Breaching peripheral tolerance promotes the production of HIV-1–neutralizing antibodies

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A subset of characterized HIV-1 broadly neutralizing antibodies (bnAbs) are polyreactive with additional specificities for self-antigens and it has been proposed immunological tolerance may present a barrier to their participation in protective humoral immunity. We address this hypothesis by immunizing autoimmune-prone mice with HIV-1 Envelope (Env) and characterizing the primary antibody response for HIV-1 neutralization. We find autoimmune mice generate neutralizing antibody responses to tier 2 HIV-1 strains with alum treatment alone in the absence of Env. Importantly, experimentally breaching immunological tolerance in wild-type mice also leads to the production of tier 2 HIV-1–neutralizing antibodies, which increase in breadth and potency following Env immunization. In both genetically prone and experimentally induced mouse models of autoimmunity, increased serum levels of IgM anti-histone H2A autoantibodies significantly correlated with tier 2 HIV-1 neutralization, and anti-H2A antibody clones were found to neutralize HIV-1. These data demonstrate that breaching peripheral tolerance permits a cross-reactive HIV-1 autoantibody response able to neutralize HIV-1.

INTRODUCTION

Advances in the isolation (Scheid et al., 2009, 2011) and screening (Walker et al., 2009) of antibody (Ab) receptors from the surface of HIV-specific B cells has led to the identification of hundreds of HIV-1 Env-specific Abs able to potently neutralize a wide breadth of HIV-1 genetic variants (Mascola and Haynes, 2013; Burton and Hangartner, 2016), and to protect against HIV-1 in both humans and animal models (Mascola et al., 2000; Balazs et al., 2011; Shingai et al., 2014; Caskey et al., 2015; Scheid et al., 2016). Characterization of these broadly neutralizing Abs (bnAbs) reveal they recognize several conserved, less immunogenic neutralizing epitopes on the HIV-1 Env protein, but they are relatively rare and develop only years after infection (West et al., 2014). Thus, a major focus of current research is to determine how to elicit these type of bnAbs more generally through vaccination (de Taeye et al., 2016). These Abs harbor several unusual features, including a long hydrophobic Ig heavy chain complementarity determining region 3 (CDR3), unusually short Ig light chain CDR3 sequences, and a high mutation burden in both CDR and framework Ig regions. Moreover, they often display polyreactive/autoreactive specificities, which includes recognition of self-antigens (Mascola and Haynes, 2013; West et al., 2014; Liu et al., 2015) such as phospholipids, ubiquitin ligase 3, and double-stranded dsDNA (Haynes et al., 2005b; Bonsignori et al., 2014; Liu et al., 2015).

The autoreactivity displayed by a subset of HIV-1 bnAbs has led to the proposal that immunological tolerance may impede the antibody response by B cells expressing these bnAbs (Haynes et al., 2005b, 2016; Verkoczy et al., 2011b). Support for this hypothesis has shown that developing B cells expressing a characterized bnAb autoreactive specificity are eliminated by central B cell tolerance in the bone marrow (Verkoczy et al., 2010, 2011a, 2013; Doyle-Cooper et al., 2013; Zhang et al., 2016). However, whether peripheral B cells expressing HIV-1–neutralizing but autoreactive specificities are similarly constrained by peripheral tolerance has been less carefully examined.

Additional evidence that autoreactive specificities are able to recognize HIV-1 Env comes from analyses of sera from autoimmune individuals (Barthel and Wallace, 1993; Mylonakis et al., 2000; Carugati et al., 2013) and autoimmune prone mice (Kion and Hoffmann, 1991; Lombardi et al., 1993) that harbor HIV-1–specific Abs in the absence of infection. Moreover, the incidence of HIV-1 infection in individuals with systemic lupus erythematosus (SLE) is lower than anticipated (Kaye, 1989; Barthel and Wallace, 1993; Palacios et al., 2002; Palacios and Santos, 2004) and recently, a SLE patient was discovered to harbor plasma able to neutralize a wide breadth of HIV-1 strains and to control HIV-1 infection in the absence of antiretroviral therapy (Bonsignori et

al., 2014). Thus, although evidence exists that autoimmune individuals harbor serum antibodies that recognize HIV-1 Env and are autoreactive, whether breaking immunological tolerance can facilitate production of HIV-1–neutralizing antibodies remains unknown.

Autoreactive B cells are generally eliminated or silenced by mechanisms of tolerance. Tolerance of B cells initially manifests in the bone marrow as central tolerance acting on immature B cells expressing their newly formed antibody as a receptor (Goodnow et al., 2005; Nemazee, 2006; Pelanda and Torres, 2006). In both humans and mice, 55–75% of newly expressed antibody receptors are autoreactive (Grandien et al., 1994; Wardemann et al., 2003), and approximately half of these are censored by central B cell tolerance (Casellas et al., 2001). Autoreactive B cells with weak autoreactivity escape central tolerance and enter the peripheral lymphoid compartments of healthy individuals and wild-type mice (Pugh-Bernard et al., 2001; Wardemann et al., 2003; Merrell et al., 2006; Koelsch et al., 2007) where peripheral tolerance renders them functionally anergic and short-lived (Cambier et al., 2007).

In this study, we addressed whether breaching immunological tolerance promotes the development of neutralizing HIV-1 antibody responses using mouse models. Mice are not a natural host for HIV-1 but can generate HIV-1–reactive Abs upon immunization, although typically unable to neutralize HIV-1. We show that autoimmune-prone, but not wild-type healthy, strains of mice can be induced by adjuvant alone to produce antibodies that neutralize tier 2 HIV-1 variants. Importantly, we further demonstrate that when immunological tolerance in wild-type healthy mice is breached experimentally, antibodies neutralizing tier 2 HIV-1 strains can be elicited and are enhanced in potency by Env immunization. Thus, our findings formally demonstrate that peripheral immunological tolerance limits the production of neutralizing HIV-1 antibody responses by B cells expressing a wild-type antibody repertoire.

RESULTS
Wild-type and B6.Sle123 mice mount a similar Env-specific IgG, but not IgM, primary antibody response
To evaluate the contribution of tolerance on the production of anti–HIV-1 antibodies, we first compared the primary anti-Env antibody response in wild-type B6 and autoimmune prone B6.Sle123 mice. B6.Sle123 is a model strain of lupus autoimmunity generated by introducing three distinct autoimmune-prone loci from the NZM2410 strain into the B6 strain (Morel et al., 1994). Similar to NZM2410, B6.Sle123 mice are characterized by impaired mechanisms of B cell tolerance and the presence of antinuclear antibodies in both females and males (Morel et al., 1994). Important to this study, B6.Sle123 mice are also congenic with B6 mice, and thus bear the same Ig variable gene segments. Age-matched (2–9-mo-old) WT B6 and autoimmune B6.Sle123 mice were immunized with alum alone or together with Env and either monomeric gp120 (ADA) or trimeric gp140 (YU2). We then measured the levels of gp120-specific IgM and IgG antibodies in serum 7 and 14 d later by ELISA. On pathogen encounter, antigen-specific IgM is the first Ig isotype produced to limit pathogen spread during the time class-switched IgG isotypes are generated and which are better suited to clear the offending pathogen, (Swanson et al., 2013). As expected, B6 mice immunized with Env + alum mounted a gp120-specific IgM response by day 14 that was >3-fold increased over preimmune levels (Fig. 1 A). In contrast, sera from naive B6.Sle123 mice already harbored high preimmunization titers of gp120-reactive IgM (∼3-fold increased over B6), but these titers did not increase further following alum or Env + alum immunization (Fig. 1 A). Polyclonal B cell activation is a feature of lupus prone mice (Klinman, 1990) that can lead to increased total Ig levels. Indeed, while total serum IgM levels did not increase with immunization in either wild-type or autoimmune prone mice (Fig. 1 B), total IgM levels were significantly higher in B6.Sle123 compared with B6 mice (Fig. 1 B).

In contrast to the IgM response, both B6 and B6.Sle123 mice mount gp120-specific IgG antibody responses after immunization with Env + alum (Fig. 1 C). All Env-specific IgG isotypes, with the exception of IgG3, were similarly and significantly increased over alum in both strains (Fig. 1 C). However, the B6.Sle123 IgG1 anti-gp120 response, while considerable, was reduced compared with that in B6 mice. Total IgG concentrations were modestly increased after Env + alum immunization relative to naive and alum-treated in B6 mice, whereas they remained unchanged in B6.Sle123 mice (Fig. 1 D). Nevertheless, total IgG levels were significantly higher in B6.Sle123 mice compared with B6 in all conditions (Fig. 1 D).

These data show that B6 mice mount typical T-dependent antigen-specific IgM and IgG antibody responses to Env immunization, whereas B6.Sle123 mice harbor preexisting high titers of gp120-reactive IgM that does not change with immunization, but also mount a normal IgG gp120-specific antibody response.

Immunization of autoimmune-prone mice with adjuvant alone induces antibodies that neutralize multiple tier 1 and 2 HIV-1 strains
We next asked whether the Env-specific antibody response elicited in mice was able to neutralize HIV-1 using a standardized in vitro TZMbl assay (Sarzotti-Kelsoe et al., 2014). Serum neutralization was tested against a panel of four HIV-1 clade B viruses representing the neutralization-sensitive tier 1 and neutralization-resistant tier 2 virus strains: SF162.LS (tier 1A), BaL.26 (tier 1B), JRFL (tier 2), and YU2 (tier 2) (Seaman et al., 2010; Chakrabarti et al., 2013). These results revealed that sera from B6 mice was essentially devoid of neutralizing activity against HIV-1 (Fig. 2), consistent with previous findings (Dosenovic et al., 2012; McCoy and Weiss, 2013). In contrast, the immune sera from ∼50% of the B6.Sle123 mice was able to neutralize tier 1A or tier 2 strains (Fig. 2
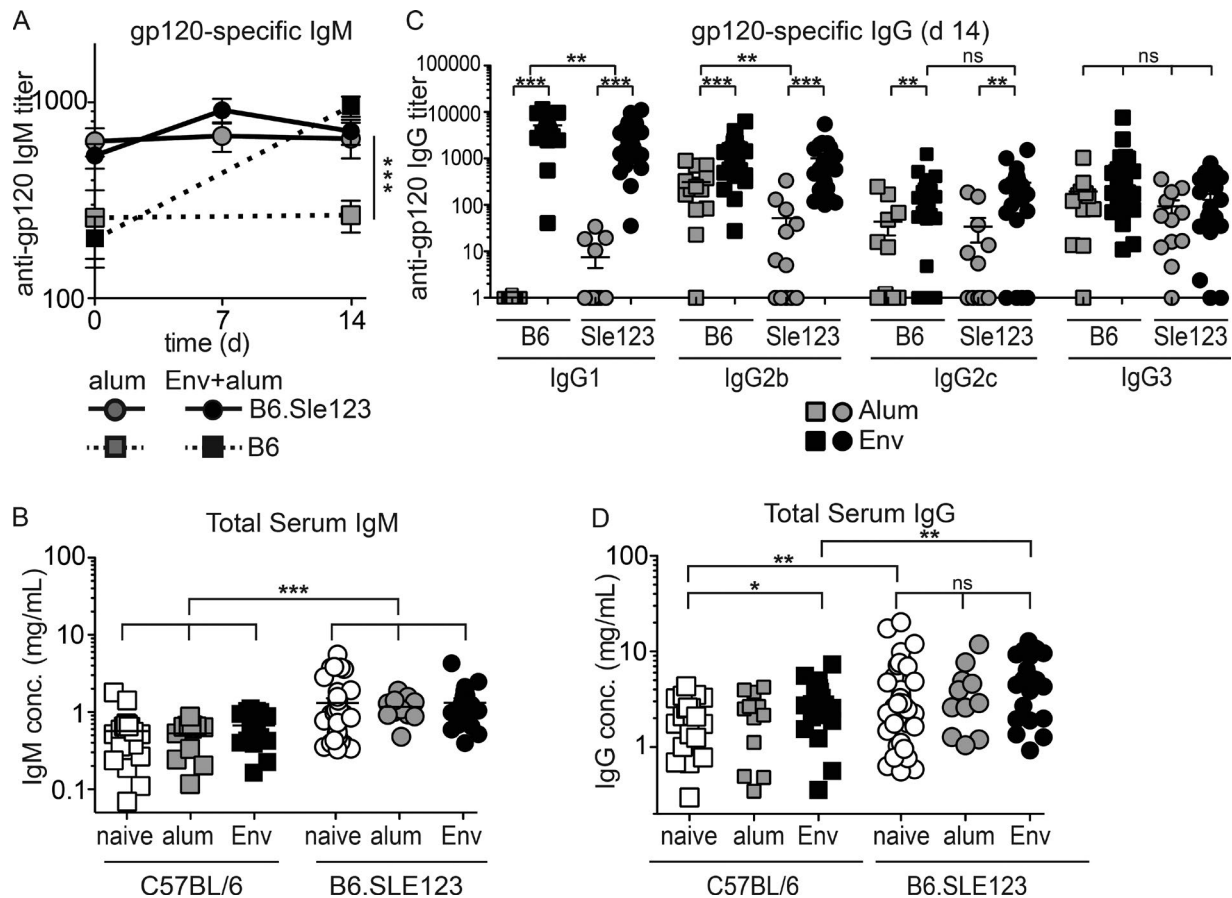
Figure 1. **B6.Sle123 and B6 mice mount a similar Env–specific IgG antibody response.** B6.Sle123 ($n = 23$ Env; $n = 11$ Alum) and B6 ($n = 24$ Env; $n = 13$ Alum) mice were immunized with alum alone (gray symbols) or with Env + alum (black symbols) and Env-specific Ig titers measured by ELISA. (A) gp120-reactive IgM is shown for B6 (squares) and B6.Sle123 (circles) mice. (B) Total IgM serum concentrations are shown for B6 (squares) and B6.Sle123 (circles) naive (open) mice and 14 d after alum alone (gray) or Env + alum (black). (C) gp120-specific IgG is shown for B6 (squares) and B6.Sle123 (circles) mice 14 d after immunization with alum alone (gray symbols) or with Env + alum (black symbols). (D) Total IgG serum concentrations are shown for B6 (squares) and B6.Sle123 (circles) naive (open) mice and 14 d after alum alone (gray) or Env + alum (black). All P-values were calculated using Student's *t* test assuming unequal variance. Each symbol represents one mouse and all data are plotted as the arithmetic mean ± SEM. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$; ns, not significant. Data shown is from five independent experiments.

