and priming neutrophils for bactericidal activity.[21,22]

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In addition, Th17 immunity may be important for addressing *S. aureus* as an intracellular pathogen[23] and IL-17A induced Th1 helper response may be of particular importance in addressing such organisms.[24] Hence we wanted to define the role of T-cell immunity in response to our vaccine antigen.

IsdB is a ~72 kDa antigen expressed on the cell surface of *S. aureus* in iron limited environments.[25-27] Its function is to capture and import heme iron from hemoglobin.[27] Due to the low iron environment of mammalian blood and tissue, IsdB is upregulated during pathogenesis in vivo.[28] IsdB is highly conserved among diverse *S. aureus* clinical isolates, both methicillin resistant and methicillin sensitive and humans, as well as mammals examined to date, have pre-existing antibody titers to IsdB,[29] although it is unknown whether these pre-existing titers offer protection. Additionally, Kuklin et al. demonstrated that immunization with IsdB formulated on amorphous aluminum hydroxyphosphate sulfate adjuvant (AAHSA) increased murine antibody titers by up to 20-fold, and non-human primate titers by 4-fold. Importantly, increased antibody titers correlated with enhanced survival in a murine disseminated challenge model.[30] Also, mAb to IsdB have in vitro opsonophagocytic (OP) activity, and efficacy in rodent challenge models.[28] Thus IsdB-specific mAb can confer protection after passive immunization, however it was of interest to determine what components of the adaptive immune system are important for protection mediated by active IsdB vaccination. To this end, a series of experiments were undertaken to dissect the immune response to IsdB in the murine lethal challenge model. Lymphocyte populations were evaluated for protective activity, and it was discovered that protection in this model is mediated by $CD4^+$ T cells, and that IL-17A plays an important role in vaccine efficacy.

Results

Lymphocytes are essential for IsdB mediated protection in a murine sepsis model. IsdB was previously shown to elicit antibody responses which correlated with protection in a murine sepsis model. However, it is possible that the antigen also elicits an effector T-cell response which may contribute to enhanced survival. To investigate the role of cell mediated immune responses during IsdB induced protection, CB-17 (WT or SCID) mice were immunized with IsdB or BSA (negative control), via intramuscular injection to the thigh, as described in the Methods section. Mice were challenged with *S. aureus* and survival was monitored for 10 d.

Since survival in this disseminated infection model correlates with antibody titer,[30] it was expected that lymphocyte (and antibody) deficient SCID mice would be susceptible to challenge. As shown in **Figure 1A**, IsdB immunized CB-17 WT mice, with an intact lymphocyte population, showed significantly higher ($p = 0.02$) survival than IsdB immunized CB-17 SCID mice, or BSA vaccinated control mice ($p = 0.02$) (data not shown). It was expected that if the SCID mice were supplemented with IsdB specific mAb, they would be protected from challenge. Monoclonal antibody CS-D7 was used to passively immunize SCID mice. This monoclonal has been previously demonstrated to significantly enhance survival in the murine sepsis model.[31] Surprisingly, SCID mice treated with CS-D7 were not protected post challenge and showed similar reduced survival as animals treated with the isotype control (MK-24), or with animals uninjected with mAb (naïve mice) (**Fig. 1B**). This experiment was performed using the Becker strain[28] as well as the SA025[31] strain as challenge inoculum, with equivalent results. Thus using either method of immunization, it was observed that an intact lymphocyte system was required for protection post IsdB immunization in the murine sepsis model of infection.

Identification of lymphocyte subset(s) critical in protection. Lymphocyte subset(s) were examined to determine which populations were critical in providing protection mediated by IsdB. Splenocytes harvested from IsdB or BSA immunized CB-17 WT mice were isolated into five major subsets: $CD19^+$,

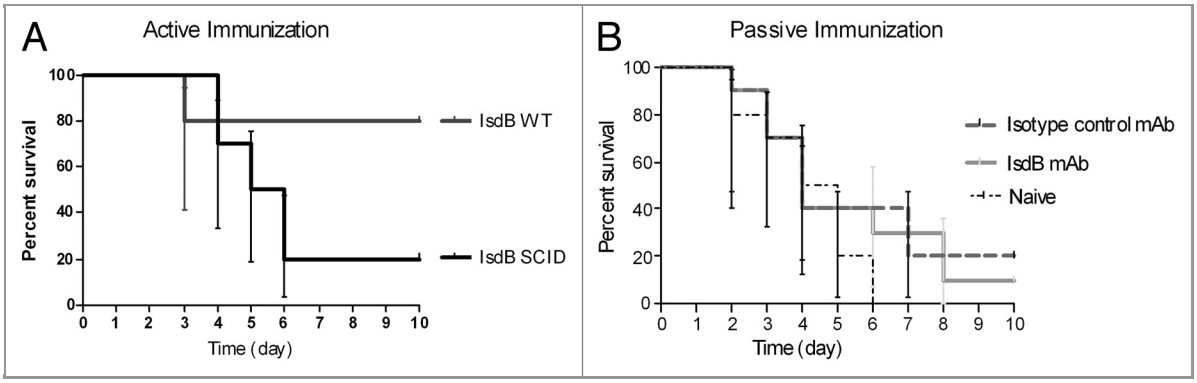

Figure 1. Survival of CB-17 (WT or SCID) mice immunized (active or passive) with IsdB. (A)WT or SCID mice (n = 10) were actively immunized i.m. with 20 μg of antigen on days 0, 7 and 21. On day 35, mice were challenged with *S. aureus* Becker (8.8×10^8 CFU) via the tail vein. Mice were monitored for survival for 10 d post challenge. Data were pooled from two independent experiments. Survival of IsdB immunized SCID mice vs. IsdB immunized WT mice, p = 0.02. Survival of IsdB immunized WT mice vs. control BSA immunized WT mice, p = 0.02 (data not shown). (B) Survival of CB-17 (SCID) mice passively immunized to IsdB and challenged via the tail vein. SCID mice (n = 10) were immunized i.p. with 400 μg of mAb (either IsdB specific CS-D7, or control MK24), or saline alone, 2 h prior to challenge with *S. aureus* SA025 (2×10^8 CFU) via the tail vein. Mice were monitored for survival for 10 d post challenge. Data were pooled from two independent experiments. Survival of IsdB immunized SCID mice vs. isotype immunized mice, p = 0.49. Error bars indicate the 95% CI.

