

1 **Suppression of the inflammatory immune response prevents the development of chronic**
2 **biofilm infection due to methicillin resistant *Staphylococcus aureus***

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14 **Running Title:** Immune response to *S. aureus* biofilm infections

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16 **Abstract**

17 *Staphylococcus aureus* is a common cause of prosthetic implant infections, which can become
18 chronic due to the ability of *S. aureus* to grow as a biofilm. Little is known about adaptive
19 immune responses to these infections *in vivo*. We hypothesized that *S. aureus* elicits
20 inflammatory Th1/Th17 responses, associated with biofilm formation, instead of protective
21 Th2/Treg responses. We used an adapted mouse model of biofilm-mediated prosthetic implant
22 infection to determine chronic infection rates, Treg frequency, and local cytokine levels in Th1-
23 biased C57BL/6 and Th2-biased BALB/c mice. All C57BL/6 mice developed chronic *S. aureus*
24 implant infection at all timepoints tested. However, over 75% of BALB/c mice spontaneously
25 cleared the infection without adjunctive therapy and demonstrated higher levels of Th2
26 cytokines and anti-inflammatory Tregs. When chronic infection rates in mice deficient in the Th2
27 cytokine, IL-4, via STAT6 mutation in a BALB/c background were assessed, the mice were now
28 unable to clear the *S. aureus* implant infection. Additionally, BALB/c mice depleted of Tregs via
29 anti-CD25 MAb were also unable to clear the infection. In contrast, the C57BL/6 mice that were
30 susceptible to infection were then able to eliminate *S. aureus* biofilm populations on infected
31 intramedullary pins once the Th1 and Th17 responses were diminished by MAb treatment with
32 anti-IL-12 p40. Together, these results indicate that Th2/Treg responses are mechanisms of
33 protection against chronic *S. aureus* implant infection, as opposed to Th1/Th17 responses,
34 which may play a role in the development of chronic infection.

35 **Introduction**

36 Nosocomial infections present a common and costly problem for the U.S. healthcare
37 system (20). *Staphylococcus aureus* is a major cause of such infections worldwide and the
38 prevalence of methicillin-resistant *S. aureus* (MRSA) strains in hospitals has risen steadily over
39 the years, contributing to increased mortality and hospital costs. *S. aureus* is the dominant
40 bacterial species involved in cases of indwelling medical device (IMD) infection, the incidence of
41 which has increased with a rise in the use of IMDs and the implantation of prosthetic joints
42 (2,9,41). When presented with such infections, often times the only treatment option is surgical
43 removal of the infected IMD, leading to significant morbidity and mortality (5,9). The
44 development of a prosthetic implant infection depends on the ability of *S. aureus* to adhere to
45 host proteins coating implants through cell wall-associated adhesins. Subsequent biofilm
46 formation by *S. aureus* makes eradication of this bacteria exceptionally difficult due to the
47 increased resistance of biofilm-embedded bacteria to host defenses (15) and antibiotics (35,37),
48 compared to their planktonic forms.

49 While *in vivo* studies are limited, there is a body of evidence that demonstrates the ability
50 of *S. aureus* to skew the host immune response by influencing cytokine production *in vitro*.
51 Staphylococcal enterotoxin B (SEB) has been shown to induce the production of IL-2 and IFN- γ
52 *in vitro* (3). Staphylococcal enterotoxin A (SEA) induces a Th1 response, associated with the
53 production of TNF- α and MIP-1b (13). Similarly, α -toxin induces IFN- γ and also increases T-bet
54 binding to DNA, which induces a Th1 response (7). Protein A is also a potent inducer of IFN- γ ,
55 TNF- α and IL-1, all cytokines associated with the Th1 response (46). This skewing, in turn,
56 influences the propensity of *S. aureus* to progress from an acute infection to a biofilm infection
57 that is chronic. During the early stages of an *S. aureus* infection, host innate immune cells, such
58 as monocytes, produce a number of pro-inflammatory cytokines, including IL-1, IL-6, IL-12 p70,
59 IL-18 and TNF- α (8,43). This cytokine milieu drives pro-inflammatory Th1 and Th17 CD4⁺ T
60 helper cells responses that can result in substantial damage to host tissues. *S. aureus* also

61 induces IL-12, IFN- γ and TNF- α production in CD4⁺ T cells, which further provokes a Th1
62 response (25). Unfortunately, Th1 responses may be ineffectual at clearing *S. aureus* in the low
63 oxygen partial pressure found deep within a biofilm (43). Th1 polarizing factors such as IL-12
64 and T-bet also serve to down-regulate the anti-inflammatory Th2 humoral immune response by
65 blocking IL-4 expression (38). This down-regulation may be detrimental to the host during IMD
66 infection, as previous studies have indicated that Th2 responses are effective at clearing biofilm
67 infections during the early stages of biofilm development (44) as well as at clearing a
68 subcutaneous infection in BALB/c mice (34). In addition, there is limited evidence that suggests
69 that Th17 responses contribute to chronic infection (4), whereas Treg responses may limit
70 catheter-related infections (19), indicating a role for these T helper cell lineages in other biofilm-
71 mediated infections, such as those associated with prosthetic implants.

72 Previous studies from our lab utilized a murine model of *S. aureus* biofilm-mediated
73 prosthetic implant infection that closely mimics key characteristics of similar infections in
74 humans: The resulting infection was chronic, localized, and recalcitrant to treatment with
75 vancomycin, despite its effectiveness at killing planktonic bacteria of the same strain. These
76 studies also demonstrated that *S. aureus* implant infections in C57BL/6 mice elicited mainly Th1
77 and Th17 cytokines and down-regulated Tregs during early stages of implant infection (36).
78 Since this inflammatory response failed to clear the infection, augmented Th2 and Treg
79 responses may play a protective role against the development of chronic *S. aureus* implant
80 infection through their ability to suppress the pro-inflammatory effects of the Th1 (38) and Th17
81 (44) responses.