and Fig. S1 A). Neutralizing activity was present in the sera of B6.Sle123 mice from 13 to 36 wk of age, but increased in mice over 4 mo of age (Fig. 2 A). To determine the specificity of this viral neutralization, neutralization was also tested for murine leukemia virus (muLV) and <1/3 of the Env + alum immune serum samples that were able to neutralize HIV-1 also demonstrated weak neutralizing activity for muLV suggesting that the elicited neutralizing antibodies were relatively HIV-1 specific (Fig. 2 A).

Because naive B6.Sle123 mice harbored preexisting high titers of Env-reactive IgM, we next asked if Env was required as an immunogen to elicit neutralization. Unexpectedly, sera from B6.Sle123 mice treated with alum alone harbored antibodies able to neutralize tier 1B BaL.26, as well as both tier 2 JRFL and YU2 strains, but not tier 1A SF162.LS (Fig. 2 C). In contrast, serum HIV-1 neutralization was rarely observed and significantly reduced in naive B6.Sle123 mice compared with

alum-treated ($P = 0.028$) or Env + alum–immunized ($P = 0.017$) mice (Fig. 2 D), and was not observed in age-matched, B6 mice injected with alum alone (unpublished data).

Mouse immune sera has been reported to neutralize HIV-1, but this was subsequently found to have erroneously resulted from an unappreciated cytotoxicity resulting from immunization with formaldehyde-fixed cells (LaCasse et al., 1999; Nunberg, 2002). It was thus important to determine if serum Ig from B6.Sle123 mice was indeed responsible for HIV-1 neutralization and to assess the specificity of this neutralization. Accordingly, Ig was purified from B6.Sle123 HIV-1–neutralizing sera and found to also display neutralizing activity against one or both tier 2 HIV-1 strains with IC_{50} <100 μg/ml (Fig. S2). In contrast, Ig purified from non-neutralizing B6 immune sera did not display any neutralizing activity. From these analyses, we conclude that the HIV-1 neutralization observed in the sera of both alum alone and
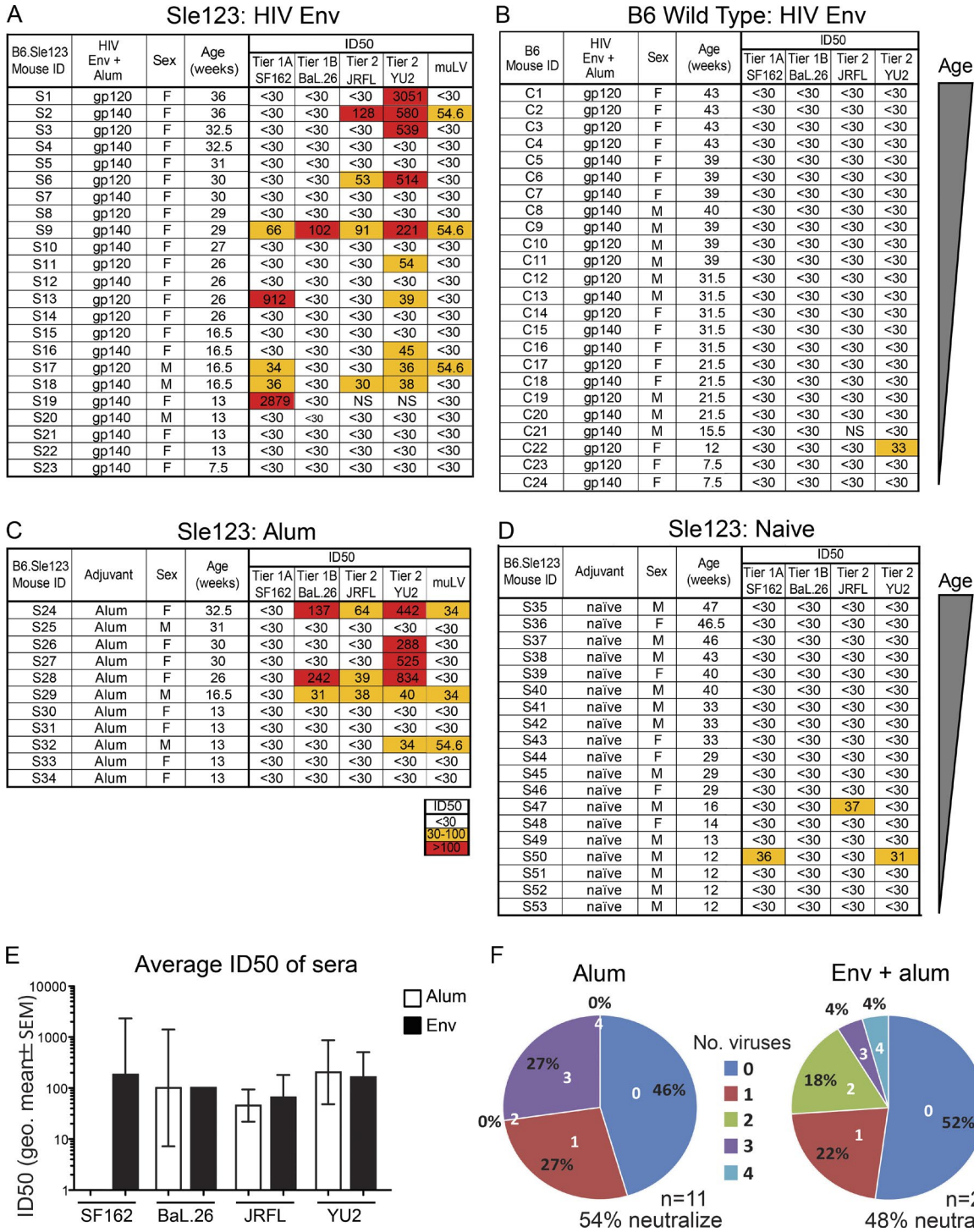
A

Sle123: HIV Env

B6.Sle123 Mouse ID	HIV Env + Alum	Sex	Age (weeks)	ID50 Tier 1A SF162	Tier 1B BaL.26	Tier 2 JRFL	Tier 2 YU2	muLV
S1	gp120	F	36	<30	<30	<30	3051	<30
S2	gp140	F	36	<30	<30	128	580	54.6
S3	gp120	F	32.5	<30	<30	<30	539	<30
S4	gp140	F	32.5	<30	<30	<30	<30	<30
S5	gp140	F	31	<30	<30	<30	<30	<30
S6	gp120	F	30	<30	<30	53	514	<30
S7	gp140	F	30	<30	<30	<30	<30	<30
S8	gp120	F	29	<30	<30	<30	<30	<30
S9	gp140	F	29	66	102	91	221	54.6
S10	gp140	F	27	<30	<30	<30	<30	<30
S11	gp120	F	26	<30	<30	<30	54	<30
S12	gp140	F	26	<30	<30	<30	<30	<30
S13	gp120	F	26	912	<30	<30	39	<30
S14	gp120	F	26	<30	<30	<30	<30	<30
S15	gp120	F	16.5	<30	<30	<30	<30	<30
S16	gp140	F	16.5	<30	<30	<30	45	<30
S17	gp120	M	16.5	34	<30	<30	36	54.6
S18	gp140	M	16.5	36	<30	30	38	<30
S19	gp140	F	13	2879	<30	NS	NS	<30
S20	gp140	M	13	<30	<30	<30	<30	<30
S21	gp140	F	13	<30	<30	<30	<30	<30
S22	gp140	F	13	<30	<30	<30	<30	<30
S23	gp140	F	7.5	<30	<30	<30	<30	<30

B

B6 Wild Type: HIV Env

B6 Mouse ID	HIV Env + Alum	Sex	Age (weeks)	ID50 Tier 1A SF162	Tier 1B BaL.26	Tier 2 JRFL	Tier 2 YU2
C1	gp120	F	43	<30	<30	<30	<30
C2	gp120	F	43	<30	<30	<30	<30
C3	gp120	F	43	<30	<30	<30	<30
C4	gp120	F	43	<30	<30	<30	<30
C5	gp140	F	39	<30	<30	<30	<30
C6	gp140	F	39	<30	<30	<30	<30
C7	gp140	F	39	<30	<30	<30	<30
C8	gp140	M	40	<30	<30	<30	<30
C9	gp140	M	39	<30	<30	<30	<30
C10	gp120	M	39	<30	<30	<30	<30
C11	gp120	M	39	<30	<30	<30	<30
C12	gp120	M	31.5	<30	<30	<30	<30
C13	gp140	M	31.5	<30	<30	<30	<30
C14	gp120	F	31.5	<30	<30	<30	<30
C15	gp140	F	31.5	<30	<30	<30	<30
C16	gp140	F	31.5	<30	<30	<30	<30
C17	gp120	F	21.5	<30	<30	<30	<30
C18	gp140	F	21.5	<30	<30	<30	<30
C19	gp120	M	21.5	<30	<30	<30	<30
C20	gp140	M	21.5	<30	<30	<30	<30
C21	gp140	M	15.5	<30	<30	NS	<30
C22	gp120	F	12	<30	<30	<30	33
C23	gp120	F	7.5	<30	<30	<30	<30
C24	gp140	F	7.5	<30	<30	<30	<30

C

Sle123: Alum

B6.Sle123 Mouse ID	Adjuvant	Sex	Age (weeks)	ID50 Tier 1A SF162	Tier 1B BaL.26	Tier 2 JRFL	Tier 2 YU2	muLV
S24	Alum	F	32.5	<30	137	64	442	34
S25	Alum	M	31	<30	<30	<30	<30	<30
S26	Alum	F	30	<30	<30	<30	288	<30
S27	Alum	F	30	<30	<30	<30	525	<30
S28	Alum	F	26	<30	242	39	834	<30
S29	Alum	M	16.5	<30	31	38	40	34
S30	Alum	F	13	<30	<30	<30	<30	<30
S31	Alum	F	13	<30	<30	<30	<30	<30
S32	Alum	M	13	<30	<30	<30	34	54.6
S33	Alum	F	13	<30	<30	<30	<30	<30
S34	Alum	F	13	<30	<30	<30	<30	<30

ID50
<30
30-100
>100

D

Sle123: Naive

B6.Sle123 Mouse ID	Adjuvant	Sex	Age (weeks)	ID50 Tier 1A SF162	Tier 1B BaL.26	Tier 2 JRFL	Tier 2 YU2
S35	naïve	M	47	<30	<30	<30	<30
S36	naïve	F	46.5	<30	<30	<30	<30
S37	naïve	M	46	<30	<30	<30	<30
S38	naïve	M	43	<30	<30	<30	<30
S39	naïve	F	40	<30	<30	<30	<30
S40	naïve	M	40	<30	<30	<30	<30
S41	naïve	M	33	<30	<30	<30	<30
S42	naïve	M	33	<30	<30	<30	<30
S43	naïve	F	33	<30	<30	<30	<30
S44	naïve	F	29	<30	<30	<30	<30
S45	naïve	M	29	<30	<30	<30	<30
S46	naïve	F	29	<30	<30	<30	<30
S47	naïve	M	16	<30	<30	37	<30
S48	naïve	F	14	<30	<30	<30	<30
S49	naïve	M	13	<30	<30	<30	<30
S50	naïve	M	12	36	<30	<30	31
S51	naïve	M	12	<30	<30	<30	<30
S52	naïve	M	12	<30	<30	<30	<30
S53	naïve	M	12	<30	<30	<30	<30

E — Average ID50 of sera

F — Alum (n=11, 54% neutralize): 0 46%, 1 27%, 2 0%, 3 27%, 4 0%. Env + alum (n=23, 48% neutralize): 0 52%, 1 22%, 2 18%, 3 4%, 4 4%. No. viruses: 0, 1, 2, 3, 4.

