MHC class I–specific antibody binding to nonhematopoietic cells drives complement activation to induce transfusion-related acute lung injury in mice

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Transfusion-related acute lung injury (TRALI), a form of noncardiogenic pulmonary edema that develops during or within 6 h after a blood transfusion, is the most frequent cause of transfusion-associated death in the United States. Because development of TRALI is associated with donor antibodies (Abs) reactive with recipient major histocompatibility complex (MHC), a mouse model has been studied in which TRALI-like disease is caused by injecting mice with anti–MHC class I monoclonal Ab (mAb). Previous publications with this model have concluded that disease is caused by FcR-dependent activation of neutrophils and platelets, with production of reactive oxygen species that damage pulmonary vascular endothelium. In this study, we confirm the role of reactive oxygen species in the pathogenesis of this mouse model of TRALI and show ultrastructural evidence of pulmonary vascular injury within 5 min of anti–MHC class I mAb injection. However, we demonstrate that disease induction in this model involves macrophages rather than neutrophils or platelets, activation of complement and production of C5a rather than activation of FcγRI, FcγRIII, or FcγRIV, and binding of anti–MHC class I mAb to non–BM-derived cells such as pulmonary vascular endothelium. These observations have important implications for the prevention and treatment of TRALI.

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Abbreviations used: Ab, antibody; BAL, bronchoalveolar lavage; i.t., intratracheal(ly); mTRALI, mouse TRALI; ROI, reactive oxygen intermediate; TRALI, transfusion-related acute lung injury.

Transfusion-related acute lung injury (TRALI) is defined as new onset or worsening of pulmonary function with acute hypoxemia and noncardiogenic pulmonary edema during or within 6 h after blood transfusion (Kopko, 2004; Chapman et al., 2009; Silliman et al., 2009; Vlaar et al., 2009). Associated clinical features include dyspnea, tachypnea, cyanosis, tachycardia, and froth in an endotracheal tube (Kopko, 2004; Toy et al., 2005; Silliman et al., 2009). Although rare (~1 case per 2,000–5,000 transfusions of blood or blood products, with a mortality rate of ~6%; Kopko, 2004; Toy et al., 2005; Silliman et al., 2009;Vlaar et al., 2009), the incidence of TRALI is increasing as clinical awareness increases and a more uniform case definition is being used. As a result, TRALI is now the leading cause of transfusion-related mortality in developed countries (Kopko, 2004; Toy et al., 2005; Silliman et al., 2009;Vlaar et al., 2009).

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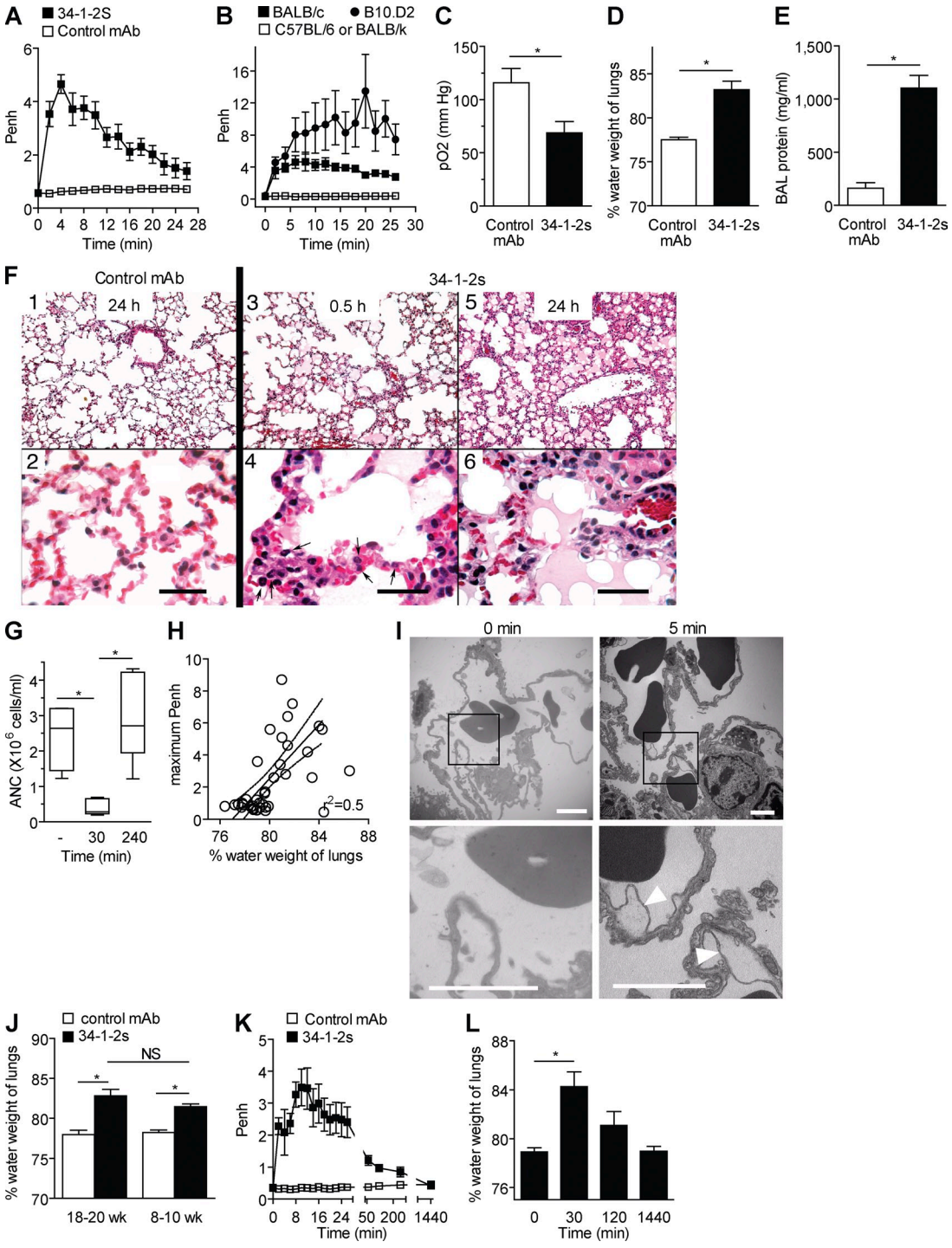