82 In this present study, we sought to elucidate the mechanisms of protection against *S.*
83 *aureus* biofilm-mediated implant infection using a mouse model of chronic *S. aureus* implant
84 infection (36) in Th1 and Th2 biased mice (11). After implantation with a pin coated with
85 adherent *S. aureus*, C57BL/6 mice could not clear the infection while BALB/c mice did and
86 possessed higher levels of IL-4 and IL-10 cytokines and Treg populations compared to C57BL/6

87 mice. This suggested protective roles for the Th2 response and reduced inflammation in
88 staphylococcal clearance. In order to modify the host immune response in the C57BL/6 mice,
89 neutralizing MAbs against inflammatory cytokines IL-6 or IL-12 p40 enabled this otherwise
90 susceptible mouse strain to clear the infection. Conversely, by reducing the anti-inflammatory
91 Treg population with anti-CD25 MAb, the infection-resistant BALB/c mice developed chronic *S.*
92 *aureus* biofilm disease. Additionally, STAT6 KO mice on a BALB/c background, which are
93 deficient in Th2 responses, are not protected from chronic *S. aureus* biofilm infection. This study
94 demonstrated that not only is the inflammatory immune response detrimental to the host in both
95 the clearance and prevention of chronic biofilm infection by *S. aureus*, but that a functional Th2
96 response is necessary for resolution of the infection.

97 Understanding the mechanisms behind the development of chronic *S. aureus* implant
98 infection mice will further our understanding of how host responses to chronic *S. aureus* implant
99 infections affect the development and clearance of *in vivo* biofilm infections. Also, one will gain
100 insight into how the host response may be manipulated by therapeutic agents to improve the
101 chances of successfully preventing and treating these infections clinically.

102

103 **Materials and Methods:**

104 *Mice:* Inbred C57BL/6 and BALB/c mice (6-8 weeks old) were purchased from Jackson
105 Laboratories (Bar Harbor, ME). STAT6 KO mice on a BALB/c background were bred in house.
106 Mice were maintained under micro-isolator conditions in the animal facilities at the University of
107 Maryland School of Medicine and the University of Maryland Dental School (Baltimore, MD), in
108 accordance with protocols reviewed and approved by the *Institutional Animal Care and Use*
109 *Committee* (IACUC) at the University of Maryland School of Medicine (Baltimore, MD).

110

111 *Bacterial strain and prep of implants:* The strain of *S. aureus* used in these experiments,
112 designated MRSA-M2, is a clinical isolate obtained from an osteomyelitis patient undergoing

113 treatment at the University of Texas Medical Branch (Galveston, TX), and has been used in a
114 number of biofilm infection models and molecular studies (6,24,36). This strain is a ST30, spa
115 type T019, and agr III strain. Autoclaved 0.25-mm insect pins (Fine Science Tools, Foster City,
116 CA) were incubated for two h in 10 ml of an overnight culture of *S. aureus* that was diluted 1:100
117 in sterile trypticase soy broth, with sufficient time and continuous flow of bacterial culture media
118 to mimic physiological conditions that favor biofilm formation (10) (36). The inoculating dose of
119 *S. aureus* was determined to be 3.0×10^5 CFU/pin (SD = 5×10^4).

120

121 *Surgical implantation:* Six to ten mice per experimental group received tibial implants. Mice were
122 anesthetized via i.p. injection of 100 mg/kg ketamine (Ketaset® - Fort Dodge Laboratories, Inc.,
123 Fort Dodge, Iowa) and 10 mg/kg xylazine (Rugby Laboratories, Inc., Rockville Center, NY). The
124 left leg of each mouse was cleansed with povidone iodine and rinsed with 70% ethanol before
125 surgical implantation of an *S. aureus*-coated pin, according to the methods previously described
126 (26) (36). Mice did not undergo any additional treatments after surgery until sacrifice, except for
127 mice receiving immunomodulatory antibodies (described below). All animal experiments were
128 performed in accordance with protocols reviewed and approved by the *Institutional Animal Care*
129 *and Use Committee* (IACUC) at the University of Maryland School of Medicine (Baltimore, MD).

130

131 *Bone cultures:* At 7, 14, 21, 28, and 49 d post-implantation, infected and uninfected BALB/c and
132 C57BL/6 mice were euthanized. Left tibiae were removed, cut into small pieces and placed in
133 300 μ l of sterile 0.85% saline per 100 μ g of bone. As previously described, bones were
134 homogenized using a Polytron PT 1200 handheld homogenizer (Kinematica, Bohemia, NY) at
135 25,000 rpm and serial 10-fold dilutions of bone homogenates were plated on sheep's blood agar
136 plates to enumerate viable *S. aureus* per g bone (16,36). At 21 d post-implantation, infected
137 BALB/c mice and STAT 6 KO mice were euthanized and left tibiae were processed and plated
138 as described above.

139

140 *Peptide Nucleic Acid-Fluorescent in situ Hybridization (PNA-FISH)*. At d 21 post-implantation,
141 pins were carefully removed from the tibiae of infected and uninfected mice to prevent
142 perturbation of biofilm mass. Pins were placed in eppendorf tubes and fixed in 2%
143 paraformaldehyde (PFA) in PBS before PNA-FISH hybridization with a FITC-labeled universal
144 bacterial probe and a rhodamine-labeled universal eukaryotic cell probe, specific for bacterial
145 and eukaryotic rRNA, respectively, as per manufacturer's instructions (Advandx, Woburn, MA).
146 Each pin was then examined with a Zeiss LSM 510 confocal scanning laser microscope (Carl
147 Zeiss, Thornwood, NY) for both green and red fluorescence using a FITC/Texas Red dual-band
148 filter and a 63X objective.

149

150 *Cytokine production assay*: To compare levels of various Th1, Th2 and Th17 cytokines at the
151 implant site, implanted tibiae were harvested from C57BL/6 and BALB/c mice at d 7 post-
152 implantation and stored at -70°C. Samples were homogenized on ice in sterile PBS containing
153 an EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Tissue
154 homogenates were centrifuged for 15 minutes at 14,000 xg at 4°C and supernatants were
155 analyzed by Ms. Lisa Hester at the Cytokine Core Laboratory at the University of Maryland
156 School of Medicine (Baltimore, MD) using quantitative sandwich ELISA. Cytokines tested
157 included murine IL-1 β , IL-2, IL-4, IL-6, IL-10, IFN- γ , and TNF- α .