$CD138^{high}B220^{int}CD19^{lo}$, $CD3^+$, $CD4^+$, and $CD8^+$, using MACS beads. These were adoptively transferred into CB-17 SCID mice via tail vein injection. Purity of the isolated populations was confirmed using flow cytometry (data not shown), and each population was > 95% homogeneous. Mice were challenged via the tail vein and monitored for survival for 10 d post challenge.

As shown in **Table 1**, CB-17 SCID mice reconstituted with a combination of $CD19^+$ plus $CD3^+$ lymphocytes (group #3) from animals immunized with IsdB were robustly protected, as compared with mice reconstituted with $CD19^+$ plus $CD3^+$ lymphocytes from BSA immunized animals ($p < 0.01$). Individual $CD19^+$ (group #2) and $CD3^+$ (group #4) lymphocytes were examined for protective activity. Surprisingly, reconstitution with IsdB immune $CD19^+$ B lymphocytes alone did not generate significant protection ($p = 0.54$) vs. the control $CD19^+$ cells. In case these lymphocytes were not producing sufficient antigen specific IgG, immune $CD138^{high}B220^{int}CD19^{lo}$ plasmacytes (group #1) were isolated and adoptively transferred. Contrary to our expectations, transfer of the IsdB immunized $CD138^{high}B220^{int}CD19^{lo}$ plasmacyte subset did not reconstitute statistically significant protection ($p = 0.2$) in the recipient mice. Thus neither IsdB immunized B cells ($CD19^+$) nor plasmacytes ($CD138^{high}B220^{int}CD19^{lo}$) were protective. However, adoptive transfer of CD3+ T cells (group #4) was found to be significantly protective ($p < 0.01$) over control. Two subsets of T cells were isolated and transferred, $CD4^+$ (group #5) and $CD8^+$ (group #6). No enhancement in survival was noted with the transfer of $CD8^+$ T cells alone ($p = 0.6$), thus suggesting that the $CD4^+$ T subset ($p = 0.06$) in the $CD3^+$ T cell population was responsible for enhanced survival of recipient mice. The adoptive transfer of lymphocyte subsets from control (BSA) mice was unable to reconstitute protective immunity in any instance.

$CD4^+$ mediated protection was antigen specific. The enhanced survival observed in SCID mice reconstituted with IsdB specific $CD4^+$ cells was investigated to determine if the response was antigen specific. Although BSA immune lymphocytes did not confer protection (**Table 1**), it was possible that IsdB immune lymphocytes could have induced a non-specific stimulation of the innate immune system leading to enhanced survival against *S. aureus* challenge. $CD4^+$ lymphocytes from IsdB immune CB-17 mice were adoptively transferred into SCID mice. The mice were challenged with a lethal dose of *S. aureus* Becker *isdB*/*harA* deletion strain (KO), or the WT parental strain. A double deletion strain was used because *harA* has high homology with *isdB*, and therefore both genes need to be deleted to ensure complete removal of this antigen.

As observed in the adoptive transfer experiments above, SCID mice replete with IsdB immune $CD4^+$ lymphocytes were significantly protected after challenge with the *S. aureus* Becker WT strain, when compared with SCID mice replete with BSA immune $CD4^+$ lymphocytes, $p < 0.003$ (**Fig. 2**). However, when similar sets of mice were challenged with the *S. aureus* Becker KO strain, the IsdB immune SCID mice did not exhibit greater survival ($p = 0.26$) than the BSA immune SCID mice. Therefore, the protection mediated by IsdB immune $CD4^+$ lymphocytes was antigen (IsdB) specific.

Lack of T cell immunity has fatal outcome while lack of B cell immunity does not. The inability of IsdB specific plasma and $CD19^+$ B cells to confer protection in the CB-17 SCID adoptive transfer model prompted us to investigate the role of B and T cells in IsdB mediated protection, using an alternative strategy. Genetically altered immunoglobulin heavy chain deficient Jh (Balb/c background) and T cell deficient nude mice (Balb/c) were immunized with IsdB, or BSA using the same protocol as above. Mice were challenged with a lethal dose ($LD_{80–90}$) of *S. aureus* Becker and survival was monitored for 10 d.

As shown in **Figure 3A**, IsdB immunized Jh (B cell deficient) mice were significantly protected after *S. aureus* challenge,

Table 1. Survival of CB-17 SCID mice after adoptive transfer of isolated lymphocyte populations and challenge via the tail vein with *S. aureus**

Cell Type	Immunity	Number of mice surviving, days post challenge											
		1	2	3	4	5	6	7	8	9	10	% survival	P value
CD138+B220+/−CD19-(Plasma cells)	BSA	10	7	5	5	3	3	3	3	3	3	**30**	0.20
	IsdB	10	10	9	7	7	6	5	5	5	5	**50**	
CD19+(B cells)	BSA	10	10	8	7	5	4	4	4	3	3	**30**	0.54
	IsdB	10	10	7	5	4	4	2	2	2	2	**20**	
CD19+CD3+(B cells+T cells)	BSA	10	7	5	3	3	1	1	1	1	1	**10**	< 0.01
	IsdB	10	10	8	8	8	8	8	8	8	8	**80**	
CD3+(Tcells)	BSA	10	10	7	4	2	2	2	2	2	2	**20**	< 0.01
	IsdB	10	10	10	8	8	8	8	8	8	8	**80**	
CD4+(Th cells)	BSA	10	10	7	6	4	4	3	3	3	3	**30**	0.06
	IsdB	10	10	10	9	7	7	7	7	7	7	**70**	
CD8+(T cells)	BSA	10	10	7	5	5	4	2	2	2	2	**20**	0.60
	IsdB	10	8	7	5	5	5	4	4	4	4	**40**	
No transfer		10	6	2	0	0	0	0	0	0	0	**0**	

*Results represent a single experiment which was repeated three times with similar results. p values were determined using Prism® Log Rank survival statistics, for pairwise comparison of each experimental group to its matching control group.