Figure 2. **B6.Sle123 mice neutralize tier 1 and 2 HIV–1 strains after alum treatment alone.** HIV-1 neutralization by (A) B6.Sle123 or (B) B6 mice 14 d after immunization with Env + alum or by B6.Sle123 mice after (C) alum treatment alone or (D) no treatment (naive). These are same mice as in Fig. 1 and data are expressed as ID_{50}, calculated as the reciprocal dilution of sera required for 50% neutralization. The number of alum and Env + alum–treated B6.Sle123 mice able to neutralize ≥1 virus (neutralizers) is statistically higher compared with similarly treated B6 mice (Env, P = 0.0007; alum, P = 0.023). Naive B6.Sle123 mice able to neutralize ≥1 virus was statistically lower than treated mice (Env, P = 0.017; alum, P = 0.028). Statistics calculated for A–D using Fisher's exact test. (E) Mean ID_{50} for neutralization of indicated tier 1 and 2 virus by B6.Sle123 treated with alum alone (open) or Env + alum (filled). Data are shown as the geometric mean ± SEM. (F) Percent of all B6.Sle123 mice treated with alum (left) or Env + alum (right) able to neutralize 0, 1, 2, 3, or 4 HIV-1 strains. Data are from five independent experiments.
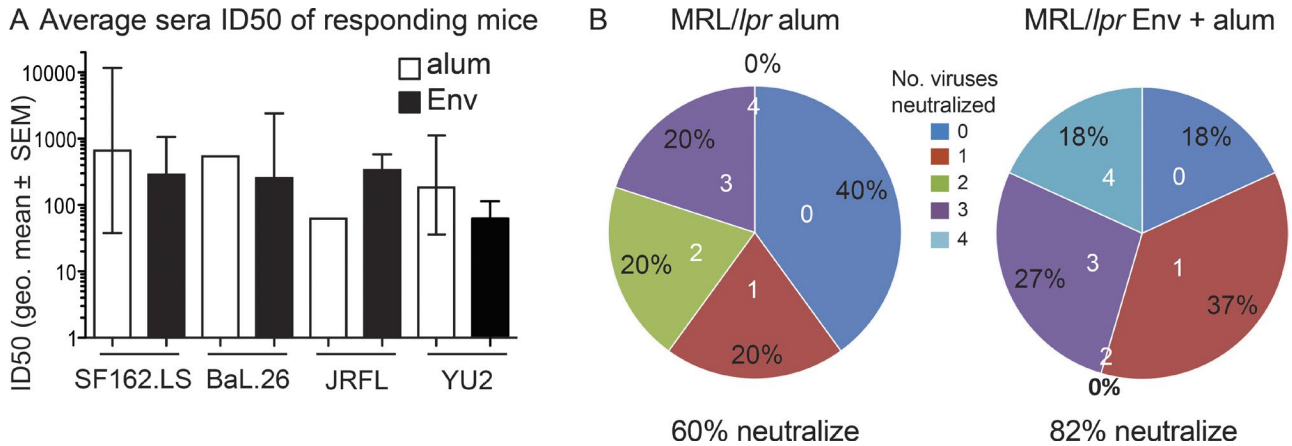
Figure 3. **Neutralization of HIV-1 is observed in MRL/lpr mice.** MRL/lpr mice (8–16 wk old) were immunized with alum ($n = 5$) or Env + alum ($n = 11$), sacrificed day 14 pi, and analyzed for neutralization. The ID_{50} is calculated as in Fig. 2. (A) The mean ID_{50} was calculated for the responders per virus for MRL/lpr Env immunized (filled bars) and alum injected (open bars) mice. Data are plotted as the geometric mean ± SEM. (B) The percentage of responding MRL/lpr alum (left, 60%) and Env (right, 82%) immunized mice was similar ($P = 0.55$). Statistics were calculated using Fisher's exact test. Data are shown from two independent experiments.

Env + alum immunized B6.Sle123 mice is relatively specific to HIV-1 and is mediated by immunoglobulin and not another serum component.

To determine if Env immunization influenced the potency of neutralization, the mean neutralization titers were calculated for both alum- and Env-immunized B6.Sle123 mice that displayed neutralization activity for each virus (Fig. 2 E). These findings show that Env immunization of B6.Sle123 mice increases the potency of neutralizing serum for tier 1A SF162.LS virus (800× increase), but not for any other HIV-1 variant. Comparing the relative breadth of neutralization between B6.Sle123 mice treated with alum alone or Env + alum revealed both treatments promoted a similar frequency (54 vs. 48%, respectively) of mice able to neutralize one or more viruses (Fig. 2 F). Thus, autoimmune B6.Sle123 mice mount an Env-specific antibody response that is able to neutralize tier 1A SF162.LS virus, whereas neutralization of tier 1B BaL.26 and tier 2 HIV-1 variants JRFL and YU2 can be elicited by alum alone.

To determine whether the elicited HIV-1–neutralizing activity was restricted to the B6.Sle123 autoimmune mouse model, we similarly treated lupus prone MRL/lpr mice (8–16 wk of age) with alum alone or with Env + alum and measured HIV-1–neutralizing activity in serum 2 wk later. In these experiments, MRL/lpr mouse sera displayed HIV-1 neutralization properties similar to those of B6.Sle123 mice (Fig. 3), but differed in that sera from MRL/lpr mice was also able to neutralize tier 1A SF162.LS after alum treatment alone (Fig. 3 A). Additionally, a higher percentage of MRL/lpr mice neutralized one or more strains of HIV-1 (60–82%; Fig. 3 B) compared with B6.Sle123 mice (48–54%; Fig. 2 F) and neutralization against all 4 HIV-1 strains, albeit weak, was observed in the sera of naive MRL/lpr, but not B6.Sle123, mice (unpublished data).

Overall, these data reveal that the treatment of autoimmune prone mice with alum alone is sufficient to induce the production of antibodies capable of neutralizing tier 1 and 2 HIV-1 strains.

HIV-1 neutralization correlates with total IgM levels and age

Approximately 50% of the treated B6.Sle123 mice produced HIV-1–neutralizing antibodies (Fig. 2). Thus, we asked if any parameters were uniquely associated with B6.Sle123 mice able to neutralize HIV-1 (neutralizers) compared with mice unable to neutralize HIV-1 (nonneutralizers). Specifically, we evaluated mouse age (Fig. 4 A), serum Ig levels (Fig. 4, B and C), and specificity to two autoantigens representing common lupus-associated autoreactive specificities (chromatin; Fig. 4 D and the RNA-binding Smith antigen; Fig. 4 E), as well as cardiolipin, a previously reported autoreactive specificity for HIV-1 bnAbs (Fig. 4 F; Haynes et al., 2005a).

From these analyses, total serum IgM levels provided a significant correlation with B6.Sle123 neutralizers compared with B6.Sle123 nonneutralizers and wild-type B6 mice (Fig. 4, B and C; and not depicted). There was a tendency for B6.Sle123 neutralizers to be older relative to B6.Sle123 nonneutralizers, although this difference was not statistically significant (Fig. 4 A). Serum IgM and IgG autoantibody titers against chromatin, Smith antigen, and cardiolipin were also not significantly different between neutralizers and nonneutralizers (Fig. 4 D–F), although the mean IgM and IgG anti-chromatin titers trended higher in HIV-1 neutralizers relative to nonneutralizers. These data show that B6.Sle123 neutralizers tend to be older and have higher serum IgM levels, but do not generally exhibit increased serum titers for three common autoantigen specificities.
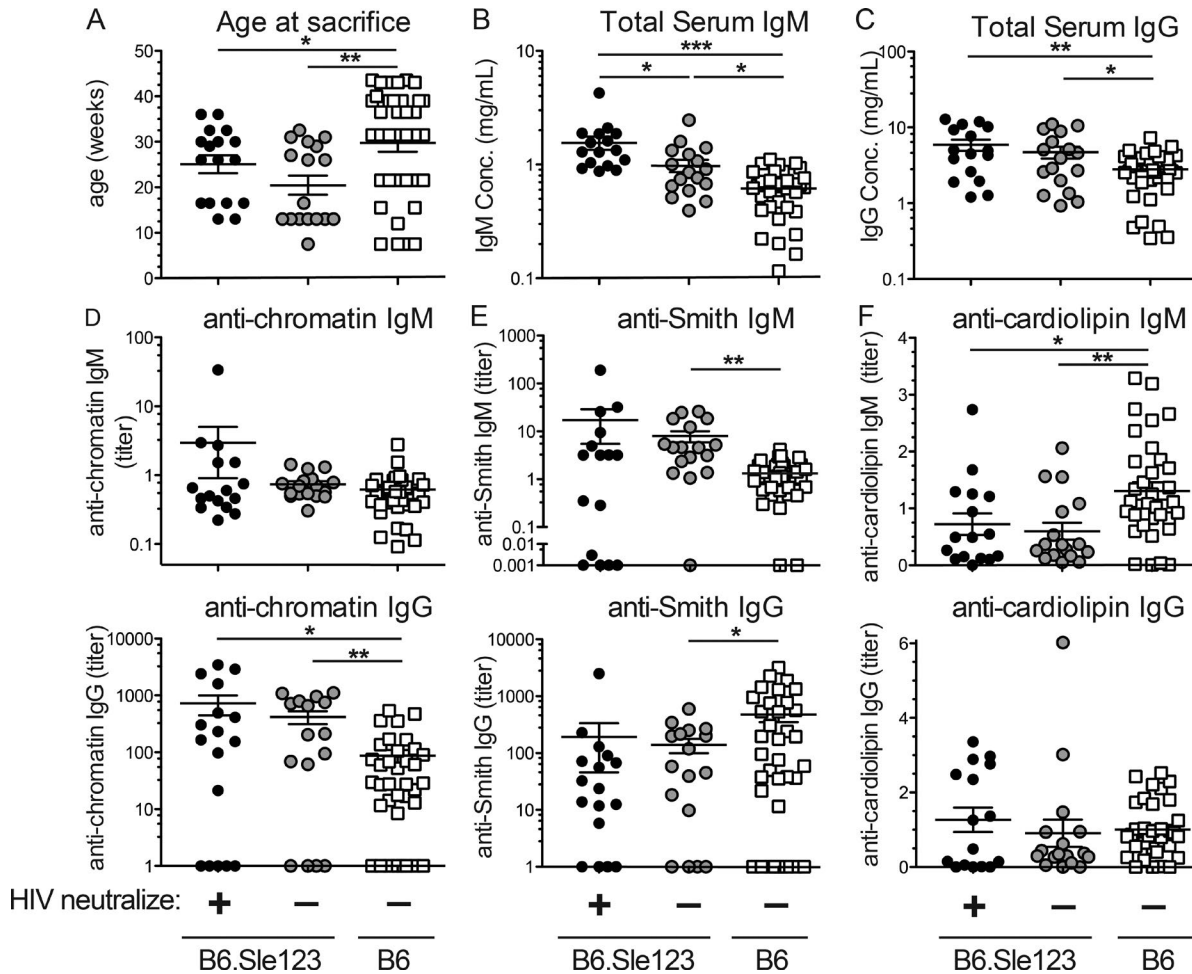
Figure 4. **HIV-1 neutralization correlates with total IgM levels.** B6.Sle123 HIV-1 neutralizers (black circles) and nonneutralizers (gray circles) from Fig. 2 were evaluated separately for (A) age at time of sacrifice and serum levels of (B) total IgM (P = 0.025) and (C) total IgG. Serum autoantibodies were also measured in B6.Sle123 neutralizers and nonneutralizers for IgM (top) and IgG (bottom) reactive with (D) chromatin, (E) Smith antigen, and (F) cardiolipin. Neutralizer is defined as mice neutralizing ≥1 strain of HIV-1. Each symbol represents one mouse and data are shown as the arithmetic mean ± SEM. All P-values were calculated using Student's t test assuming unequal variance. *, P < 0.05; **, P < 0.001; ***, P < 0.0001. Only total IgM levels are significantly different between B6.Sle123 neutralizers and nonneutralizers (P = 0.025).