158

159 *Treg frequency*: To compare Treg frequency between C57BL/6 and BALB/c mice, 1×10^6
160 draining LN cells were aliquoted into FACS tubes (Becton Dickinson, Bedford, MA) and a Treg
161 FACS staining kit (eBioscience, San Diego, CA) was used to determine the frequency of Tregs
162 in draining LN cells. Cells were surface stained with FITC-labeled anti-mouse CD4 and PE-
163 labeled anti-mouse CD25 MAbs, and stained intracellularly with PE-Cy5-labeled anti-mouse
164 Foxp3 MAb in accordance with the manufacturer's protocol. Results were expressed as the

165 percentage of Foxp3⁺CD25⁺CD4⁺ T cells in C57BL/6 versus BALB/c mice. Cells were analyzed
166 using an LSR II flow cytometer (BD Biosciences, San Jose, CA).

167

168 *In vivo Treg neutralization:* To determine if Tregs play a role in bacterial clearance, BALB/c mice
169 were injected i.p. with 1.0 mg of the anti-CD25 MAb, PC61.5.3 (BioXCell, West Lebanon, NH),
170 to neutralize natural Tregs *in vivo*. Control mice received i.p. injections of rat IgG1. MAbs were
171 injected two hours before surgical implantation of an *S. aureus*-coated pin into the left tibia, as
172 previous described. Mice received subsequent i.p. injections of 1.0 mg MAb at d 7 and 14 post-
173 implantation to maintain reduced Treg frequencies for the duration of the experiment. Mice were
174 euthanized at d 21 post-implantation, and tibiae were harvested, processed, and serial dilutions
175 of bone homogenates plated on blood agar plates to enumerate CFUs/g bone, as previously
176 described.

177

178 *Histology and Immunohistochemistry:* Tibiae from infected and uninfected control C57BL/6 and
179 infected BALB/c mice were evaluated by histology and immunohistochemistry in order to
180 observe differences between the histology and infiltrating neutrophils. Tibiae were removed at 7
181 d post-implantation from each mouse strain and fixed in formalin. Tibiae were then decalcified
182 and paraffin embedded sections were stained with hematoxylin and eosin. Additional paraffin
183 embedded sections were immunostained using the anti-Ly-6g MAb, Gr-1 (eBioscience, San
184 Diego, CA), to detect neutrophil infiltration into the implant site. A peroxidase-conjugated
185 secondary Ab and colorimetric substrate was used to visualize labeled cells. All tissue sections
186 were counterstained with hematoxylin. Each section was examined using an Omega 4000 light
187 microscope (Alpha and Omega Microscopes, Gaithersburg, MD) with a 10X objective. All tissue
188 processing and staining was performed by Ms. Elizabeth Smith at the University of Maryland,
189 Baltimore Center for Vascular and Inflammatory Disease Histology Core.

190

191 *Immunomodulation:* To determine if neutralization of Th17 and/or Th1 responses increased
192 rates of bacterial clearance, C57BL/6 mice were injected i.p. with 1.0 mg of the anti-IL-6 MAb,
193 MP5-20F3, or the anti-IL12 p40 MAb, C17.8, (BioXCell, West Lebanon, NH) to neutralize the
194 effects of these cytokines on the production of Th17 and Th1/Th17 responses *in vivo*,
195 respectively. Control mice received i.p. injections of either rat IgG1 or rat IgG2a isotype controls
196 Abs or no additional treatment. MAbs were injected two hours before surgical implantation of an
197 *S. aureus*-coated pin into the left tibia, as previous described. Mice received subsequent i.p.
198 injections of 1.0 mg MAb at d 7 and 14 post-implantation to maintain the neutralization of IL-6
199 and IL-12 for the duration of the experiment. Mice were euthanized at d 21 post-implantation,
200 and tibiae were harvested, processed, and serial dilutions of bone homogenates plated on blood
201 agar plates to enumerate CFUs/g bone, as previously described.

202

203 *Statistical analysis.* Mean and SD were calculated and analyzed using Student's *t*-test with a *p*
204 value of <0.05 to determine statistical significance. Experiments determining the percentage of
205 mice still infected at various time points post-implantation were analyzed using Fishers Exact
206 test with a *p* value of <0.05 for statistical significance.

207

208 **Results:**

209

210 *Chronic S. aureus implant infection is less prevalent in BALB/c versus C57BL/6 mice.* Tibiae
211 from Th2-biased BALB/c and Th1-biased C57BL/6 mice receiving implants of *S. aureus*-coated
212 pins were harvested and processed at d 7, 14, 21, 28, and 49 post-infection. Viable *S. aureus*
213 was cultured from homogenized bone tissue, as previously described. 100% of C57BL/6 mice
214 were still infected at all post-infection time points tested. Initially, at d 7 and 14 post-
215 implantation, there was no significant difference in the percentage of infected BALB/c and
216 C57BL/6 mice (Fig. 1a). However, the percentage of BALB/c mice infected at d 21 post-infection
217 dropped significantly to 41.67%. By d 49, there was an even more dramatic decrease in
218 infection levels, with viable *S. aureus* cultured from only 25% of BALB/c tibiae (Fig. 1a). Biofilm
219 formation was not evident on implanted pins removed from uninfected C57BL/6 mice receiving
220 sterile control pins (Fig. 1b), as determined by confocal scanning laser microscopy.
221 Interestingly, biofilm formation was also not evident on implanted pins removed from any of the
222 infected BALB/c mice examined (Fig. 1d). This was in sharp contrast to implanted pins removed
223 from infected C57BL/6 mice, which clearly demonstrate *S. aureus* biofilm formation on the pin
224 surface (Fig. 1c).

225

226 *S. aureus implant infection results in increased Th2 cytokines in BALB/c but not C57BL/6 mice.*

227 Cytokine levels were compared between infected BALB/c and C57BL/6 mice in order to
228 determine the character of the immune response that may be responsible for the differential
229 susceptibility to *S. aureus* biofilm infection observed. At d 7 post-implantation, tibiae were
230 removed from infected BALB/c and C57BL/6 mice, homogenized, and supernatants of bone
231 homogenates were then analyzed for cytokine production using quantitative sandwich ELISA.
232 Results indicate that both mouse strains produce at least some level of all of the Th1, Th2 and
233 Th17 cytokines tested, including IL-1 β , IL-2, IL-4, IL-6, IL-10, IFN- γ , and TNF- α . However, the
234 major Th2 cytokines, IL-4 and IL-10, were significantly higher in infected BALB/c compared to

235 C57BL/6 mice (Fig. 2), indicating a more robust Th2 response in the BALB/c mouse strain.
236 These data support the hypothesis that a host Th2 response plays a protective role against the
237 development of chronic *S. aureus* implant infection.