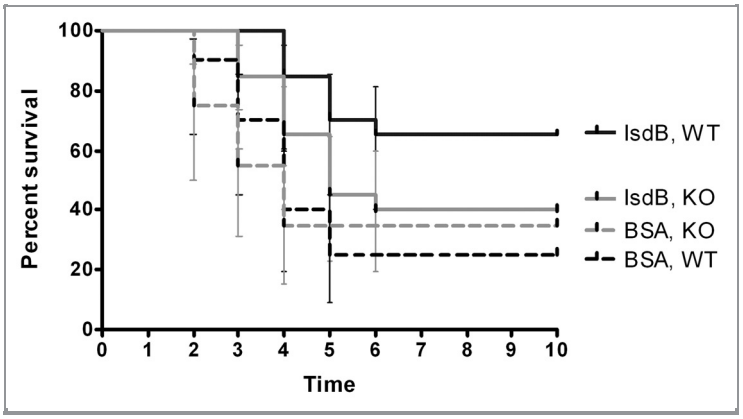

Figure 2. Survival of CB-17 SCID mice replenished with IsdB or BSA immunized $CD4^+$ T cells. $CD4^+$ T cells from CB-17 mice immunized with either IsdB, or BSA were adoptively transferred into SCID mice (n = 10) via the i.v. route, and the mice were challenged with either WT *S. aureus* Becker, or KO Becker (*isdB/harA*$^{-/-}$)(LD_{80-90}; 4.9×10^8–8.7×10^8) via the tail vein. Mice were monitored for survival for 10 d post challenge. Survival of mice challenged with WT *S. aureus* Becker, p = 0.003 for IsdB immunized SCID mice vs. BSA immunized SCID mice. Survival of mice challenged with KO *S. aureus* Becker, p = 0.26 for IsdB immunized SCID mice vs. BSA immunized SCID mice. Error bars indicate the 95% CI values.

compared with the BSA control Jh mice (p = 0.04) which were not protected. The control naive (unimmunized) Jh mice were also not protected. The IsdB immunized nude (T cell deficient) mice (**Fig. 3B**) were not protected after *S. aureus* challenge in comparison to the BSA immunized control nude mice (p = 0.88). These data were in agreement with our prior adoptive transfer experiments, indicating a critical role for T lymphocytes, but not B lymphocytes nor plasma cells, in mediating protection after immunization with IsdB, in the murine sepsis model. Titers to IsdB were measured for each group of mice. Jh mice had no IgG titer to IsdB (**Fig. 3C**) while nude mice had IsdB titers post immunization, although lower than the expected titer for WT mice (**Fig. 3D**).

IsdB Immunization primarily induced secretion of IL-17A but not IFN-g or IL-4. As observed in the adoptive transfer experiments, the CD4+ T cells were the cell population which conferred protection after challenge with *S. aureus*. To define the cytokine response induced by vaccination, spleens were harvested from IsdB and BSA immunized CB-17 WT mice two weeks post boost (day 28). The splenocytes were stimulated ex vivo for 20 h with IsdB peptide pools or IsdB intact protein

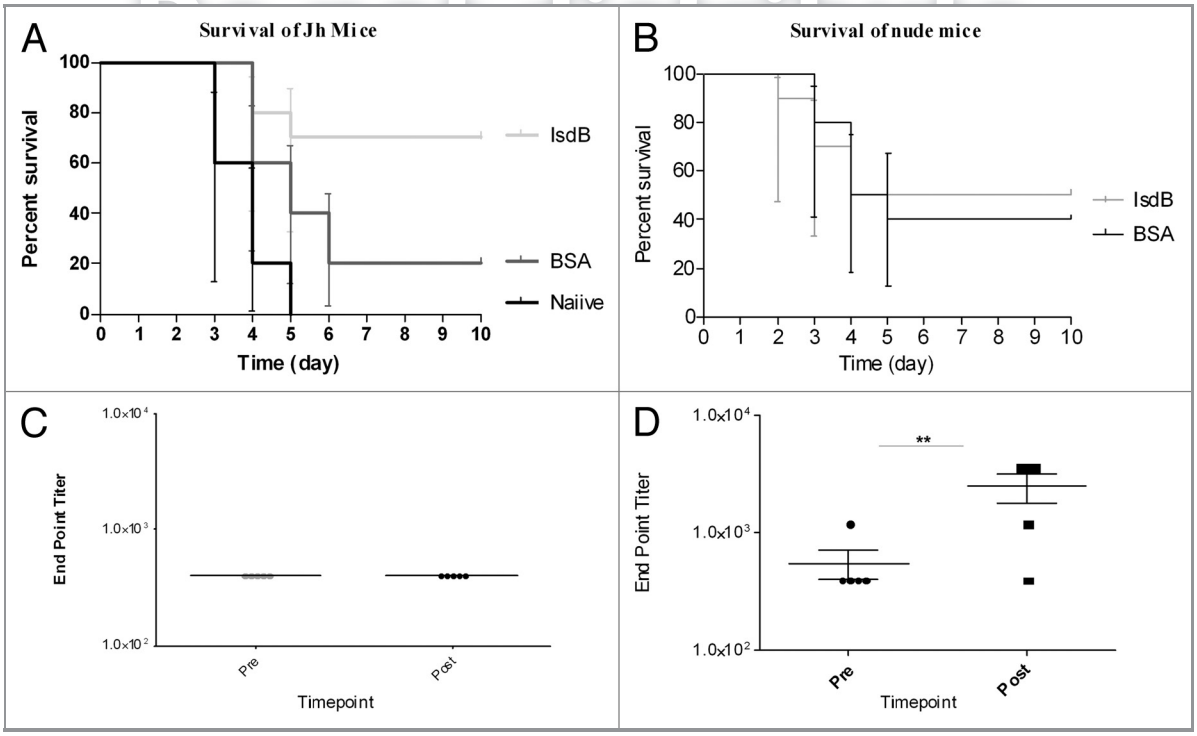

Figure 3. Survival of IsdB immunized B and T cell deficient mice challenged via the tail vein. IgH deficient Jh mice [(A) n = 10] or T-cell deficient nude mice [(B) n = 10] were actively immunized i.m. with IsdB or BSA and challenged with *S. aureus* Becker, (6.4×10^8 CFU, LD_{80-90}) via the tail vein, as described in the Methods section. Mice were monitored for survival for 10 d post challenge. IsdB immunized Jh mice survival compared with control BSA immunized Jh mice (p = 0.04). T-cell deficient nude mice immunized with IsdB survival compared with BSA immunized nude mice (p = 0.88). Jh (C) and nude (D) mice were bled prior to IsdB vaccination on day 0, and post vaccination on day 28, and titer of IsdB antibody response measured using an ELISA, as described in the Methods section.