Figure 1. mTRALI resembles human TRALI. (A) BALB/c male mice (four mice per group) were injected i.v. with 34-1-2s (mouse IgG2a anti–H-2D^d/K^d mAb) or mouse IgG2a isotype control mAb (δIA6.2) and evaluated for the next 26 min for changes in breathing pattern suggestive of dyspnea (shown as increases in Penh). Data are representative of more than five experiments. (B) BALB/c (H-2^d), B10.D2 (H-2^d), BALB/k (H-2^k), and C57BL/6 (H-2^b) background male mice (four mice per group) were injected i.v. with 34-1-2s. Penh was evaluated. Pooled graph is representative of two BALB/k, three C57B/6, three B10.D2, and three BALB/c experiments. (C) BALB/c male mice (10 mice per group) were injected i.v. with 34-1-2s or control mAb. Arterial blood pO_2 was measured 15 min later. Results are pooled from two experiments. (D) BALB/c male mice (five mice per group) were injected i.v. with 34-1-2s or control mAb; 30 min later, lungs were harvested, and percent water content was measured. Data are representative of three experiments. (E) BALB/c male mice (five mice per group) were injected i.v. with 34-1-2s or control mAb; 30 min later, BAL fluid was collected, and its protein content was measured. Data are representative of two experiments. (F) Representative H&E-stained sections of lung from BALB/c male mice injected i.v. with control mAb (1 and 2) or 34-1-2s (3–6). Lungs were harvested 0.5 (3 and 4) or 24 h (1, 2, 5, and 6) later. Arrows are pointing to neutrophils. (G) BALB/c male mice (five mice per

Laboratory features associated with TRALI can include transient acute leukopenia (Kopko and Popovsky, 2004), antibodies (Abs) in donor plasma that react with recipient HLA class I or II, granulocytes or monocytes (Kopko et al., 2003; Gajic et al., 2007; Chapman et al., 2009; Silliman et al., 2009), and increased donor plasma concentration of substances, such as lipid mediators, that can activate recipient neutrophils (Silliman et al., 1997, 1998; Gajic et al., 2007; Fung and Silliman, 2009). These laboratory features suggest two principle etiologies of TRALI that might act together or independently: (1) Ab activation of leukocytes that damages recipient lungs (Kopko et al., 2003; Silliman et al., 2009; Shaz et al., 2011) and (2) increased concentrations of platelet-derived vasoactive mediators that develop during blood storage and directly increase pulmonary vascular permeability or induce leukocytes to release mediators that have this effect (Silliman et al., 2009; Shaz et al., 2011). Although TRALI has occurred in individuals who appeared to have no predisposing risk factor before transfusion (Engelfriet et al., 2001; Toy et al., 2005), it occurs most commonly in individuals who have disorders, such as septic shock, recent surgery, or disseminated intravascular coagulation that may act additively or synergistically with Abs or mediators delivered by transfusion to acutely increase pulmonary vascular permeability (Gajic et al., 2007; Chapman et al., 2009; Fung and Silliman, 2009; Silliman et al., 2009; Vlaar et al., 2009; Shaz et al., 2011). Such preexisting disorders may prime leukocytes or vascular endothelial cells to react more potently to antileukocyte Abs and/or mediators.