238

239 *S. aureus* implant infection results in chronic infection in STAT 6 KO BALB/c but not wt BALB/c
240 mice. Previous studies from our lab demonstrated that BALB/c mice are less susceptible to the
241 development of chronic *S. aureus* implant infection than C57BL/6 mice. In order to determine if
242 this difference in disease outcome is due to the production of an effective Th2 response, STAT6
243 KO mice, which lack a key component of the signalling pathway required to mount a Th2
244 response, wt BALB/c, and STAT6 KO mice were implanted with *S. aureus*-coated pins. Tibiae
245 from both wt and KO mice were harvested and processed on d 21 post-implantation and viable
246 *S. aureus* was cultured from homogenized bone tissue. Results indicate that STAT6 KO mice
247 have lost the ability clear an *S. aureus* implant infection before chronic disease develops, as
248 viable *S. aureus* can be cultured from the tibiae in 100% of these mice at d 21 post-implantation,
249 in contrast to only 42.1% of wt BALB/c mice (Fig. 3). These data further support our hypothesis
250 that a Th2 response is protective against chronic *S. aureus* implant infection. Although 100% of
251 the STAT6 KO mice were still infected at d 21, this mutant mouse strain had similar CFU counts
252 in the tibiae at d 21 to wt BALB/c mice and 100-fold lower CFU counts compared to C57BL/6
253 mice, although these numbers were not statistically significant (data not shown). This may
254 indicate a possible compensatory mechanism in STAT6 KO mice, such as higher Treg levels, or
255 suggest that the robust inflammatory Th1 and Th17 responses observed in C57BL/6 mice may
256 further exacerbate the severity of the infection.

257

258 *Tregs protect BALB/c mice from developing chronic infection after implantation of an S. aureus-*
259 *coated pin.* The role that Tregs play in protecting BALB/c mice from the development of chronic
260 implant infection was evaluated by determining the relative population levels of this T cell subset

261 in each mouse strain. Briefly, draining LN from infected C57BL/6 and BALB/c mice were
262 harvested at 7 and 21 d post-infection and single cell suspensions of LN cells were prepared.
263 LN cells were FACS surface stained for CD4 and CD25 and for intracellular FoxP3. Results
264 indicate that the percentage of CD4⁺CD25⁺ T cells expressing Foxp3 is significantly lower at
265 both d 7 and 21 post-implantation in C57BL/6 versus BALB/c mice (Fig. 4a). To further evaluate
266 the protective role of Tregs in BALB/c mice, tibiae from infected BALB/c receiving i.p. injections
267 of anti-CD25 MAb to neutralize Tregs *in vivo*, or an isotype control were harvested and
268 processed at d 21 post-infection. Viable *S. aureus* was cultured from homogenized bone tissue,
269 as previously described. There was a significant difference between BALB/c mice receiving anti-
270 CD25 MAb and mice receiving isotype control. When injected with rat IgG1, approximately 41%
271 of BALB/c mice were still infected at d 21 post-implantation, which is consistent with data from
272 previous studies on untreated BALB/c mice. In contrast, the percentage of BALB/c mice still
273 infected at d 21 post-implantation after treatment with anti-CD25 MAb increased to 87.5% (Fig.
274 4b).

275
276 *Implantation of an S. aureus-coated pin leads to increased neutrophil infiltration at the implant*
277 *site in C57BL/6, but not BALB/c mice at d 7 post-implantation:* Because down-regulation of Tregs
278 results in increased infection rates, it was hypothesized that the Tregs may promote the delay in
279 neutrophil infiltration into areas of localized staphylococcal infection. In order to observe the
280 neutrophil invasion early in the infection in the two mouse strains, paraffin-embedded tibia
281 sections from infected C57BL/6 mice and infected BALB/c mice were stained with hematoxylin
282 and eosin (H&E) and compared to tibial sections from uninfected C57BL/6 mice. A separate set
283 of sections were immunostained with an anti-Ly-6g MAb to visualize neutrophil infiltration, a key
284 indicator of inflammation and an active Th17 response. Results demonstrate the infiltration of
285 large numbers of neutrophils to the implant site of C57BL/6 mice, as indicated by the large
286 number of PMNs observed in hematoxylin and eosin-stained images (Fig. 5a) and also visible in

287 IHC images as dark brown cells (Fig. 5b). A large area of involucrum formation is also clearly
288 observable, and is a result of bone remodelling resulting from chronic infection and inflammation
289 at the implant site. In contrast, results from BALB/c mice indicate a lack of both neutrophil
290 infiltration and involucrum formation at the implant site (Fig. 5c and 5d), which instead resemble
291 the implant site of C57BL/6 mice receiving sterile implants (Fig. 5e and 5f). Naked eye
292 observation of tibiae from infected BALB/c mice also suggests decreased neutrophil infiltration
293 and inflammation, as indicated by a lack of involucrum formation and pus compared to tibiae
294 from infected C57BL/6 mice (data not shown).

295
296
297

298 *Treatment of C57BL/6 mice with anti-IL-12 p40, but not anti-IL6 MAb results in decreased levels*
299 *of chronic infection.* To determine the roles of pro-inflammatory Th1 and Th17 responses in
300 exacerbation of chronic biofilm infection and the potential to control chronic infection by curbing
301 these responses, C57BL/6 mice were treated pre-surgically and for the duration of the
302 experiment with either anti-IL-6 or IL-12 p40 MAbs to neutralize Th17 or both Th1/Th17
303 responses, respectively. Treatment with these cytokine-neutralizing MAbs *in vivo* also allowed
304 the simultaneous evaluation of these products as potential immunomodulatory agents. Control
305 mice were treated with either rat IgG1 or rat IgG2a as isotype controls. Viable *S. aureus* was
306 cultured from homogenized bone tissue, as previously described, at d 21 post-infection. With
307 anti-IL-6 treatment, 85.7% of mice were still infected by d 21 compared to either untreated mice
308 or mice treated with isotype control where 100% were still infected, although this difference was
309 not significant (Fig. 6a). In contrast, treatment of mice with anti-IL-12 p40 Ab resulted in a
310 significant reduction in the percentage of C57BL/6 mice still infected at d 21 compared to
311 untreated mice, from 100% for untreated mice to 62.5% for mice treated with anti-IL-12 MAb
312 (Fig 6b). Untreated mice and mice treated with isotype control in both experiments showed
313 comparable levels of infection, 100%.