(as described in the Methods section) and cytokine secretion by CD4+ T cells was measured by Elispot assay and ICS assay.

Intracellular cytokine staining (ICS) using intact IsdB protein revealed a significant increase in the frequency of IL-17A secreting CD4+ T cells post IsdB immunization (**Fig. 4A and S1**), as compared with BSA immunized control mice (**Fig. 4B and S1**). A minimal increase in IFNγ-secreting CD4+ T cells was also noted upon stimulation (**Fig. 4A**). No response from IL4 secreting

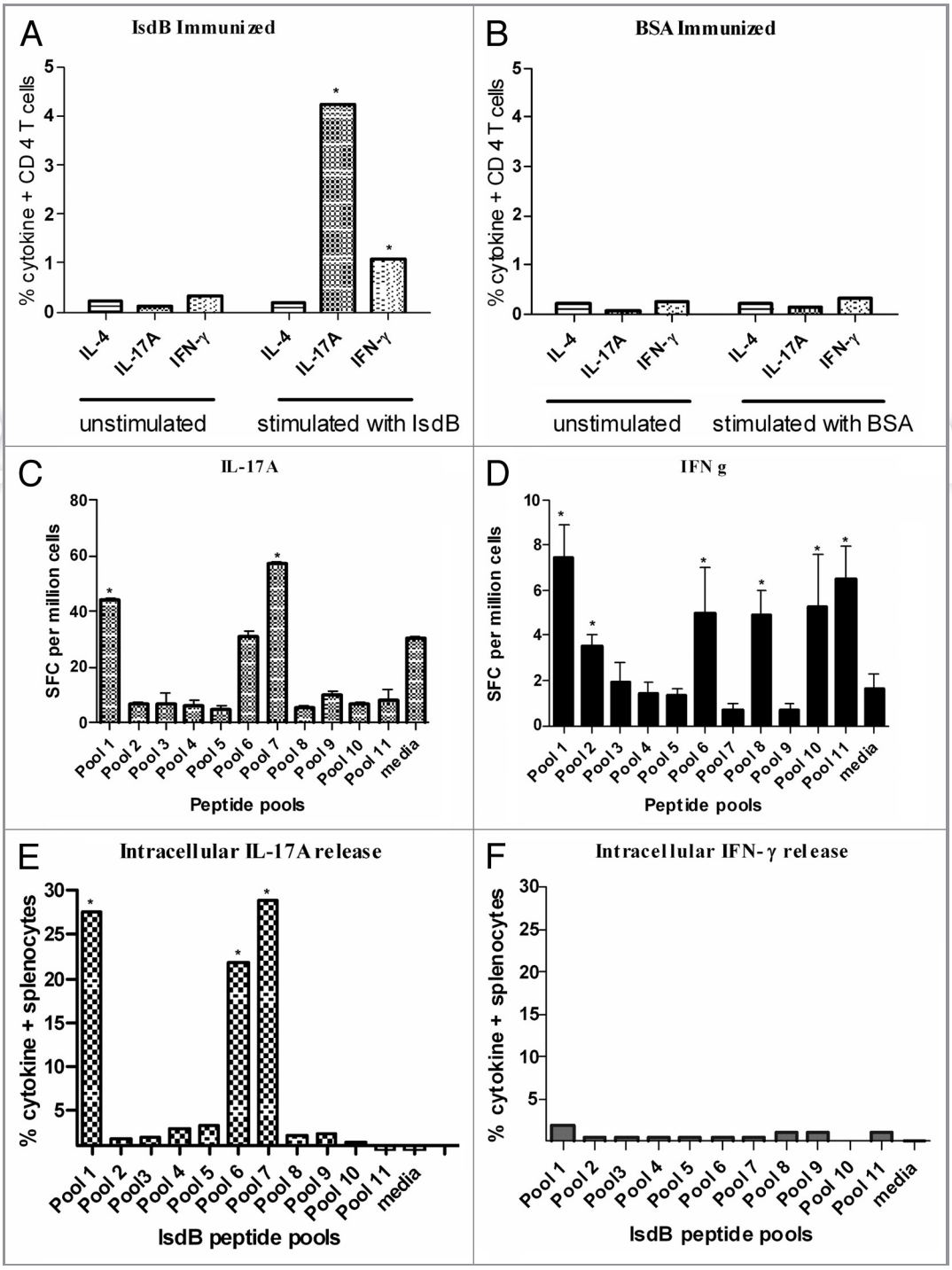

Figure 4. ICS and ELISpot evaluation of IsdB immune splenocytes for cytokine secretion. Splenocytes were isolated from IsdB (A) or BSA (B) immune CB-17 mice and stimulated for 20 h in vitro with IsdB or BSA, or left unstimulated. IL17A, IFNγ and IL4 secreting CD4 T cells were identified by flow cytometry as described in the Methods section. Overlapping 15 mer peptide pools from IsdB were used to stimulate splenocytes from IsdB immune CB-17 mice. Splenocytes were subsequently analysis by ELISpot for the production of IL17A (C) or IFNγ (D) release. ICS was used to confirm ELISpot results by evaluating which IsdB peptide pools stimulated intracellular secretion of either IL17A (E) or IFNγ (F).