B6.Sle123 mice that neutralize HIV-1 have higher titers of histone H2A–reactive IgM. To further assess potential autoreactive specificities that correlate with the ability to neutralize HIV-1, sera from B6.Sle123 neutralizers, nonneutralizers and wild-type B6 mice were interrogated with an autoantigen array of 95 known autoantigens (Li et al., 2007). The results of this analysis for common lupus-associated autoantigens are shown in Fig. 5 A. Neutralizers and nonneutralizers exhibited similar serum levels of IgG autoantibodies reactive with H2A, H2B, or total histones, as well as IgM antibodies against H2B and total histones, DNA-associated autoantigens, or the La, Ro/SSA, or Smith RNA–binding proteins (Fig. 5 A). Among all autoantigens tested, only IgM anti-histone H2A titers were significantly elevated in B6.Sle123 neutralizers relative to nonneutralizers. This finding was further confirmed by ELISA showing that neutralizers had significantly higher serum

titers of H2A-reactive IgM compared with the nonneutralizers (Fig. 5 B; P = 0.005). In contrast, the IgM anti-H2B was similar between both cohorts of B6.Sle123 (Fig. 5 B; P = 0.17). These results demonstrate that IgM anti-H2A serum titers correlate with the ability of B6.Sle123 mice to neutralize HIV-1.

Experimental breach of immunological tolerance in C57BL/6 mice facilitates the production of HIV-1–neutralizing antibodies. Our data show that autoimmune-prone strains of mice treated with alum produce HIV-1–neutralizing antibodies, and this activity correlates with increased anti-H2A IgM autoantibody titers. B cells capable of producing antinuclear antibodies are present in the blood and peripheral lymphoid compartments of both healthy individuals and wild-type mice (Li et al., 2011). These autoreactive B cells are gen-
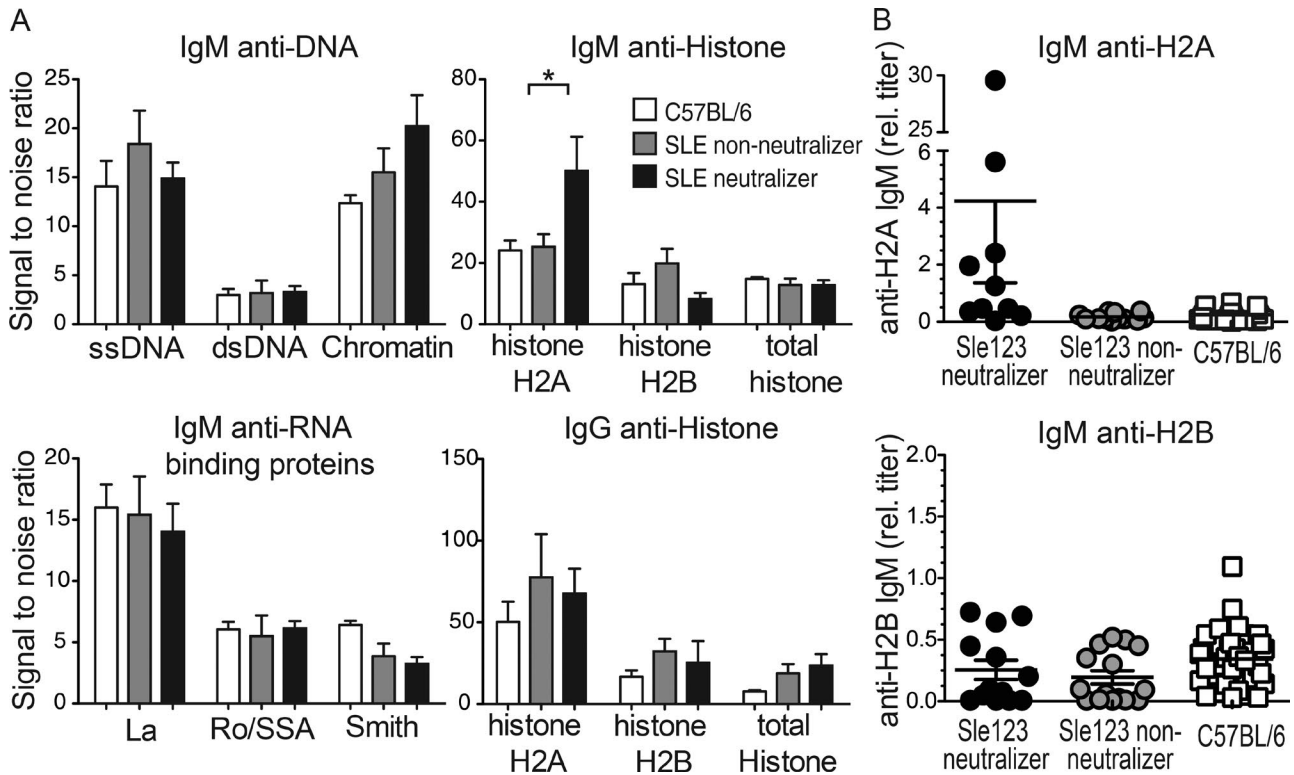
Figure 5. B6.Sle123 HIV–1 neutralizers harbor elevated levels of IgM anti–histone H2A. (A) Sera from B6 (open), B6.Sle123 nonneutralizers (gray), and neutralizers (black) were interrogated with an autoantigen array and results for the IgM reactive with the indicated anti-DNA antigens (top left), RNA-binding proteins (bottom left), and anti-histone antigens (top right), as well as IgG anti-histone antigens (bottom right) are shown. Relative serum titers of (B) IgM anti-H2A (left) and anti-H2B (right) measured by ELISA in B6.Sle123 neutralizers and nonneutralizers. Each symbol represents measurements for one mouse, and all data are plotted as the arithmetic mean ± SEM. P-values were calculated using the Mann-Whitney nonparametric test; *, P < 0.05; **, P < 0.001. Histone H2A IgM titers are significantly higher in B6.Sle123 neutralizers versus nonneutralizers (P = 0.005).

erally anergic, but at times can participate in an antibody response to foreign antigens (Sabouri et al., 2014; Reed et al., 2016). Thus, we next considered if peripheral autoreactive B cells able to neutralize HIV-1 exist in wild-type mice but are normally restrained by immunological tolerance. To address this, we experimentally compromised immunological tolerance in adult B6 mice (Fig. 6 A) by treatment with the isoprenoid alkane, 2,6,10,14-tetramethylpentadecane (pristane), which promotes lupus-like autoantibodies within 1 mo and disease, including renal pathology and proteinuria, within 5–6 mo in various wild-type mouse strains (Satoh and Reeves, 1994; Satoh et al., 2000; Kuroda et al., 2004; Summers et al., 2010). Indeed, 30 d after pristane treatment of adult B6 mice we observed measureable titers of chromatin reactive serum IgM and IgG by ELISA (unpublished data). At this time point, mice were immunized twice 2 wk apart with alum alone or with Env (trimeric YU2 gp140) + alum and sera tested for neutralization before and after immunization.

1 mo after pristane treatment alone, ~42% of B6 mice harbored weakly neutralizing sera for a tier 2 HIV-1 strain (36% for JRFL, 31% for YU2, and 14% for both), but surprisingly not for tier 1 HIV-1 strains (Fig. 6, B and E). Two

immunizations with either alum alone or Env + alum increased the percentage of tier 2 neutralizers twofold (68% for JFRL; 75% for YU2) and induced a minority of mice (18%) to also neutralize tier 1 viruses (Fig. 6, C and E). Further, pristane-treated mice immunized twice with alum or Env + alum had significantly higher neutralization titers against tier 2 HIV-1 strains than mice treated with pristane alone (Fig. 6, C and F; and Fig. S1 B). Importantly, the neutralization of tier 2 HIV-1 strains after two immunizations of either alum or Env + alum again appeared relatively specific to HIV-1, as <20% of the HIV-1–neutralizing sera also (weakly) neutralized muLV (Fig. 6 C).

Pristane-treated mice given alum alone neutralized tier 2 HIV-1 at similar frequencies (75%) as those immunized with Env + alum (Fig. 6 C). To understand if Env immunization influenced the neutralizing antibody response, both cohorts of pristane-treated mice were compared and analyzed individually for potency of HIV-1–neutralizing activity. These data revealed that immunization with Env resulted in a significant increase in the ID_{50} relative to the sera from mice treated with alum only, which was comparable to mice treated only with pristane (Fig. 6 G). Thus, Env immunization increases
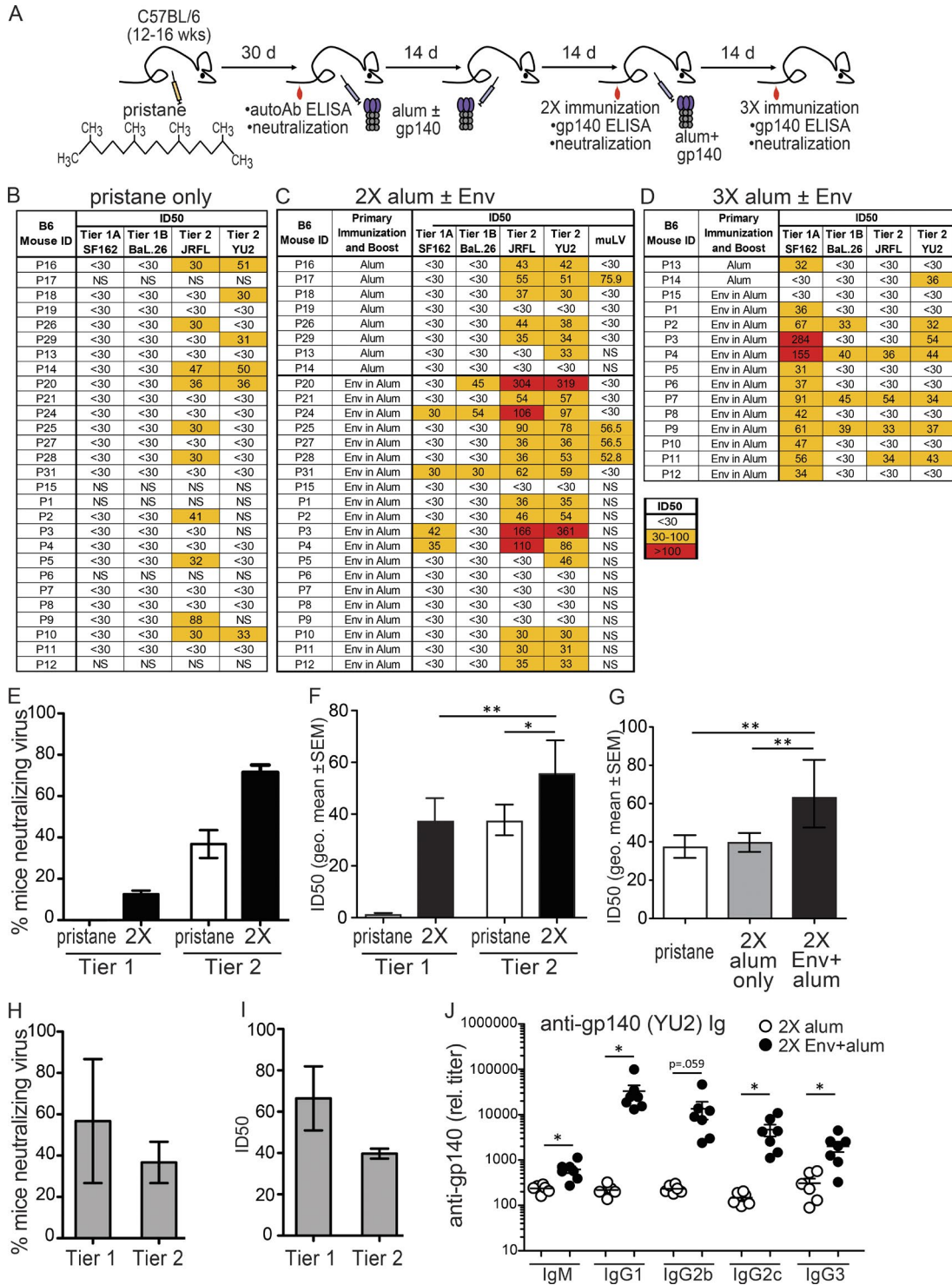
Figure 6. Wild-type mice neutralize tier 2 HIV-1 after an experimental breach in immunological tolerance. (A) Schematic of experimental protocol. Serum neutralization of tier 1 and 2 HIV-1 strains by B6 mice treated with (B) pristane alone for 30 d or after (C) 2 (2X) or (D) 3 (3X) subsequent immunizations with alum alone or Env + alum as indicated. Data are from the same mice serially bled. NS, insufficient sera available for analysis. (E) Percentage of mice able to neutralize tier 1 and 2 HIV-1 viruses after pristane only (open bars) or after 2X Env + alum immunizations (black bars). (F) Mean ID$_{50}$ for the mice that neutralized tier 1 and 2 HIV-1 viruses after pristane only (open bars) or after 2X Env + alum immunizations (black bars). Data are plotted as the geometric mean ± SEM. (G) Mean ID$_{50}$ are shown for B6 mice able to neutralize tier 2 HIV-1 strains after pristane alone (open bars), after two immunizations with alum alone (gray bars) or after 2 immunizations with Env + alum (black bars). (H) Percentage of mice able to neutralize tier 1 and 2 HIV-1 viruses after 3