314

315 **Discussion:**

316

317 Biofilm-mediated *S. aureus* infection is a costly and traumatic complication for patients
318 receiving indwelling medical devices (2,41). Growth in the biofilm state allows *S. aureus* to
319 successfully evade the host immune response and establish a chronic infection that is
320 recalcitrant to clearance (12). In this present study, significantly lower numbers of Th2 biased
321 BALB/c mice were shown to progress from acute to chronic *S. aureus* infection, as indicated by
322 the 75% infection clearance rate in these mice whereas none of the Th1 biased C57BL/6 mice
323 were able to accomplish microbial eradication. BALB/c mice were shown to produce a more
324 robust Th2 cytokine response, a higher frequency of Tregs, and reduced neutrophil infiltration
325 following *S. aureus* implant infection compared to C57BL/6 mice. By disrupting the Th2 pathway
326 or reducing Treg numbers, BALB/c mice are no longer able to clear this chronic infection. In
327 contrast, C57BL/6 mice showed improved bacterial clearance after treatment with anti-IL-12 p40
328 MAb, which neutralizes both Th1 and Th17 inflammatory responses *in vivo*.

329 Initially, at d 7 and 14 post-implantation, the percentage of infected BALB/c mice did not
330 differ from the percentage of infected C57BL/6 mice (Fig. 1a). However, the percentage of
331 BALB/c mice infected at d 21 and d 49 post-infection dropped significantly (Fig 1a). In addition,
332 biofilm formation was not evident on implanted pins removed from infected BALB/c mice (Fig.
333 1d), as determined by confocal scanning laser microscopy. This was in contrast to implanted
334 pins removed from infected C57BL/6 mice, which demonstrate *S. aureus* biofilm formation on
335 the pin surface (Fig. 1c).

336 The cytokine profile at the implant site of infected BALB/c mice was compared to that of
337 infected C57BL/6 mice to determine if cytokine production patterns were different between
338 these two strains. At d 7 post-implantation, both C57BL/6 and BALB/c mice showed production
339 of Th1, Th2, and Th17-associated cytokines, including IL-1 β , IL-2, IL-4, IL-6, IL-10, IFN- γ , and
340 TNF- α (Fig. 2). This is not entirely unexpected; BALB/c and C57BL/6 are both wild-type mouse
341 strains capable of mounting all three types of T helper immune responses, and many types of

342 infections elicit a combination of these responses. While most of these cytokines were produced
343 at statistically similar levels, BALB/c mice produced statistically significantly higher levels of the
344 Th2 cytokines IL-4 and IL-10 compared to C57BL/6 mice (Fig 2), cytokines which are important
345 in the initiation and maintenance of a Th2 response. IL-4 is the main inducer of the Th2
346 response and simultaneously inhibits the initiation of a pro-inflammatory Th1 response (17). IL-
347 10 inhibits both Th1 and Th17 responses through the inhibition of IFN- γ , IL-12, IL-23, and IL-6
348 (49). These data demonstrate that BALB/c mice that are effective at clearing chronic infections
349 have a Th2 biased response. However, once this Th2 response is diminished, BALB/c mice are
350 no longer able to clear the infection, as demonstrated by our studies utilizing a STAT6 KO
351 mouse strain (17,42) (Fig. 3).

352 While a functional Th2 response was required for clearance, a Treg response might also
353 play a role. Tregs are a key part of the adaptive immune response, regulating potentially
354 detrimental inflammatory immune responses (47). Previous studies from our lab demonstrated
355 that Tregs are down-regulated at d 7 during *S. aureus* implant infection in infected C57BL/6
356 mice. Therefore, this down-regulation may enable the inflammatory response to progress
357 unabated and contribute to the development of a chronic infection (36). To determine if Tregs
358 aid in protection against chronic *S. aureus* implant infection in BALB/c mice versus C57BL/6
359 mice, draining LN cells from infected C57BL/6 and BALB/c mice were analyzed for Foxp3
360 expression by FACS at d 7 and 21 post-implantation. At both d 7 and d 21 post-implantation,
361 draining LN from BALB/c mice contained a higher percentage of Foxp3 expressing CD4⁺ T cells
362 compared to LN from C57BL/6 mice (Fig.4a). In order to determine if these cells play a
363 functional role in disease protection, BALB/c mice were treated with the anti-CD25 MAb PC61
364 or isotype control to neutralize Tregs *in vivo* before implantation of an *S. aureus*-coated pin and
365 throughout the duration of the experiment. Results demonstrated that at d 21 BALB/c mice
366 treated with anti-CD25 lost the ability to effectively clear an *S. aureus* implant infection before
367 progression to chronic disease. Only 41% of infected BALB/c mice control group were still

368 infected at d 21 post-implantation, compared to 87% of BALB/c mice after anti-CD25 treatment
369 (Fig. 4b). These data provide evidence that Tregs play a protective role against the
370 development of chronic *S. aureus* implant infection. These data further suggest that promoting
371 Treg down-regulation during the early stages of implant infection may be a strategy employed
372 by *S. aureus* to thwart the host immune response in order to form a biofilm. It is possible that
373 Tregs contribute to the prevention of chronic *S. aureus* biofilm-mediated infection in BALB/c
374 mice by curbing Th1 and Th17 responses, both of which have been implicated in heightened
375 inflammation and chronic infection. Pro-inflammatory cytokines and lysed neutrophils damage
376 local host tissues and can lead to decreased vascular sufficiency and an increased amount of
377 devitalized bone and tissue available for *S. aureus* attachment and biofilm development at the
378 infection site (25).