$CD4^+$ T cells was observed. Additionally we also evaluated the secretion of IL-17A and IFN-g from immune splenocytes using IsdB peptide pools in an ELISpot assay (**Fig. 4C and D**). It was observed that IsdB peptide pools 1 (a.a. 42–93) and 7 (a.a. 242–333), stimulated robust secretion of IL-17A from splenocytes (**Fig. 4C**). Pools 1 (a.a.42–93), 10–11(a.a.402 to 457) stimulated IFN γ response, however, this was not as robust as the IL-17A response (**Fig. 4D**). Alternatively, secretion of IL17A, IFN-γ and IL4 from splenocytes using IsdB peptide pools was also evaluated in an ICS assay. Similar to Elispot results, stimulation with pools 1, 6 and 7 resulted in robust IL-17A secretion from splenocytes (**Fig. 4E**; **Fig. S2**) however we did not detect a robust IFN-γ response upon stimulation with the overlapping IsdB peptide pools using ICS technique (**Fig. 4F**; **Fig. S3**).

IsdB does not mediate protection in p19KO mice. Based upon the data above, it appeared that IsdB mediated immunity induced a robust Th17 (IL-17A), as opposed to a Th1 (IFN-γ), or Th2 (IL4) response in the CB-17 mice. It was therefore possible that stimulation of the Th17 response contributed to protection in mice in the sepsis model. To investigate this possibility, IL-23p19 KO mice (C57Bl/6 background) were utilized. Genetic deletion of the IL-23p19 subunit prevents production of the cytokine IL-23, and thus the formation of IL-17 and IL 22-secreting $CD4^+$ T-cells.[21,32] IL-23p19 KO mice, or the parental WT C57Bl/6 were immunized with either IsdB or BSA as previously described, and challenged with *S. aureus*.

As indicated in **Figure 5**, IL-23p19 KO mice immunized with IsdB had a similar survival rate as mice immunized with BSA (p = 0.77). Although both IsdB and BSA immunized groups of mice had a higher survival rate than the naïve, unimmunized mice, there was no difference in survival between the two immunized groups.

Protection in mice is mediated by IL-17A and not IFN-γ. Results from the IL-23p19 KO mouse strain supported the possibility of Th17 cells contributing to the IsdB vaccine-mediated protection of Balb/c mice in the sepsis model. If this were so, then IL-17A could be an important cytokine involved in, or mediating, the IsdB vaccine protection.[33,34] Since IL23p19KO mice are also deficient in IL22 producing CD4T cells, the role of both IL17A and IL22 in protection was evaluated using neutralizing mAbs to each of these cytokines (**Fig. 6**; **Fig. S4**). IsdB immunized Balb/c were administered saline, or 400 μg of either IL-17A, IFN-γ, IL22 neutralizing mAb, or isotype control mAb per mouse, through the i.p. route. Two hours post administration of saline or mAb, mice were challenged through the tail vein and survival was monitored for 10 d.

When IsdB immunized Balb/c mice were injected with saline (IsdB), or were injected with isotype control mAb, prior to *S. aureus* challenge, they had equivalent survival in this model (60% and 55% respectively) (**Fig. 6A**). IsdB-immunized mice administered neutralizing antibody to IL-17A had a significant decrease in survival, when compared with IsdB-immunized mice administered isotype control mAb (p = 0.004). IsdB immunized

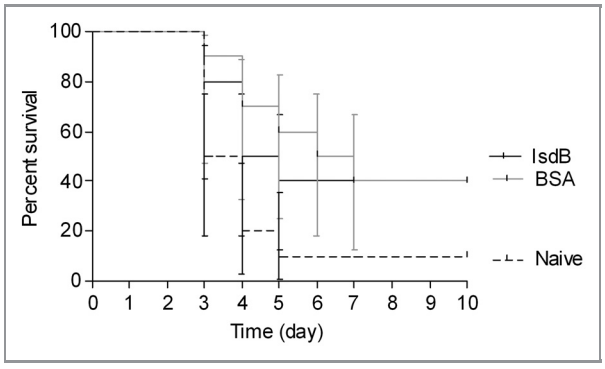

Figure 5. Survival of p19KO mice immunized with IsdB or BSA and challenged via the tail vein. p19KO mice (n = 10) were immunized i.m. with 20 μg of antigen on days 0, 7 and 21. On day 35 mice were challenged with *S. aureus* Becker (8×10^8 CFU) via the tail vein. Mice were monitored for survival for 10 d post challenge. Survival of IsdB immunized p19KO mice vs. BSA immunized p19KO mice, p = 0.77. Error bars = 95% CI values.