the potency of the neutralizing antibody response against tier 2 HIV-1 strains in pristane-treated wild-type mice.

A cohort of pristane-treated mice were immunized a third time with Env + alum to explore if an additional boost would further increase the potency or breadth of HIV-1 neutralization. Interestingly, after a third immunization 87% of mice could neutralize tier 1A SF162.LS (Fig. 6, D and H), but with a concomitant loss of ability to neutralize tier 2 HIV-1 (Fig. 6 I). These data suggest that multiple Env immunizations of pristane-treated mice ultimately leads to an antibody response focused on gp120 neutralization epitopes specific for the easily neutralized tier 1 SF162.LS HIV-1 variant over tier 2 strains.

To confirm that pristine-treated B6 mice maintain the ability to mount an antigen-specific antibody response as reported (Weinstein et al., 2008), we measured Env-specific IgM and IgG isotypes after Env immunization. These results show that pristane-treated B6 mice immunized with Env mounted antigen (Env)-specific IgM and IgG antibody responses that were comparable after 2 or 3 Env immunizations and were considerably elevated over alum alone (Fig. 6 J and not depicted).

Together, these data show that an experimental breach of immunological tolerance in wild-type mice promotes the production of antibodies able to neutralize tier 2 strains of HIV-1. Importantly, these mice retain the ability to mount Env-specific antibody responses that further increase the percentage of mice neutralizing tier 2 HIV-1 variants and the effectiveness of these antibodies. However, continued Env immunization ultimately focuses the antibody response on gp120-neutralizing epitopes restricted to tier 1 HIV-1 strains.

Neutralization of tier 2 HIV-1 strains by Env immunized pristane-treated mice correlates with elevated levels of IgM anti-histone H2A. Long chain hydrocarbons are known to act as adjuvants (Wilner et al., 1963), and pristane has been shown to promote polyclonal B cell activation and antibody secretion in mice (Kuroda et al., 2004). Thus, we measured total Ig serum levels 1 mo after pristane treatment of B6 mice, and after serial Env immunizations. These analyses showed that total IgM levels increased significantly 30 d after pristane treatment and again after two Env immunizations (Fig. 7 A). A third Env immunization, however, did not further increase total IgM levels significantly (Fig. 7 A). Pristane treatment also led to a significant increase in total IgG levels that were also increased after two Env immunizations (Fig. 7 B). In contrast to IgM, a third Env immunization did further increase IgG serum levels (Fig. 7 B), consistent with an ongoing class-switched Env-specific IgG response.

Elevated serum levels of IgM anti-H2A correlate with HIV-1 neutralization in alum-treated autoimmune-prone B6.Sle123 mice (Fig. 5). Thus, we measured IgM anti-H2A titers in pristane-treated B6 mice before and after Env immunization. These results show that anti-H2A IgM titers increase modestly 30 d after pristane treatment alone relative to naive mice and are significantly increased further after two Env immunizations (Fig. 7 C). After a third Env immunization of pristane-treated B6 mice, however, IgM anti-H2A autoantibody titers declined significantly (Fig. 7 C). The reduced IgM anti-H2A autoantibody titers after 3 Env immunizations correlated with a reduced frequency and potency of tier 2 HIV-1 neutralization and with increased neutralization of tier 1 HIV-1 strains (Fig. 6 D). Stratification of pristane-treated mice into tier 2 neutralizers and nonneutralizers revealed that tier 2 neutralizers again harbor significantly higher titers of anti-H2A IgM compared to tier 2 nonneutralizers (Fig. 7 D). In contrast, anti-histone H2B IgM titers were comparable between these cohorts (Fig. 7 E). These findings alongside our data on autoimmune B6.Sle123 mice demonstrate that anti-histone H2A IgM titers are significantly associated with the ability of mouse sera to neutralize tier 2 HIV-1 strains.

Histone H2A–reactive IgM monoclonal antibodies isolated from B6.Sle123 mice neutralize tier 2 strains of HIV-1 and recognize Env. Our data thus far demonstrate a significant correlation between the presence of serum tier 2 HIV-1 neutralization and increased titers of histone H2A–reactive IgM in both a genetically predisposed and pristane-induced autoimmune mouse model. To better understand the basis for this correlation, we generated hybridomas with splenocytes isolated from B6.Sle123 mice immunized 14 d previously with Env + alum and that displayed serum neutralization of tier 2 HIV-1 strains (Fig. 2 A). Monoclonal antibodies derived from these hybridomas were screened for reactivity to histone H2A by ELISA and subsequently characterized for additional autoreactive specificities, as well as specificity to HIV-1 Env, including HIV-1 neutralization. From these hybridomas, five IgM and two IgG mAbs were found to be H2A reactive (Fig. 8 B). Importantly, two of these five histone H2A–reactive IgM mAbs (Sle P4E4 and Sle O4C5, shown in red in Fig. 8), isolated from different mice, displayed relatively potent neutralizing activity against both tier 2 strains of HIV-1, JRFL, and YU2, but not tier 1 SF162.LS or BaL.26 (Fig. 8 A and Fig. S1 C).

We further assessed the specificities of these purified mAbs against chromatin autoantigens and HIV-1 Envelope proteins by ELISA, using the same range of concentrations tested in the TZMbl assay (0.023–50 µg/ml). These data revealed that these neutralizing IgM antibodies also dis-

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Env + alum immunizations. (I) Mean ID_{50} for the mice that neutralized tier 1 and 2 HIV-1 viruses after 3X Env + alum immunizations. (J) gp140 YU2-specific ELISAs for IgM and IgG between 2X alum (open circles) and 2X Env + alum (black circles) immunized mice. Data are representative of two independent experiments. P-values were calculated by Student's *t* test assuming nonequal variance; *, $P < 0.05$; **, $P < 0.001$.
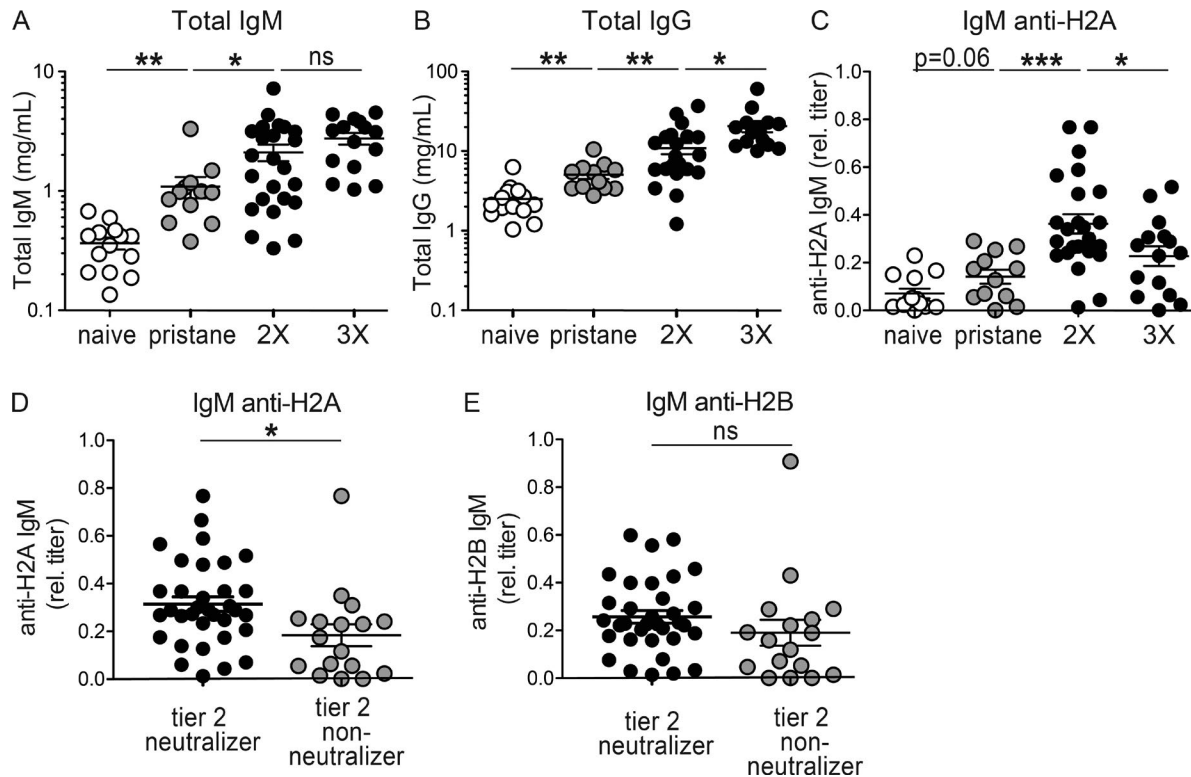
Figure 7. Elevated IgM anti–histone H2A titers correlate with tier 2 HIV-1 neutralization by pristane treated wild–type C57BL/6 mice. Total serum concentrations of (A) IgM, (B) IgG, and (C) relative titers of serum IgM anti-H2A are shown for naive (open circles) B6 mice or B6 mice treated 30 d with pristane only (gray circles) and subsequently immunized 2X or 3X (black circles) with alum alone or Env + alum. Serum from individual B6 mice (regardless of treatment with pristane alone, alum alone, or Env + alum) were separated based on neutralization of ≥1 tier 2 HIV-1 strains and measured for (D) IgM anti-H2A or (E) IgM anti-H2B relative titers. Mice neutralizing only tier 1 strains were included in the tier 2 nonneutralizer group (mostly 3X). Each symbol represents measurements for one mouse, and all data are plotted as the arithmetic mean ± SEM. All P-values were calculated using Student's *t* test assuming unequal variances. *, P < 0.05; **, P < 0.001; ***, P < 0.0001; ns, not significant. Data are representative of two independent experiments.

played slight reactivity against histone H2B and chromatin (Fig. 8 B), suggesting these antibodies may recognize a quaternary epitope on dsDNA/H2A/H2B nucleosomes. Lastly, we tested the hybridomas for reactivity against HIV-1 Envelope proteins to potentially explain why only two out of the five histone H2A-reactive IgM antibodies neutralized HIV-1. From these analyses, we found that only the two neutralizing histone H2A-reactive IgM antibodies recognized both gp140 (YU2) and the gp120 CD4-binding site (CD4bs, RSC3; Fig. 8 B). IgM recognition of the CD4bs was found to be specific, as we saw a considerable loss of reactivity with a mutated (null) CD4bs (ΔRSC3), as reported with other CD4bs-specific bnAbs, such as VRC01 (Wu et al., 2010). From these mAbs, we conclude that a subset of polyreactive IgM antibodies recognize both histone H2A and neutralizing epitopes on the HIV-1 Envelope protein.