379 In addition to the deleterious effects of Treg down-regulation, Th17 activation may also
380 have a role in promoting the mobilization of neutrophils, and play an important role in bridging
381 innate and adaptive immunity (48). Although the Th17 response is important in preventing life-
382 threatening bacterial infections, such as infections of the lung and bacterial sepsis, Th17
383 responses have limited effectiveness in long-term protection from bacterial infections (32).
384 Neutrophils are limited in their ability to clear biofilm-embedded *S. aureus*, and tissue damage
385 resulting from pro-inflammatory cytokines, reactive oxygen species and lysosomal enzymes that
386 are released upon neutrophil lysis and death, can lead to localized vascular insufficiency and a
387 predisposition towards biofilm development and chronic infection (25). Cytokine data from
388 previous studies in our lab indicated the elevated production of Th17 cytokines in C57BL/7 mice
389 receiving *S. aureus*-coated implants (36). To determine if neutrophil infiltration at the implant
390 site is involved in chronic infection, we performed immunohistochemical analysis of paraffin-
391 embedded tibial sections removed from infected and uninfected C57BL/6 and infected BALB/c
392 mice at d 21 post-implantation. Immunostaining tibial sections with a MAb directed against the
393 neutrophil marker Ly-6g demonstrated a large influx of neutrophils into the implant site of

394 C57BL/6 mice, which also showed a large area of involucrum formation (Fig. 5a and 5b). This
395 was in contrast to the implant site of BALB/c mice (Fig 5c and 5d), which closely resembled that
396 of uninfected control mice (Fig 5e and 5f), in terms of low neutrophil numbers and a lack of
397 involucrum formation. These results suggest that long-term neutrophil infiltration and activation
398 are associated with biofilm formation and chronic disease.

399 Data from this study and previous studies in our lab have suggested that Th1 and Th17
400 responses are associated with chronic *S. aureus* implant infection, and may play a detrimental
401 role for the host (36). We sought to neutralize these responses *in vivo* not only to definitively
402 demonstrate that these responses are mechanisms of disease progression and exacerbation,
403 but to also demonstrate that targeted neutralization of these responses can potentially be useful
404 therapeutically in the prevention of chronic *S. aureus* implant infection. To do this, C57BL/6
405 mice were treated with the anti-IL-6 MAb, MP5-20F3, the anti-IL-12 p40 MAb, C17.8, or isotype
406 control Abs. Treatment with anti-IL-6 MAb resulted in a slight decrease in the percentage of
407 C57BL/6 mice still infected at d 21 post-implantation, but neutralization of IL-6 alone did not
408 result in significantly reduced infection rates (Fig. 6a). IL-6 is required for the differentiation of
409 the Th17 lineage, so it is possible that even though Th17 may play a detrimental role in biofilm
410 infection, targeting this response alone is not enough to curb the incidence of chronic infection.
411 In contrast, C57BL/6 mice treated with anti-IL-12 p40 MAb that reduces both Th1 and Th17
412 responses did show a significant reduction in the percentage of mice still infected at d 21 post-
413 infection (Fig. 6b). IL-12 p40 is shared by both IL-12 and IL-23. IL-12 is a polarizing cytokine
414 that induces the Th1 response, and IL-23 is required for the maintenance of Th17 cells (32).
415 Because neutralization of IL-12 p40 resulted in significantly lower infection rates, it is possible
416 that there is a cumulative effect that results from the combination of Th1 and Th17 responses,
417 both of which may be required to exacerbate chronic infection. Th1 and Th17 responses both
418 lead to the release of a myriad of pro-inflammatory cytokines and migration and activation of
419 neutrophils (8,25,51). Therefore, by suppressing these inflammatory host responses, conditions

420 that promote biofilm formation in the host are limited and *S. aureus* may be less able to develop
421 into a chronic biofilm infection.

422 These data also indicate that neutralization of IL-12 p40 may be used prophylactically to
423 prevent chronic implant infection in patients before surgical implantation of an indwelling
424 medical device. The administration of intravenous anti-IL-12 p40 MAb has been shown in
425 clinical trials to be generally well-tolerated by patients (22), making anti-IL-12 p40 treatment a
426 realistic option for patients in the future.

427 The deleterious effects of Th1 and Th17 in this particular model and the contribution to
428 development of a chronic biofilm infection runs in stark contrast to a number of other studies.
429 For example, cell-mediated and innate immune responses have been shown to play vital roles
430 in conferring protection in several non-biofilm infection models (1,18,23,27,28,33), including Th2
431 responses not associated with biofilm formation (21). Also, in other studies, neutrophils are the
432 dominant cell type elicited during *S. aureus* infection and that they play a key role in protection
433 against non-biofilm infections (31,39,40). Even in acute superficial biofilm infection model (50),
434 attenuation of the inflammatory response contributes to persistence.

435 These differences between the studies can be explained by differences in types of
436 infections (biofilm and chronic versus planktonic and acute) as well as the immunological
437 properties in the various compartments of the host where the infection occurs. For those
438 infections within or just below the skin as well as non-biofilm septic infections, a robust innate
439 and adaptive inflammatory response seems necessary for clearance. However, in other
440 locations such as the intramedullary bone infection model in mice (36) cases of osteomyelitis
441 (29), endocarditis (45), and endophthalmitis (14), robust response walls the infection off to
442 prevent systemic spread but also forms a nidus of infection that promotes chronic and
443 progressive tissue damage and occasional escape and seeding of other areas. Also, even in
444 cases such as osteomyelitis without an indwelling medical device, the early acute inflammation

445 may also produce the devitalized tissue necessarily needed by *S. aureus* to attach and form
446 biofilms and a chronic infection (30).

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448 Once *S. aureus* has colonized an implant and chronic infection has developed, the only
449 treatment option available is removal of the infected implant, a costly and traumatic procedure.
450 Understanding how host adaptive immune responses contribute to *S. aureus* biofilm-mediated
451 implant infection may lead to the development of better strategies employed by clinicians to
452 treat and prevent these types of infections. The results of this study not only contribute to a
453 greater understanding of the role of host immunity in the development of biofilm infections, but
454 these findings also suggest a new form of adjuvant therapies that may promote effective
455 clearance of *S. aureus* biofilm infections, either alone or in combination with antimicrobial
456 therapy and vaccination strategies.