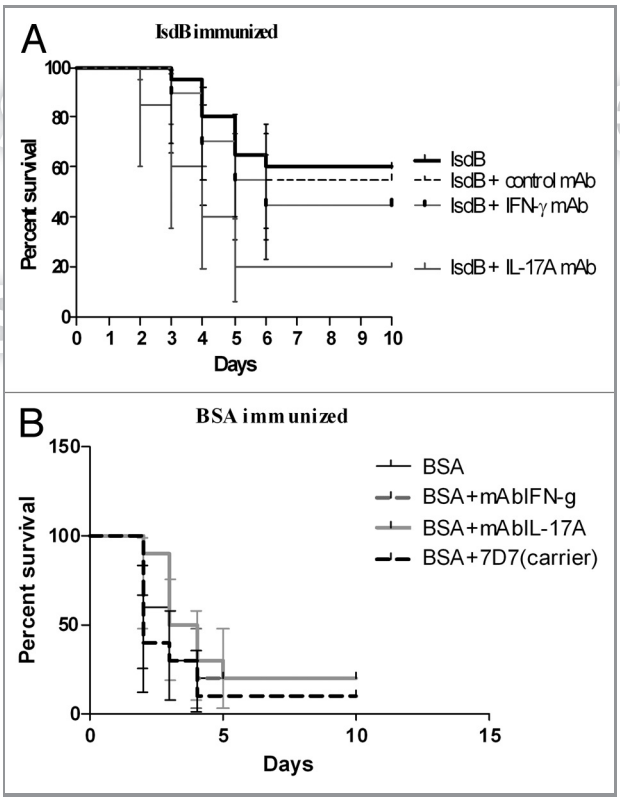

Figure 6. Survival of Balb/c mice immunized with IsdB (A) or BSA (B) and challenged in the presence of neutralizing mAbs. Balb/c mice (n = 10) were immunized i.m. with 20 μg of antigen on days 0, 7 and 21. On day 35 mice were injected ip with 400 μg of neutralizing IL17A, IFN-γ or isotype control mAb, or an equal volume of saline. After 2 h, mice were then challenged with *S. aureus* Becker (7.8×10^8 CFU) via the tail vein. Mice were monitored for survival for 10 d post challenge. Data shown is from a single experiment repeated two times with equivalent results. (A) Survival of IsdB immunized mice injected with IL17A mAb vs. isotype control mAb, p = 0.004; survival of IsdB immunized mice injected with IFNγ mAb vs. isotype control mAb, p = 0.48. (B) Survival of BSA immunized mice injected with IL17A mAb vs. isotype control mAb, p = 0.17; survival of BSA immunized mice injected with IFN-γ mAb vs. isotype control mAb, p = 0.18. Error bars = 95% CI.

mice administered IL-17A neutralizing mAb had the same survival rate as for negative control BSA immunized mice (20%). Administering IFN γ neutralizing mAb to IsdB immune mice resulted in a somewhat lower survival than for isotype control mAb injected mice, however, the difference was not significant (p = 0.48). BSA immune mice served as the negative control for this experiment. As shown in **Figure 6B**, there was no significant difference in survival in BSA immunized mice with saline nor any antibody injected (p = 0.05). Additionally, we found that IL22 does not play a major role in this model, as there was no significant drop in survival of IsdB immunized mice, challenged with a lethal *S. aureus* dose, comparing the IL22 neutralizing mAb to an isotype control mAb (p = 0.59, **Fig. S4**) This experiment indicates that there is a direct link between IsdB immunization, IL-17A production, and efficacy in the murine model of lethal sepsis.

Discussion

The humoral immune response to *S. aureus* is believed to be important for protection from infection, and as such, has been extensively investigated.[35-39] However this response may not necessarily contribute to clearance of bacteria or reduction of disease.[15,40] Evidence is mounting that antibody plays a limited role in the clearance of multiple bacterial pathogens. For example, the clearance of *Streptococcus pneumoniae* colonization may not depend on the humoral immune response,[41-43] and antibodies are not important in the clearance of *Helicobacter pylori* in a murine challenge model.[44,45] Investigation of human genetic linked disease has implicated a role for $CD4^+$ Th17 cells in the natural clearance of *S. aureus*. Patients with hyperimmunoglobulin E syndrome (HIES), or Job syndrome, suffer from repeated *S. aureus* and *C. albicans* infections of the skin and mucosal surfaces.[46] Recently mutations in the signal transducer and activator of transcription 3 (STAT3) intracellular regulator were described in these patients, and the resulting defective STAT3 was linked to dramatically decreased or missing IL-17A-producing $CD4^+$ cells (i.e., Th17 cells).[47-49] A significant decrease in IL-17A was observed in these patients, which could account for the lack of protection against repeated *S. aureus* and *C. albicans* infections.[22] Th17 cells are thought to mediate protection from intracellular bacteria, extracellular bacteria and fungal infections[21,32,50,51] particularly at mucosal surfaces.[22] IL-17A secreted by Th17 cells stimulates release of chemokines and granulocyte colony stimulating factors, thus recruiting neutrophils to sites of pathogen invasion.[50] Although IL-17A reduction was linked to the repeated *S. aureus* and *C. albicans* infections in Job syndrome patients, autoantibodies to IL-17A and IL-17F, as observed in autoimmune polyendocrine syndrome type I (APS-1) patients do not lead to *S. aureus* infections. These patients exhibit repeated *C. albicans* infections, but no unusual level of infections with other pathogens.[52] Perhaps the difference between the two syndromes resides with the intact Th17 activity in the APS-1 patients, which might provide some activation of mucosal cells and keratinocyte cationic peptide defenses and thus provide some residual immunity for pathogens like *S. aureus* at these sites. In any event, the connection between STAT3, Th17, IL-17A and *S. aureus* immunity is a complex and evolving story.[53]

Mice models have been used to investigate the role of Th17 in natural immunity to *S. aureus*. In the absence of both IL-17A and IL-17F in a cytokine deletion murine strain ($Il17a^{-/-}IL17f^{-/-}$),[54] formation of spontaneous mucocutaneous abscesses was observed around the nose and mouth. These abscesses contained *S. aureus*, indicating the involvement of IL-17A/F cytokines in the natural control of *S. aureus* infections. However after active iv challenge with *S. aureus*, no difference was observed in survival, or kidney bacterial burden, between the $Il17a^{-/-}IL17f^{-/-}$ strain and the wild type progenitor strain. Therefore, IL-17A/F were not critical immune modulators during disseminated *S. aureus* infection, but only for local mucocutaneous infection.[54] It is important to note that the mice in these experiments were un-immunized. The role of IL-17A in mucocutaneous protection from *S. aureus* was confirmed in work from a second group.[55] These investigators demonstrated that mice deficient in $CD3^+$ γδT cells exhibited cutaneous lesions significantly larger than observed in wild type mice. Reconstituting the mice with a single dose of IL-17A could restore the impaired immunity of the deletion strain back to that of the wild type strain.