DISCUSSION

A subset of HIV-1 bnAbs have been characterized to recognize both a neutralizing Env epitope and a self-antigen (Haynes et al., 2005a; Yang et al., 2013; Bonsignori et al., 2014;

Liu et al., 2015). Our data extend these findings to include an additional HIV-1–neutralizing polyreactive antibody that recognizes histone H2A. Based on these previous findings, it has been postulated that tolerance may pose a hurdle for Env-specific autoreactive B cells from mounting an HIV-1 antibody response. Indeed, it has been shown that central B cell tolerance can prevent the development of certain HIV-1–neutralizing clones (Haynes et al., 2005a,b; Verkoczy et al., 2011b). We have examined an important correlate of this hypothesis by investigating if breaching immunological tolerance is able to promote protective HIV-1 humoral immunity. Here, using lupus prone mouse models, we confirm that immunological tolerance indeed limits wild-type B cells from producing Env-specific antibodies able to neutralize tier 2 HIV-1 strains. We extend these findings by further formally demonstrating that a breach in peripheral tolerance can lead to the production of HIV-1–neutralizing antibodies in mice with wild-type immune systems.

Previous studies have evaluated the role of tolerance in the regulation of B cells that express autoreactive HIV-1 bnAb specificities (i.e., 2F5, 4E10) by generating Ig knock-in

A

Name	Isotype	SF162.LS IC50	BaL.26 IC50	JRFL IC50	YU2 IC50
Sle P4E4	IgM	>50	>50	5.7	6.8
Sle O4C5	IgM	>50	>50	32.4	4.7
Sle O5E2	IgM	>50	>50	>50	>50
Sle P4F2	IgM	>50	>50	>50	>50
Sle P4E2	IgM	>50	>50	>50	>50
Sle O6F6	IgM	>50	>50	>50	>50
Sle O7H11	IgG	>50	>50	>50	>50
Sle P1D1	IgG	>50	>50	>50	49.9

B
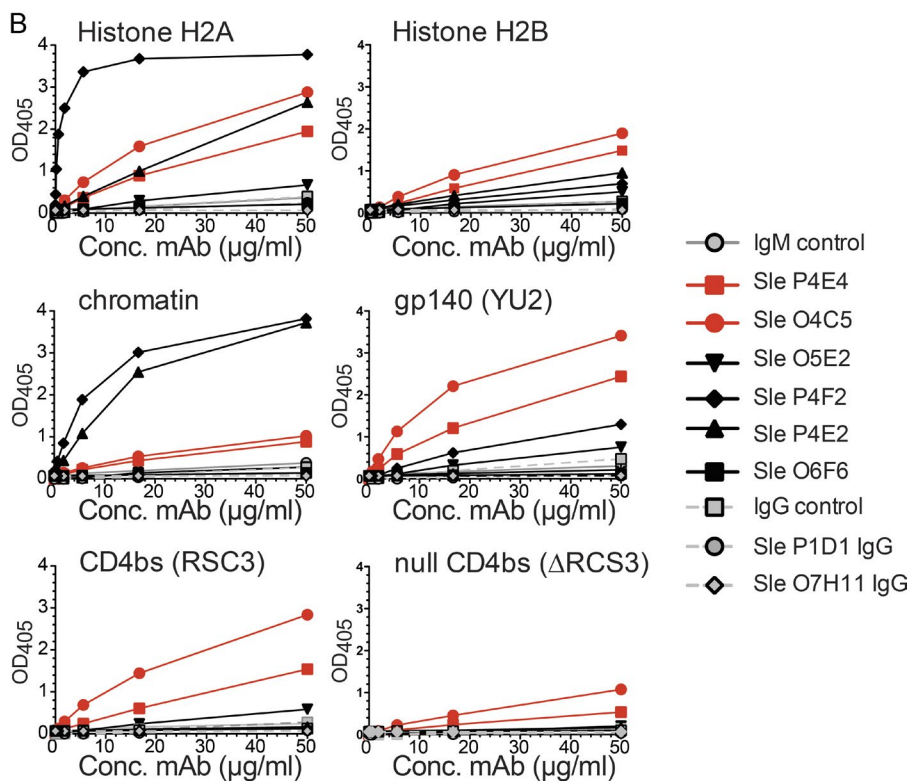
Figure 8. **Histone H2A–reactive IgM monoclonal antibodies, isolated from B6.Sle123 mice, neutralize tier 2 strains of HIV–1.** Hybridomas were generated from splenocytes isolated from B6.Sle123 mice that displayed serum tier 2 HIV-1 neutralization ($n = 2$). (A) Purified monoclonal IgM and IgG antibodies ($n = 8$) were tested for HIV-1 neutralization against 4 strains of HIV-1 and reported as IC_{50} (the concentration of antibody required for 50% neutralization). (B) Specificities of the monoclonal antibodies were tested using ELISA against the histone H2A, histone H2B, and chromatin nuclear antigens in addition to HIV-1 gp140 (YU2) Env and the CD4bs (RSC3) epitope, as well as a CD4bs-negative control (ΔRSC3). The two neutralizing IgM mAbs (P4E4 and O4C5) are shown with red symbols, and lines and non-neutralizing mAbs shown with solid black symbols and lines for IgM and gray symbols and dashed lines for IgG. IgM and IgG control antibodies were used to determine the background of the assay and for which ODs above this line were considered positive. Data are representative from three independent experiments.

mice. Specifically, the rearranged Ig genes encoding these specificities have been introduced into the Ig loci, such that all developing B cells express the autoreactive HIV-1 bnAb as a surface antigen receptor (Verkoczy et al., 2010, 2011a, 2013; Doyle-Cooper et al., 2013; Zhang et al., 2016). Results from these studies have shown that B cells with these HIV-1 bnAb autoreactive specificities are predominantly censored by central tolerance in the bone marrow that ultimately precludes their further development. Recently, a small number of germline 2F5-expressing splenic B cells displaying an anergic phenotype have been reported to mount a gp41-specific antibody response, although whether these antibodies were able to neutralize HIV-1 was not shown (Zhang et al., 2016). Together, these studies have documented that central toler-

ance prevents the development of B cells expressing an autoreactive broadly neutralizing HIV-1 specificity. However, autoreactive B cells that have escaped central B cell tolerance also exist in the peripheral B cell pool of both humans and mice where they have been characterized to be functionally anergic (Pugh-Bernard et al., 2001; Wardemann et al., 2003; Merrell et al., 2006; Koelsch et al., 2007). Whether peripheral tolerance normally limits the ability of these cells from mounting a protective HIV-1 antibody response has not yet been established.

We addressed this question with a different approach by initially asking if autoimmune prone strains of mice with known deficits in tolerance can mount HIV-1 Env-specific antibody responses that could be neutralizing. Our findings

revealed that although both healthy wild-type and autoimmune-prone mice mount Env-specific antibody responses, only autoimmune mice produce HIV-1–neutralizing antibodies. About half of Env immunized autoimmune prone, but not wild-type, mice generated serum antibodies able to neutralize tier 2 HIV-1 strains. Although B6.Sle123 mice mount normal primary Env-specific IgG antibody responses, Env was not required as an immunogen for HIV-1 neutralization; adjuvant treatment alone was sufficient to elicit tier 2 HIV-1–neutralizing antibodies. Thus, we conclude that in this autoimmune prone mouse strain, alum promotes autoreactive and Env-reactive B cells expressing neutralizing antibodies to differentiate into antibody-secreting cells.

A second autoimmune strain, MRL/*lpr*, also produced tier 2 HIV-1–neutralizing serum antibodies after alum treatment and also, in contrast to B6.Sle123, as naive mice (unpublished data). We consider that this difference between autoimmune strains likely reflects the fact that naive MRL/lpr mice harbor relatively high titers of autoreactive antibodies in sera and develop lupus at significantly younger ages than B6.Sle123 mice. Regardless, together our findings further demonstrate certain autoreactive specificities are able to neutralize HIV-1. Our findings using mouse models is also consistent with a recent study by Bonsignori et al. (2014) on an individual with SLE who was infected with HIV-1 before autoimmune diagnosis but controlled her viral load without antiretroviral therapy. The plasma from this individual also displayed multi-clade broadly neutralizing HIV-1 activity and an autoreactive (anti-dsDNA) Env-specific antibody (CH98) with similar breadth and potency was isolated from a memory B cell (Bonsignori et al., 2014). Thus, although impaired tolerance in both humans and mice has already been associated with the presence of Env-specific antibodies (Kion and Hoffman, 1991; Barthel and Wallace, 1993; Lombardi et al., 1993; Mylonakis et al., 2000; Carugati et al., 2013), our results demonstrate that Env-specific antibodies produced as a consequence of a breach in tolerance have the potential to provide humoral protection against HIV-1.

It is interesting that a sizeable proportion of bnAbs with specificities to the conserved CD4-binding site on gp120 or MPER on gp41 also recognize self-antigens. Whether this reflects a mechanism evolved by HIV-1 to avoid adaptive immunity is not clear. However, endogenous retroviruses have comprised a sizable portion of the mammalian genome for millions of years and feasibly altered the evolution of our immune system. Thus, some retroviral-encoded antigens may indeed be considered self by the vertebrate immune system and antibodies capable of recognizing these antigens may be released when tolerance is compromised and possibly accounting for the weak muLV neutralizing in both the genetically prone and experimentally induced autoimmune mouse models.

It was important to unequivocally demonstrate that serum antibody was responsible for the observed HIV-1 neutralization in our assays, because the ability of serum antibody to neutralize HIV-1 has previously mistakenly been reported

(LaCasse et al., 1999; Nunberg, 2002). Accordingly, we used independent approaches to document antibody-mediated HIV-1 neutralization that included showing that purified serum IgG and two IgM mAbs isolated from immunized B6.Sle123-derived hybridomas were able to neutralize tier 2 HIV-1. Thus, although the tier 2 HIV-1–neutralizing titers from sera were relatively weak, this neutralizing activity was specific and generated using a suboptimal immunogen after one or two immunizations of B6.Sle123 and pristane-treated wild-type mice, respectively.

A major finding presented in this study demonstrates that pristane treatment of wild-type B6 mice followed by Env immunization elicits tier 2 HIV-1–neutralizing antibodies. Pristane has been characterized to compromise immunological tolerance in wild-type mice leading to the production of autoantibodies against nuclear antigens beginning about 1 mo after treatment with increasing titers accumulating thereafter (Satoh and Reeves, 1994; Satoh et al., 2000). As autoimmune prone mice treated with alum alone produced tier 2 HIV-1–neutralizing antibodies, we expected alum to also elicit HIV-1–neutralizing antibodies in pristane-treated B6 mice. Although this was the case, we additionally show that Env immunization furthered the breadth and potency of neutralizing activity. This indicates enhanced neutralizing activity results from an Env-driven antibody response in mice with impaired immunological tolerance. These data support proposals suggesting that a break in immunological tolerance before HIV-1 Env vaccination may be a first step to eliciting bnAbs (Verkoczy et al., 2011b; Dosenovic et al., 2012; Zhang et al., 2016). Indeed, relaxing transitional B cell selection by administering B cell activating factor (BAFF) before Env immunization in wild-type mice also increased serum autoantibodies which correlated with the presence of tier 1 HIV-1–neutralizing antibodies (Dosenovic et al., 2012). Our study extends these findings by formally demonstrating a pristane-induced break in peripheral tolerance promotes the production of tier 2 HIV-1–neutralizing antibodies. Further, we identify a novel histone H2A autoreactive specificity that is also able to neutralize tier 2 HIV-1 strains.