457

458 **Acknowledgments**

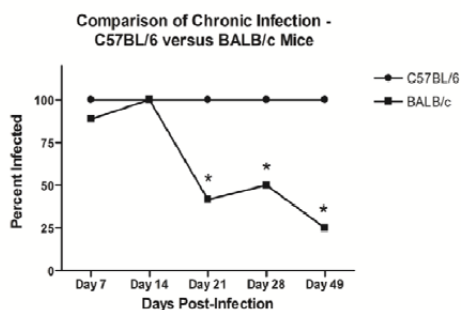
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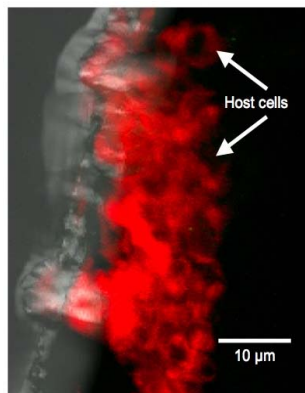
469 **Figure 1:**

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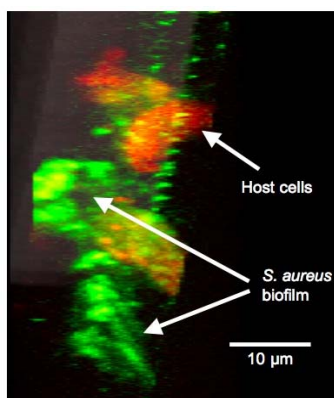
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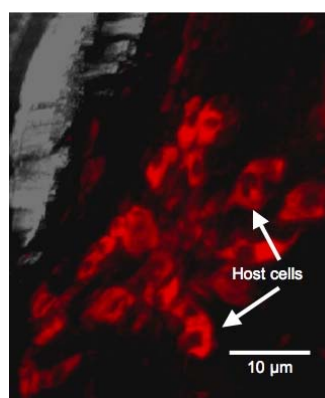
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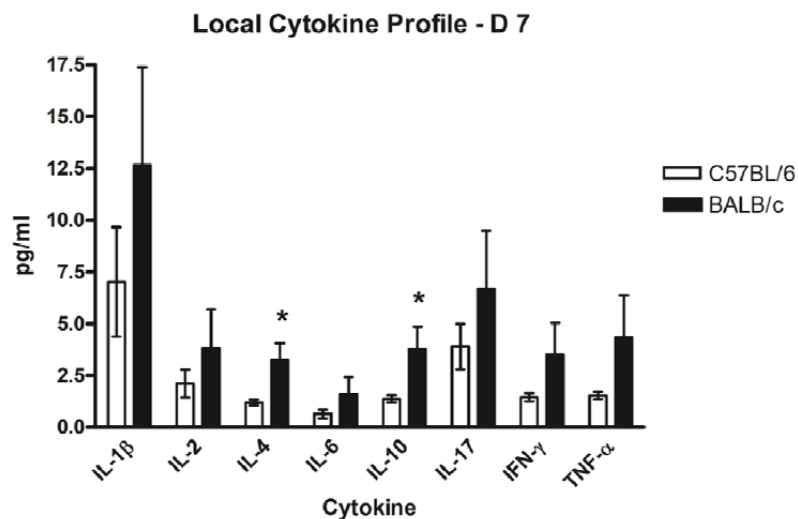


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478 **Figure 1.** Comparison of chronic, biofilm-mediated infection between C57BL/6 and BALB/c
 479 mice. (A) Percent of C57BL/6 versus BALB/c mice still infected at 7, 14, 21, 28, and 49 d after
 480 receiving an *S. aureus*-coated pin. The host immune response was unable to clear the infection
 481 in any C57BL/6 mice, in which 100% of the mice were still infected at all time points. In contrast,
 482 41.67%, 50.00%, and 25.00% of BALB/c mice were still infected at d 21, 28 and 49,
 483 respectively. Confocal scanning laser microscopic images of pins removed at d 21 post-
 484 implantation from C57BL/6 mice receiving either sterile pins (B) or *S. aureus*-coated pins (C), or
 485 BALB/c mice receiving *S. aureus*-coated pins (D) and labeled using a FITC-labeled universal
 486 bacterial probe and a rhodamine-labeled universal eukaryotic cell probe, specific for bacterial
 487 and eukaryotic rRNA, respectively. In contrast to C57BL/6 mice, biofilm formation is not evident
 488 on the pin removed from the infected BALB/c mouse. (n= 5-8 mice per group, experiments
 489 performed in triplicate, * denotes $p < 0.05$ BALB/c compared to C57BL/6 by Fishers exact test).

490 **Figure 2:**

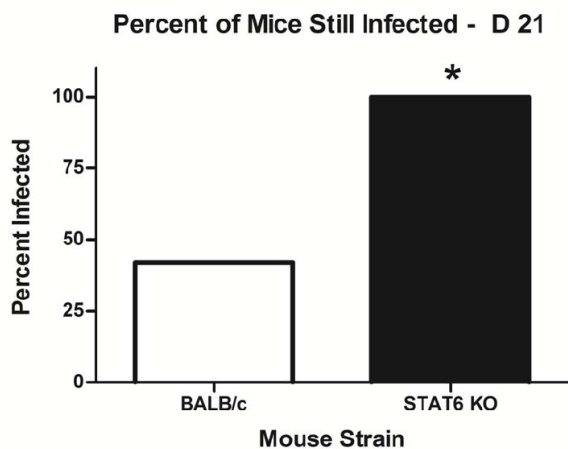


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Figure 2. Local cytokine profile at implant site. Tibiae were removed from C57BL/6 and BALB/c receiving *S. aureus*-coated pins. Supernatants from bone homogenates were analyzed for cytokine production at d 7 post-implantation as described in Materials and Methods. Significant up-regulation of IL-4 and IL-10 in BALB/c mice indicate a predominantly Th2 type response in this mouse strain, suggesting a role in protection from chronic implant infection (n= 5-8 mice per group, experiments performed in triplicate, * denotes $p < 0.05$ compared to controls by Student's *t*-test). Bars represents SEM.

503 **Figure 3:**

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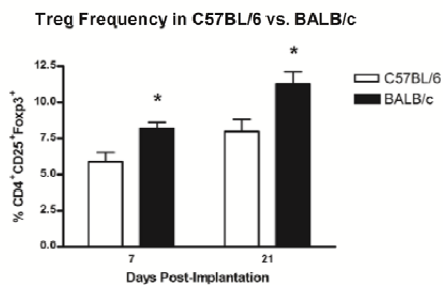
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Figure 3. Comparison of chronic, biofilm-mediated infection between BALB/c and STAT6 KO mice. Tibiae from BALB/c and STAT6 KO mice receiving *S. aureus*-coated implants were removed at d 21 post-implantation and serial dilutions of bone homogenates were plated on blood agar plates. The host immune response was unable to effectively clear the infection in STAT6 KO mice, of which 100% were still infected at d 21 post-implantation. In contrast, only 42.1% of BALB/c mice were still infected at d 21. (n= 6-10 mice per group, experiments performed in duplicate, * denotes $p < 0.05$ compared to controls by Fishers exact test).