IsdB has significant vaccine activity when administered to mice, and IsdB specific antibodies generated by active immunization can confer protection.[28,30,56] In light of the expanding evidence supporting the importance of T cells in immunity to bacteria, we initiated experiments to explore the role of lymphocytes in IsdB mediated protection. To this end, lymphocyte populations were investigated to determine which population(s) conferred protection. In the absence of any lymphocytes (in SCID mice), no protection was observed after IsdB immunization, which was not unexpected, because these mice are defective for IgG production. However, protection could not be reconstituted by passive immunization with IsdB specific mAb in the absence of lymphocytes. The dependence of mAb mediated protection on having an intact lymphocyte system may be explained by the fact that Abs promote a more effective inflammatory response in the presence of T cells. Infact there are several other instances in which passive mAb efficacy requires an intact immune system.[57,58] Thus, lymphocytes played a critical role in protection mediated by anti-IsdB mAb in our model. The identity of the critical population of lymphocytes was investigated through passive transfer of isolated lymphocyte sub-populations into SCID mice. The active cells were determined to be T cells ($CD3^+/CD4^+$), whereas B lineage cells ($CD19^+$ or $CD138^hB22^{+/-}CD19^{/-}$) provided little or no protection. This was an unexpected finding, and was confirmed through active vaccination and challenge of B-cell deficient, and T-cell deficient mice. B-cell deficient immunized mice were protected from challenge, whereas T-cell deficient mice were not. These data suggest that IgG and B-cells do not play a primary role in protection mediated by IsdB. We have previously shown that increasing antibody titers after vaccination with IsdB correlate with protection in a murine model of disseminated staph infection.[30] However a side by side evaluation of T-cell responses was not done in those experiments. In light of these data, it is likely that the rising antibody titers were

a consequence of a robust CD4 T helper cell response. In fact, antibody titers have been used as surrogate markers of protection[59] and shown to be elevated during polarized T helper responses.[60] Thus, IsdB vaccine efficacy in this model of disseminated infection was in fact mediated by IL-17A-secreting CD4 T lymphocytes. Additionally, protection mediated by T cells was antigen specific, as demonstrated in SCID mice passively immunized with IsdB specific $CD4^+$ T cells and challenged with the *S. aureus isdB*$^-$/*harA*$^-$ deletion strain. In that case, mice were not protected against challenge. These data confirm that T cell mediated protection was not due to non-specific immune stimulation stimulated by IsdB immune T cells. This experimental result is thus consistent with earlier findings in which Balb/c mice were actively vaccinated with IsdB and were not protected after challenge by the *S. aureus isdB*$^-$/*harA*$^-$ deletion strain.[30]

$CD4^+$ T cells were identified as the effectors for IsdB mediated protection, in the adoptive transfer experiments. These cells were examined for the expression of cytokines to determine which T-helper population was likely mediating protection. Analysis of IsdB immune $CD4^+$ cells revealed minimal or no increase in the frequencies of IFNγ (Th1) and IL-4 (Th2) secreting CD4 T cells upon stimulation with IsdB, whereas IL-17A (Th17) secreting cells made upto 4% of the $CD4^+$ population. These results were confirmed using ELISpot and ICS analysis. To confirm that Th17 cells were important in IsdB mediated efficacy, mice deficient in IL-23 (IL-23p19 KO) were immunized and challenged with *S. aureus*. These mice cannot produce functional IL-23, which is critical in differentiation of Th17 cells.[32] Consequently, protection was not observed in this strain of mice. Additionally, anti-IL-17A mAb eliminated IsdB mediated protection in Balb/c mice ($p = 004$), whereas anti-INFγ mAb had much less effect ($p = 0.48$). Neither mAb affected survival in BSA immunized mice ($p = 0.17$ or $p = 0.18$ respectively). Since IL23p19KO mice are also deficient in the production of IL22, mice were also evaluated for survival post challenge by neutralizing only IL22, but not IL17A, in vivo. Here, upon challenge with *S. aureus* we found no difference in the survival of mice treated with anti IL22 mAb or not treated with the (IL 22) mAb ($p = 0.59$).

Taking the data in total, the protection using the IsdB vaccine in this disseminated lethal challenge model is mediated through a Th17 response, in which IL-17A plays an important role. Neither immune B cells nor immunoglobulin support efficacy. Other vaccine antigens that have been reported to enhance efficacy against lethal *S. aureus* challenge, in a disseminated murine model or kidney infection model, through Th17 stimulation; are the *Candida albicans* adhesin Als3p[14,19] and *S. aureus* fibrinogen binding clumping factor A (Clf-A).[58] Als3p antigen protects mice from both *C. albicans* and *S. aureus* challenge in the absence of B lymphocytes.[14] IsdB differs from Als3p in that antibodies to IsdB, when passively administered, can provide protection from a lethal *S. aureus* challenge.[28,30,56] Data elucidating the underlying protective murine immune responses to these antigens may shed light on the necessary mechanism of adaptive immune response to induce protection to *S. aureus* in humans.[58,61] There are several observations which imply that antibodies may not be sufficient for protection against *S. aureus* infection. For example, antibodies targeted to several *S. aureus* surface antigens have demonstrated preclinical efficacy, including polyclonal anti-capsule type 8 and type 5,[62-64] polyclonal and monoclonal anti- ClfA[65,66] and Fab to an ABC transporter.[67] However, human clinical trials resulted in a disappointing lack of statistically significant efficacy.[68-70] Taken together, the role of vaccine induced Th17 response should be evaluated as a biomarker for vaccine efficacy, along with antibody response, for future vaccine development. IsdB induces both humoral and cellular immune responses which may both contribute to efficacy.

Methods

Ethics statement. All animal work was performed in strict accordance with the recommendations in the Guide for care and use of Laboratory Animals of the National Institutes of Health. The protocol was approved by Institutional Animal Care and Use Committee (IACUC, APS# 10079975730248), Merck Research Labs, West Point, PA.

Bacterial strains: bacteria. Bacteria used in this investigation were as follows: *S. aureus* Becker-MSSA (obtained from Prof Chia Lee, University of Arkansas), *S. aureus* Becker *isdB harA* deletion mutant-MSSA, and SA025-MRSA (Merck clinical isolate).[31] Bacteria were grown on tryptic soy agar (TSA), or tryptic soy broth (TSB) overnight, pelleted and stored as frozen 15% glycerol stocks. For use in experiments, bacteria were thawed, pelleted, and resuspended in the appropriate buffer or medium. Bacterial CFU were quantitated by serial dilution and plating on TSA, with growth overnight at 37°C.