How alum elicits HIV-1–neutralizing antibodies in the B6.Sle123 autoimmune mouse strain is not clear. We note, however, that when used alone, alum is able to induce several proinflammatory cytokines, such as IL-1β, IL-5, and IL-6 (McKee et al., 2009). Further, alum and other adjuvants, including pristane can induce autoimmune-like features in healthy individuals (Vera-Lastra et al., 2013). We propose that alum and pristane treatment generate a proinflammatory setting that promotes poly-/autoreactive B cells, which are normally restrained by tolerance mechanisms, to differentiate and secrete autoantibodies that are also able to neutralize HIV-1.

Not all autoimmune-prone mice produced HIV-1–neutralizing antibodies (∼50% of B6.Sle123 and 60–80% of MRL/*lpr*). Among the parameters tested, the only significant correlation with tier 2 HIV-1–neutralizing activity of B6.Sle123 mice were with elevated levels of serum IgM and

titels of IgM anti-H2A histone protein. Importantly, pristane-treated B6 mice also elicited tier 2 HIV-1–neutralizing antibody responses and harbored elevated titers of anti-H2A IgM compared with nonneutralizers. Thus, these data reveal a significant correlation between titers of IgM H2A-reactive autoantibodies and tier 2 HIV-1 neutralization. We confirmed this correlation by isolating histone H2A-reactive IgM mAbs that also recognize HIV-1 Env and the gp120 CD4 binding site and are able to neutralize tier 2 HIV-1. The exact epitope or posttranslational modification of histone H2A recognized by these mAbs has not yet been identified. Autoreactive specificities in other Env-specific antibodies have been shown to increase the apparent affinity of a bnAb for the virion by virtue of heteroligation, the recognition of both Env and a putative self-antigen (e.g., phospholipid) on the virion surface (Mouquet et al., 2010). It is difficult to envision how an anti-H2A specificity may promote heteroligation in this manner. Alternatively and most likely, the histone H2A specificity may reflect an example of molecular mimicry where HIV-1 Env has evolved to mimic an epitope on histone H2A as a mechanism of immune camouflage implemented via immunological tolerance.

Mice are not a physiological host for HIV-1 and eliciting neutralizing activity for tier 1 HIV-1 has been a general challenge in mouse models. When achieved in wild-type mice, typically after multiple Env immunizations, neutralization has been limited in breadth to neutralization sensitive, lower tier HIV-1 strains (McCoy and Weiss, 2013; Hu et al., 2015). However, tier 2 HIV-1 strains are more similar to founder viruses that dominate new infections, which a successful vaccine would be expected to protect against (de Taeye et al., 2016). Immunization of rabbits and macaques with more stable, native-like Env trimers (Sanders et al., 2002; Beddows et al., 2005; Burton and Hangartner, 2016; de Taeye et al., 2016) can elicit autologous tier 2 HIV-1–neutralizing antibodies (de Taeye et al., 2015, 2016; Sanders et al., 2015). However, immunization of wild-type mice with similar Env trimers have only elicited tier 1 HIV-1–neutralizing antibodies thus far (Hu et al., 2015). Considering these findings, our immunization of pristane-treated wild-type B6 mice twice with a (relatively unstable) Env trimer that elicits tier 2 HIV-1–neutralizing antibodies is noteworthy.

The nature of the B cells in pristane-treated wild-type mice that give rise to tier 2 HIV-1–neutralizing activity remains unclear. In humans, the anti-gp160 antibody response to HIV-1 has been shown to predominantly arise from poly-/autoreactive mature B cells (Mouquet et al., 2011; Mouquet and Nussenzweig, 2012). In mice, transitional, anergic and marginal zone B cell subpopulations have all been characterized to express a poly-/autoreactive antibody repertoire (Chen et al., 1997; Carey et al., 2008), and we previously reported that marginal zone B cells from naive wild-type B6 mice often express a gp120-reactive antibody receptor that also displays autoreactivity toward nuclear antigens (Pujanauski et al., 2013). Which of these B cell populations,

if any, is responsible for tier 2 HIV-neutralizing activity and whether Env-elicited HIV-1–neutralizing B cells can develop into memory B cells remains to be determined but can be addressed with mouse models. Regardless, our study shows that immunological tolerance limits the ability of these autoreactive precursor B cells to produce Env-specific antibodies capable of neutralizing tier 2 HIV-1 strains.

MATERIALS AND METHODS
Mice
Wild-type C57BL/6J, B6.Sle123 (B6.NZM-*Sle1/Sle2/Sle3*$^{NZM2410/Aeg}$/LmoJ), and MRL/lpr (MRL/MpJ-*Faslpr*/J) were purchased from The Jackson Laboratory and bred in specific pathogen–free conditions in the animal facility at the University of Colorado-Anschutz Medical Campus (Aurora, CO) or at the Biological Resource Center at National Jewish Health (NJH; Denver, CO). Male and female mice were used between 7 and 45 wk of age. All experiments were approved and performed in accordance with the University of Colorado Denver and NJH Institutional Animal Care and Use Committee.

HIV–1 Envelope protein production and purification
HIV-1 Envelope proteins were made and purified as described previously (Pujanauski et al., 2013). Monomeric gp120 (ADA) and trimeric gp140 (YU2) were produced by transient transfection of COS7 cells (ATCC) with 5 µg of gp120 or gp140 plasmid (provided by T.M. Ross, University of Georgia, Athens, GA) and Lipofectamine (Invitrogen). gp120 and gp140 were purified as before (Pujanauski et al., 2013), using a column made with agarose-bound lectin (Vector Laboratories). HIV-1 Envelope protein, bound to the column, was washed using PBS, and eluted with 1M methyl mannopyranoside (Sigma-Aldrich). The eluted protein was buffer exchanged and concentrated into sterile PBS using 15 ml capacity centrifugal 30-kD cutoff filters (Ambicon). Protein purity was checked by Western blot with murine anti-gp120 antibody (ImmunoDx) and SDS-PAGE.

Immunizations and sera collection
Mice were immunized i.p. with 50 µg of either monomeric gp120 or trimeric gp140 using Alu-Gel-S (Thermo Fisher Scientific) as an alum adjuvant (Bower et al., 2004, 2005). Dose solutions were prepared by diluting HIV-1 Envelope proteins into PBS at the proper concentration and an equal volume of Alu-Gel-S was added. For alum-only injections, Alu-Gel-S was mixed 1:1 with sterile PBS. Immunogens were made at least 4 h before dosing, and were incubated at 4°C while rotating to achieve an even suspension. For pristane treatment, 500 µl of pristane (2,6,10,14-Tetramethylpentadecane; Sigma-Aldrich) was injected i.p. Blood was collected from mice by tail vein bleeds before immunization and by cardiac puncture at time of sacrifice 14 d after immunization, unless otherwise noted. Sera was prepared by collecting blood in 1.1-ml serum microtubes containing Z gel (Sarstedt) and incubating for 45 min at room

temperature to allow proper coagulation. Serum was collected by centrifugation at 10,000 g for 4 min at room temperature, pipetted into Eppendorf tubes, and stored at −20°C.

ELISA for HIV-1 Envelope–reactive Ig and total Ig quantitation in sera

To detect HIV-1 Envelope reactive Ig, ELISAs were done as previously described (Pujanauski et al., 2013). 96-well Nunc-Immuno MaxiSorp plates (Thermo Fisher Scientific) were coated with 2 µg/ml of gp120 (ADA) or gp140 (YU2) in PBS overnight at 4°C. Plates were washed three times with 0.1% Tween-20/PBS, blocked for 2 h at room temperature with 1% BSA in PBS, and washed again. To capture Envelope-reactive antibodies, mouse sera samples were diluted starting at 1:10 in PBS, and subsequently serially diluted threefold for a total of eight dilutions per sample. 50 µl of mouse serum dilutions were added to coated ELISA plates and incubated for at least 2 h at room temperature or overnight at 4°C. Plates were washed three times and incubated with alkaline phosphatase (AP)-conjugated goat anti–mouse isotype-specific (IgM, IgG, IgG1, IgG2b, IgG2c, IgG2a, or IgG3) detection antibody (Southern Biotech) diluted 1:2,000 in blocking buffer for 1 h at 37°C. Plates were washed and color was developed by adding 1 mg/ml of 4-nitrophenyl phosphate disodium salt hexahydrate (Alkaline Phosphatase Substrate; Sigma-Aldrich) diluted in developing buffer (1M diethanolamine, 8.4 mM MgCl$_2$, and 0.02% NaN3, pH 9.8). Absorbance values were read at 405 nm on VersaMax ELISA reader (MDS Analytical Technologies). Titers were calculated from the serial dilutions by calculating the reciprocal serum dilution that gives half of the maximal OD405 response. Titer calculations were performed using GraphPad Prism software (v5) by first log transforming the reciprocal dilutions values followed by calculating EC$_{50}$ values using the sigmoidal dose response with variable slope equation. Samples that did not reach the maximal OD$_{405}$ response at any dilution, compared with the positive control, were set below the limit of detection.

Total IgM and IgG concentrations in mouse sera were quantitated using a sandwich ELISA as described previously (Swanson et al., 2010). 96-well plates were coated with either goat anti–mouse IgM or goat anti–mouse Ig(H+L) (Southern Biotech) diluted at 1/100 in PBS. Plates were blocked, washed, and developed as for the HIV-1 Envelope ELISAs. To capture total Ig, mouse sera samples were diluted starting at 1:1500 in PBS, and subsequently serially diluted threefold for a total of eight dilutions per sample. Standard curves containing known concentrations of mouse IgM or IgG (Southern Biotech) were added to each plate and a four parameter equation was fitted using VersaMax software. Total serum Ig concentrations were quantitated from the standard curve equations using OD$_{405}$ values in the linear absorbance range.

ELISA for autoantigen reactive Ig in sera

Chromatin and Smith Ig ELISAs were performed as previously reported (Fournier et al., 2012; Larson et al., 2012).

In brief, to detect chromatin-reactive Ig, plates were coated with 10–15 µg/ml of calf thymus chromatin in PBS, 1 mM EDTA, 0.05% NaN$_3$ buffer, overnight at 4°C. Chromatin was either a gift from L. Wysocki (National Jewish Health, Denver, CO) and John Cambier (University of Colorado Denver, Aurora, CO). After coating, plates were washed once with 0.5% Tween 20/PBS, blocked for 2 h at 37°C with 1% BSA, 1 mM EDTA, 0.05% NaN$_3$ in PBS, and washed three times with 0.5% Tween 20/PBS. To capture chromatin-reactive antibodies, mouse sera samples were diluted starting at 1:30 in blocking buffer, and subsequently serially diluted threefold for a total of eight dilutions per sample. 50 µl of mouse serum dilutions were added to coated ELISA plates and incubated for at least 2 h at room temperature or overnight at 4°C. Plates were washed three times and incubated with alkaline phosphatase (AP)-conjugated goat anti–mouse isotype-specific (IgM or IgG) detection antibody (Southern Biotech) diluted 1:1,000 in blocking buffer for 1 h at 37°C.

For Histone H2A- and H2B-reactive IgM ELISAs, plates were coated with 1 µg/ml of human recombinant histone H2A or H2B (New England Biolabs) in PBS pH 7.4. Plates were washed, blocked with 1% BSA in PBS, developed, and serum samples diluted, as described for chromatin-reactive Ig ELISAs. For Smith antigen-reactive Ig ELISAs, plates were coated with 1 µg/ml of Smith antigen (Meridian Life Sciences) diluted in 0.05M carbonate buffer, pH 9.5, overnight at 4°C. Plates were washed, blocked, and developed, and serum samples diluted, as described for chromatin-reactive Ig ELISAs. For cardiolipin-reactive Ig ELISAs, 50 µg/ml of cardiolipin sodium salt (Sigma-Aldrich) was diluted in 100% molecular grade ethanol (Thermo Fisher Scientific) and allowed to evaporate completely overnight at 4°C (Dosenovic et al., 2012). Blocking buffer (1% BSA/PBS) was added the next day after coating. Plates were washed, blocked, and developed, as described for HIV-1 Envelope-reactive ELISAs. All serum samples for autoantigen ELISAs were diluted, starting at 1:30 and subsequently serially diluted threefold for 8 total dilutions.