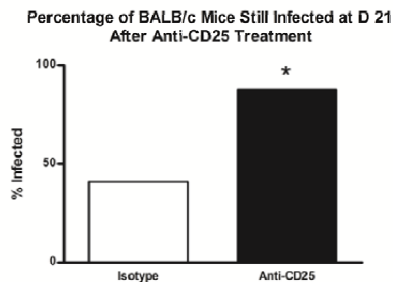
515 **Figure 4:**

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521 **Figure 4.** Effect of Tregs on development of chronic infection in C57BL/6 versus BALB/c mice.
 522 (A) Draining lymph nodes were removed from infected C57BL/6 and BALB/c mice at d 7 and 21
 523 post-implantation. Single cell suspensions were intracellularly stained for Foxp3 as described in
 524 Materials and Methods. Treg frequency, expressed as the ratio of Foxp3 expression in CD4⁺
 525 lymphocytes, of infected C57BL/6 was significantly lower compared to BALB/c mice at both d 7
 526 and d 21 post-implantation. (B) BALB/c mice were treated with i.p. injection of the anti-CD25
 527 MAb PC61 or isotype control before implantation of an *S. aureus*-coated pin and at d 7 and 14
 528 post-implantation, as described in Materials and Methods. Treatment with anti-CD25 reversed
 529 the protection against chronic implant infection normally observed in BALB/c mice (n= 8-13 mice
 530 per group, single experiment performed, * denotes $p < 0.05$ compared to controls by Student's *t*-
 531 test or Fishers exact test). Bars represent SD.

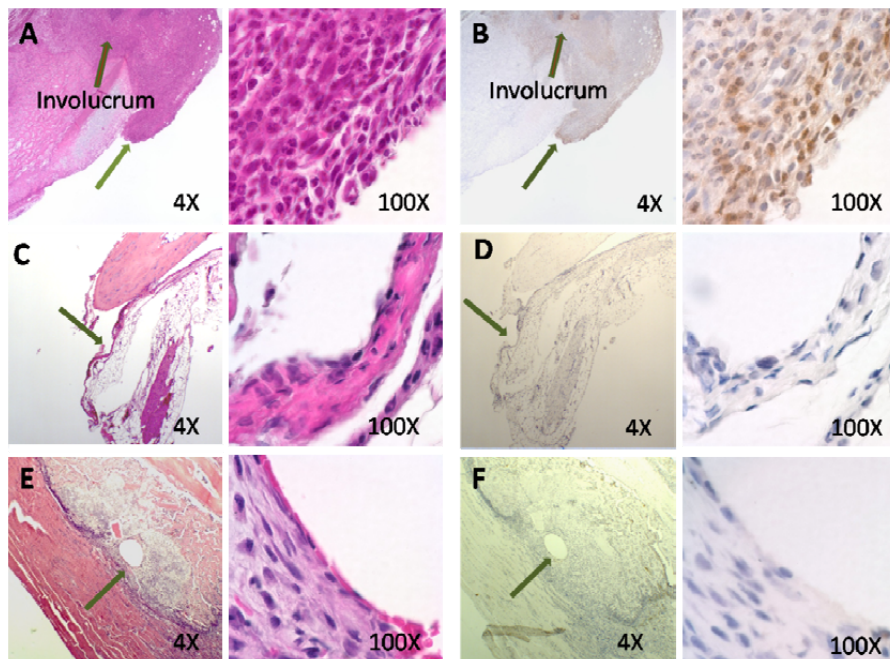
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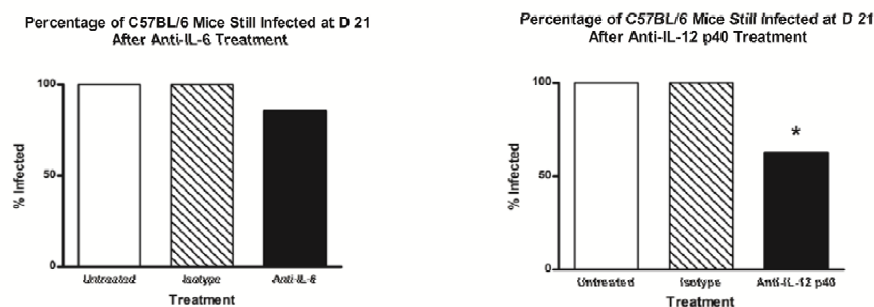
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Figure 5:



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Figure 5. Local neutrophil infiltration at implant site. Tibiae were removed from C57BL/6 and BALB/c receiving *S. aureus*-coated pins at 7 d post-implantation. Paraffin-embedded sections were stained with hematoxylin and eosin, or immunostained with anti-Ly-6g MAb and peroxidase-labeled secondary Ab and examined using a light microscope. Areas of pin insertion are noted by arrows. Neutrophil infiltration (seen as a brown stain) and involucrum formation are evident at the implant site in C57BL/6 mice (A and B), but not BALB/c mice (C and D), which appear similar to uninfected control mice (E and F). H&E staining of d 7 tibiae was performed in triplicate. Anti-Ly-6g immunostaining was performed as a single experiment.

550 **Figure 6:**

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Figure 6. Effect of anti-IL-6 and anti-IL-12 p40 treatment on development of chronic infection in C57BL/6. (A) C57BL/6 mice were treated with i.p. injection of the anti-IL-6 MAb MP5-20F3, injection of isotype control, or were left untreated before implantation of an *S. aureus*-coated pin and at d 7 and 14 post-implantation, as described in Materials and Methods. Treatment with MP5-20F3 did not significantly decrease the percentage of C57BL/6 that developed chronic implant infection. (B) A separate set of C57BL/6 mice were treated with i.p. injection of the anti-IL-12 MAb C17.8, injection of isotype control, or were left untreated before implantation of an *S. aureus*-coated pin and at d 7 and 14 post-implantation, as described in Materials and Methods. Treatment with C17.8 resulted in a significant decrease in the percentage of C57BL/6 that developed chronic implant infection compared to untreated mice. Although mice from untreated and isotype treated mice had the same rates of infection (100%) (n= 2-13 mice per group, * denotes $p < 0.05$ compared to controls by Fishers exact test).

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