Murine strains. Mice were purchased from Taconic Farms Inc. CB-17 WT and CB-17 SCID mice were used for adoptive transfer studies. The CB-17 strain of mice is genetically similar to Balb/c except for the presence of immunoglobulin heavy chain, Igh-1b allele of the C57BL/Ka strain. These mice were chosen because of the availability of the SCID counterpart on the CB-17 background.[4] Additionally, immunoglobulin heavy chain deleted Jh (Balb/c) and nude (Balb/c) mice from Taconic farms were used for lethal challenges. C57BL/6 mice genetically deficient in IL-23p19 (B6.129-IL23p19^{tm1Dnax}) also called IL-23p19 KO, were obtained from Merck Research Labs, Palo Alto, CA) All animals were housed in a specific-pathogen-free environment and were negative for pathogens in routine screening.

Murine lethal challenge model**:** Active and passive immunization and challenge**.** Active and passive immunization were used to evaluate IsdB-mediated protection in a disseminated lethal challenge model. The challenge model was previously described.[28] Briefly, mice were actively immunized three times with IsdB (20 μg per dose) formulated with amorphous aluminum hydroxyphosphate sulfate adjuvant (AAHSA) or bovine serum albumin (BSA, negative control) formulated with AAHSA. The doses were administered as two 50 μL intramuscular injections on days 0, 7 and 21. The mice were bled on day 28, and sera were screened for reactivity to IsdB by ELISA. On day 35 the mice were challenged with an $LD_{80–90}$ dose of *S. aureus* (from TSA culture, 4.9×10^8 to 8.7×10^8 CFU/ mouse) by intravenous tail vein injection, and survival was monitored for 10 d, as most fatalities were

encountered between 2 and 6 d post challenge. Unless otherwise noted, *S. aureus* strain Becker was used throughout for lethal challenges. In some experiments, 400 ug/mouse of a neutralizing rat anti-mouse IL-17A antibody (JL7.1D10, Merck Research Labs), a neutralizing rat anti-mouse IFN-γ antibody (XMG 1.2, Merck Research Labs), and mouse anti-human IL-22mAb (100% crossreactive with mouse IL-22, R&D Systems, Catalog # 782IL) or isotype control mAb (7D7, Merck Research Labs) were administered i.p. to immunized mice 2 h prior to iv bacterial challenge.[71] For passive IsdB immunization, CB-17 (WT and SCID) mice were injected with 400 μg of IsdB specific mAb (CS-D7) or irrelevant isotype matched control (MK-24) via the i.p. route, 2 h prior to lethal challenge with *S. aureus* SA025, as previously described.[31] Lethal challenge was done via tail vein using SA025 (TSA culture, 1.9×10^8 to 8.7×10^8 CFU) via tail vein injection. Survival was monitored for 10 d post challenge.

ELISA assays. To measure anti-IsdB titers an ELISA was performed as previously described.[30] IL-17A levels in the serum were determined using mouse IL-17A ELISA kit (Invitrogen, Catalog #KMC 3021) as per manufacturer's instructions (data not shown).

ELISpot assays. The gamma interferon (IFN-γ) and IL-17A spot ELISA (ELISpot) assays were performed using kits (Mabtech, Catalog # 3321–2A and 3521–2A respectively). Briefly, peptides spanning the entire length of IsdB were synthesized (JPT PeptideTech) and pooled such that each pool had a final concentration of 1mM. Peptides were synthesized as 15mers with 11 amino acid overlap and covered aa 1–645 of SACOL 1138 (IsdB). Splenocytes (2.5×10^5) were incubated with 10 μM peptide pools, for 18–20 h. Cells were suspended in 100 μl RPMI supplemented with 10% fetal bovine sera (Atlanta Biologicals), 10 mM HEPES, 2 mM glutamine, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1} (P/S) in duplicate wells. Spot-forming cells (SFCs) were detected with a biotin–avidin alkaline phosphatase conjugate, using an immunospot analyzer (Cellular Technology).

Lymphocyte isolation and adoptive transfers. Lymphocyte subsets, namely $CD4^+$ T-cells, $CD8^+$ T-cells, $CD3^+$ T-cells, $CD19^+$ B-cells, and $CD138/B220/CD19^+$ plasma cells were purified from spleens of CB-17 mice using immuno magnetic selection (Miltenyi Biotec) as per the manufacturer's directions. Briefly, cells were labeled with a cocktail of cell surface markers except the marker for the population of interest. The labeled cell population was passed through a magnetic column and the negative fraction (unbound cells) was collected. A small sample from the eluted purified population was labeled with the CD marker of interest, and analyzed on a FACS Calibur flow cytometer (BD Biosciences) to assess the purity(data not shown). 10^7 isolated lymphocytes from IsdB immunized or BSA immunized mice were transferred into each recipient mouse via the tail vein. Lethal i.v. challenge with *S. aureus* was performed 2 h post transfer of lymphocyte subsets.

Cytokine analysis. The cytokine secretion analysis was done using intracellular cytokine staining kits (BD Biosciences, Catalog # 51-2041AK). Briefly, $1–2 \times 10^6$ lymphocytes were incubated with or without peptide pools (10 μg/mL) or with or without intact protein (IsdB, 25 μg/ml) for 2 h. Cells were treated with brefeldinA for an additional 4 h then washed, permeabilized and fixed using the cytofix/cytoperm reagent. PE-conjugated mouse IFN-γ, IL4 and IL-17A mAbs were used to measure cytokine secretion. PE-conjugated isotype control cocktail as well as purified blocking antibody cocktail were used to measure background. Additionally, in some instances cells were also stained with APC labeled anti-CD4 mAb (BD Biosciences, 553051) to determine the frequency of $CD4^+$ $cytokine^+$ cells. Forward- vs. side-scatter profiles were used to define the live splenocyte populations, and gates were set based on antibody isotype controls and cells that stained positive for the protein of interest. Samples were collected on a FACSCalibur flow cytometer (BD Biosciences), and analysis was performed using Treestar Flow Jo (Ashland) software.

Statistical methods. For comparison of survival in the murine lethal challenge experiments individual experiments were analyzed using the Prism software (Prism for Windows, version 5.01, GraphPad Software), and choosing the Log rank, Mantel Cox test statistical method for testing statistical significance. Probability (P) values < 0.05 were considered significant. In some cases, data were pooled from identical experiments for analysis.

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Supplemental Material

Supplemental materials may be downloaded here:
www.landesbioscience.com/journals/vaccines/article/18946

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