Autoantigen ELISAs were read at OD405 nm and titers calculated as described for HIV-1 Envelope-reactive Ig ELISAs. All titers were normalized to a positive control serum sample added to each plate to attain a relative titer. In this case, the positive control sera was pooled from old (>16 wk) lupus prone MRL/lpr and B6.Sle123 mice. Each positive control aliquot had previously been tested for chromatin, Smith, and cardiolipin-reactive IgM and IgG, and the appropriate aliquot used for each ELISA.

Autoantigen protein array

Sera samples from six B6.Sle123 neutralizers, six B6.Sle123 nonneutralizers, and three B6 nonneutralizers were sent to UT Southwestern for IgM and IgG binding analysis on the 95 autoantigen protein array panel II. Samples were prepared and data analyzed by the UT Southwestern Microarray core as described previously (Li et al., 2006; Chong et al., 2012).

In brief, serum samples are treated with DNase I before incubation on the 95 autoantigen array chip, and mouse IgM and IgG were detected with fluorescent antibodies. Array chips are read using GenePix 4400A Microarray scanner, and data were analyzed by GenePix 7.0 software. Fluorescent intensities for each autoantigen are averaged, normalized to internal controls, and data are reported as both normalized signal intensity and the signal to noise ratio (SNR).

TZMbl neutralization assays

Neutralization assays using TZMbl cells with mouse sera were performed as described previously (Sarzotti-Kelsoe et al., 2014) and as per the Montefiori website (Duke University). TZMbl cells were received through the National Institutes of Health AIDS Reagent Program (Division of AIDS, NIAID), and all assays were performed in BSL2^{+} and BSL3 facilities, in accordance with UCD Biosafety protocols. Before assaying mouse sera collected from immunized and control B6.Sle123, MRL/lpr, and B6 mice, sera were heat inactivated at 55°C for 45 min. Sera were vortexed and centrifuged immediately before diluting for the neutralization assay. Mouse sera samples were run in duplicate for each viral assay using white opaque, 96-well CulturPlates (Perkin Elmer). Heat-inactivated mouse sera was diluted starting at 1:15 in DMEM, followed by 1:3 serial dilutions for a total of eight dilutions in 100 µl total volume. In cases where purified monoclonal antibodies were tested, purified antibody samples were diluted to a starting concentration of 50 µg/ml, and serially diluted 1:3 down the plate for a total of eight dilutions in 100 µl total volume. HIV-1 strains were diluted at the predetermined viral titer (see HIV-1 production and titration section) in DMEM. 50 µl of diluted virus was added to each well containing mouse sera dilutions and allowed to incubate for 2 h at 37°C, 5% CO_2. Given the 1:2 dilution adding virus, the final starting dilution of mouse sera was 1:30. TZMbl cells were trypsinized, washed, and 10,000 cells/100 µl in complete DMEM + 10% FBS were added to each well. Each plate was run with one column (8 wells) of cell only control wells (150 µl media + 100 µl cells) and one column of virus controls (100 µl DMEM + 50 µl diluted virus + 100 µl cells). Cells were incubated for 2 d at 37°C, 5% CO_2. After incubation, 100 µl of supernatant was removed, replaced with 100 µl luciferase substrate (Britelite plus Reporter Gene Assay system; Perkin Elmer), and pipetted up and down to lyse cells. Plates were read on a Victor X light luminescence plate reader (Perkin Elmer) after 2 min after cell lysis, but within 15 min.

Serum neutralization titers are expressed as inhibitory dose 50 (ID_{50}), the reciprocal of the serum dilution that produces 50% virus neutralization, as compared with the virus controls. Percent neutralization was calculated using the luminescence Excel macro, provided by D. Montefiori online and modified by P. Gao (Duke University, Durham, NC). The equation used to calculate percent neutralization is (average RLU virus control − RLU sample well)/(average RLU virus control − average RLU cell control) × 100. Percent neu-

tralization data were used when the RLU values for virus only controls were >10-fold above background, and the standard deviation was within 30% for the cell only and virus only controls, as well as between sample duplicates. Each day a neutralization assay was run, a positive control antibody, VRC01, was run in duplicate to validate the assay. VRC01 was diluted starting at 2 µg/ml and serially diluted 1:3 for a total of 8 dilutions. The IC_{50} was calculated as the antibody concentration needed to give 50% neutralization. The IC_{50} obtained for each virus strain was in good agreement with previously published IC_{50} values (Wu et al., 2010), and consistent across neutralization assays run on different days (standard deviation ±30%).

Neutralizing antibody titers against Friend muLV was performed as previously described (Santiago et al., 2008) by co-incubating serial dilutions of mouse sera with 50 infectious units of Friend muLV before infection of *Mus dunni* cells. Sera from Friend muLV-infected mice were used as a positive control. IC_{50} values were computed based on a one-site sigmoidal fit (GraphPad Prism).

HIV-1 production and viral titration

Pseudo-viruses (SF162.LS, JR-FL, and BaL.26) were produced by transient co-transfection of 5 µg/ml of gp160 encoding plasmid and 10 µg/ml of SG3Δenv plasmid in 293T cells. The SG3Δenv, BaL.26, and SF162.LS plasmids were all obtained through the National Institutes of Health AIDS Reagent Program, Division of AIDS, NIAID, NIH (Cheng-Mayer et al., 1997; Stamatatos et al., 2000; Wei et al., 2002, 2003; Li et al., 2006). The JR-FL plasmid was a gift from P. Clapham at the University of Massachusetts (Gonzalez-Perez et al., 2012). YU2 full-length virus (Gene Bank M93258) was also produced by transient transfection of 293T cells using 20 µg/ml of plasmid. The YU2 plasmid was obtained through the NIH AIDS Reagent Program (Li et al., 1991, 1992).

Transfections were done with 293T cells, growing at 50–70% confluency, in T75 flasks. DNA was diluted in 0.13 M $CaCl_2$ containing HBS buffer (Hepes buffered saline), pH 7.0, and incubated at room temperature for 30 min to allow DNA aggregates to form. DNA was added to cells, with fresh media (complete DMEM + 10% FBS). Cells were incubated at 5% CO_2 at 37°C, media was replaced 16 h later, and viral supernatants collected 24 h later. Viral supernatants were filtered through 0.2 µm syringes, aliquoted, and stored at −80°C. Each virus was titrated before use, as previously reported (Sarzotti-Kelsoe et al., 2014). In brief, viral supernatants were thawed, diluted starting at 1:3 in DMEM, and serially diluted 1:3 for a total of 8 dilutions, and 100 µl total volume in white opaque, 96-well CulturPlates (Perkin Elmer). TZMbl cells were infected by adding 10,000 cells to each well in 100 µl of complete DMEM + 10% FBS. 2 d later, cells were lysed, luciferase substrate added, and read as for the TZMbl assay described in the preceding section. Data are given by the instrument in relative light units (RLUs) and plotted against

reciprocal dilutions to calculate the dilution of virus needed to produce a mean of 150,000 RLUs in lysed TZMbl cells. This was the viral dilution (titer) used to infect cells in the TZMbl neutralization assays.

VRC01 production and purification

VRC01 was produced by transiently co-transfecting 293F cells (Invitrogen) using 250 µg of heavy and 250 µg of light chain plasmids and 293Fectin reagent (Invitrogen). The VRC01 heavy and light chain plasmids (CMVR VRC01 H + L) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Wu et al., 2010). Cell supernatants were collected after 5 d by centrifugation and filtered through a 0.22-µm filter (Nalgene). VRC01 was purified over a protein G column, buffer exchanged into PBS, and stored at −20°C. VRC01 was validated using ELISA and found to bind the CD4bs (RSC3), with minimal binding to null CD4bs (ΔRSC3), as reported (Wu et al., 2010). RSC3 and ΔRSC3 were obtained from the NIH AIDS Reagent Program.

Generation of B cell hybridomas and purification of monoclonal antibodies

B cell hybridomas were generated from B6.Sle123 mice immunized with Env + alum and that displayed serum neutralization of tier 2 HIV-1 strains, as previously described (Fournier et al., 2012; Pujanauski et al., 2013). Cell supernatants from hybridomas were screened at least twice for histone H2A-reactivity 7 d after selection. Monoclonal antibodies were considered histone H2A–reactive if they had an OD_{405} > 1.0 after successive screening using two different blocking proteins: bovine serum albumin (Thermo Fisher Scientific) and fish skin gelatin (Sigma-Aldrich). For negative Ig controls, hybridoma supernatants that tested negative for histone H2A were selected at random. Hybridomas were cultured in complete RPMI 1640 medium with 15% FBS, depleted of bovine IgG by passage over a protein G column, until IgM and IgG levels were in sufficient concentrations in supernatants for purification.

IgG monoclonal antibodies were purified from hybridoma supernatants using a protein G column. IgM monoclonal antibodies were purified with affinity chromatagraphy using an anti–mouse IgM mAb (rat IgG2a; clone R33-24.12) covalently coupled to Sepharose beads. Both IgM and IgG monoclonal antibodies were eluted from columns using 0.1M glycine-HCl, pH 2.8, and pH neutralized using 1M Tris-HCl, pH 9.0. Purified antibodies were concentrated and buffer exchanged into PBS, pH 7.4 at least three times using 15 ml capacity centricons with 30 kD filter cutoff (Ambicon). Mouse IgM and IgG concentrations were quantified using sandwich ELISA, as described in Materials and methods. SDS-PAGE analysis indicated no contamination of the monoclonal IgM antibodies with the rat monoclonal IgG antibody coupled to Sepharose. Concentrations were adjusted to 50 µg/ml for testing using the TZMbl assay for

HIV-1 neutralization and using ELISA for antibody specificities, as described.

Purification of IgG from sera using protein G beads

To purify IgG from the sera of B6.Sle123 and B6 mice, we used a previously described protocol for depletion of IgG from sera (Melchers et al., 2012). In brief, 120 µl of sera + 200 µl of 50% (vol/vol) of protein G agarose beads (Thermo Fisher Scientific) + 680 µl PBS (pH 7.4) were incubated overnight at 4°C while rotating. The next day, samples were centrifuged at 5,000 g for 2.5 min, and supernatant was collected into another microcentrifuge tube, and repeated if necessary until no beads were left. Protein G beads were washed with 500 µl of 1X RIPA/Tween-20 buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1%NP-40, 0.25% sodium deoxycholate, 0.05% Tween-20, protease inhibitors) at least five times. After each wash, samples were centrifuged at 5,000 g for 2.5 min. IgG was eluted from the beads by adding 1,100 µl of IgG elution buffer (0.1M glycine-HCl, pH 2.8), and tubes were inverted to mix. To neutralize the elution buffer and bring the pH back to 7.0, 100 µl of 1M Tris-HCl pH 9.5 was added. Samples were centrifuged at 5,000 g for 2.5 min to remove beads. The supernatants were collected, concentrated, and buffer exchanged at least 10 times into PBS (pH 7.4). IgG concentrations were measured and quantitated by sandwich ELISA, as described above, and the purified IgG was shown to be pure using SDS-PAGE. Purified IgG samples were tested for neutralization by the TZMbl assay, described above, with a starting concentration of 100 µg/ml, and serially diluted threefold for a total of 8 dilutions in 100 µl total volume. The IC_{50}, defined as the concentration of antibody required for 50% neutralization, was calculated using the TZMbl neutralization Excel macro and reported for the purified antibody samples.

Statistics and data analysis

Statistics were calculated using GraphPad Prism software (v5). Statistical significance was analyzed using a two-tailed Student's t test assuming unequal variances or Mann-Whitney test when the variances were not a Gaussian distribution. Neutralization data between immunization groups and controls was analyzed using Fisher's Exact Test. $P \leq 0.05$ was considered significant and denoted as *, $P < 0.05$; **, $P < 0.001$; and ***, $P < 0.0001$. If no statistics are reported, data are not statistical significant ($P > 0.06$). Data were graphed as the geometric mean ± SEM.

Online supplemental material

Fig. S1 shows tier 1 and tier 2 HIV-1 neutralization curves by serum from B6.Sle123 and C57BL/6 mice from Figs. 1 and 6. Fig. S2 shows tier 2 HIV-1 neutralization by IgG purified from alum ± Env immunizations of B6.Sle123 and C57BL/6 mice.

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