Previously reported studies have modeled human Ab–induced TRALI by injecting mice of the H-2^d haplotype with 34-1-2s, a mouse IgG2a mAb to H-$2D^d$ and H-$2K^d$ (MHC class I antigens; Looney et al., 2006, 2009). Injection of this mAb rapidly induces pulmonary vascular leak that increases lung water content and is associated with temporary neutropenia (Looney et al., 2006). Studies with this system suggested a pathogenic mechanism that involves FcγRs, neutrophils, and platelets, with generation of reactive oxygen intermediates (ROIs) that cause pulmonary vascular leak by damaging vascular endothelium (Looney et al., 2006, 2009). However, these studies did not address two important features of this model: (1) 34-1-2s induces TRALI-like disease in normal male but not female mice, although human TRALI occurs with equal frequency in both genders, and (2) other IgG2a anti–H-2^d mAbs fail to induce detectable disease. These features raised doubts about the pathogenic mechanism that was reported and led us to perform additional experiments that injected mice of the same strain (BALB/c) with the same anti–MHC class I mAb. Based on the results of these experiments, we now report that the TRALI-like syndrome induced by this mAb is mediated primarily by complement, particularly C5a, rather than by FcγR, and by peripheral blood monocytes, rather than platelets or neutrophils. We also find that anti–MHC class I mAb must react with non-BM–derived cells, such as vascular endothelial cells, to cause disease. In addition, we demonstrate direct ultrastructural damage to pulmonary vascular endothelium by 5 min after mAb injection, confirm the importance of ROIs in disease pathogenesis, provide an explanation for the susceptibility of male but not female mice, show that prestimulation of the inhibitory FcR, FcγRIIb, suppresses TRALI, and demonstrate that anti–H-2^d mAbs that are individually incapable of inducing TRALI-like disease can provoke this disorder when injected together. In addition to providing information that may be important for TRALI prevention or treatment, these results identify potential artifacts that can confuse investigations of the pathogenesis of other immune-mediated disorders.

RESULTS

Anti–H-2^d mAb rapidly disrupts pulmonary vascular basement membranes to induce TRALI-like disease in H-2^d mice

Injection of BALB/c and other H-2^d haplotype mice with 34-1-2s, a mouse IgG2a mAb which binds to H-$2D^d$ and H-$2K^d$ (Ozato et al., 1982), caused an H-2^d–specific change in breathing pattern (Fig. 1, A and B) shown by barometric plethysmography as increased "enhanced pause" (Penh), which reflects a selective increase in the duration of the late phase of expiration (Hamelmann et al., 1997). This was accompanied by decreased blood oxygenation (pO_2; Fig. 1 C), increased lung water content (Fig. 1 D), leakage of protein into the airways (Fig. 1, E and F), and temporary redistribution of blood neutrophils to the lungs (Fig. 1, F and G). Changes in breathing pattern were observed by 2 min after mAb injection (Fig. 1, A and B) and correlated closely with increases in lung water (Fig. 1 H) and development of shock and hemoconcentration (not depicted). Physical disruption of the capillary walls, characterized by separation of endothelial cells from the underlying

group) were injected i.v. with 34-1-2s. Blood was drawn before or 30 or 240 min after challenge, and absolute neutrophil count (ANC) was measured. Data are representative of three experiments. (H) BALB/c male mice (10 mice per group) were injected i.v. with 34-1-2s or control mAb. Maximum Penh during the subsequent 26 min was determined. Lungs were harvested 30 min after injection and evaluated for percent water content. r^2 for percent water weight of lungs versus maximum Penh = 0.521. Data are representative of two experiments. (I) Representative electron micrographs of lungs harvested from BALB/c male mice before or 5 min after i.v. injection of 34-1-2s. Black boxes in top pictures outline the enlarged area below. White arrowheads point to areas of separation of vascular endothelium from basement membrane. Bars: (F) 70 µm; (I) 2 µm. (J) BALB/c male mice ages 8 and 18 wk (four mice per group) were injected i.v. with 34-1-2s or control mAb, and lungs were harvested for evaluation of percent water content 30 min later. Data are representative of two experiments. (K) BALB/c male mice (five to six mice per group) were injected i.v. with 34-1-2s or control mAb, and Penh was evaluated over a 1,440-min period. Data are representative of two experiments. (L) BALB/c male mice (five to six mice per group) were injected i.v. with 34-1-2s or control mAb, and lungs were harvested for evaluation of percent water content 0, 30, 120, and 1,440 min later. Data are representative of two experiments. *, $P < 0.05$ using a nonparametric two-tailed *t* test. Mean ± SEM is shown.

basement membrane, was apparent by 5 min after mAb injection, was not associated with platelet thrombi or neutrophil infiltrates (Fig. 1 I), and was never observed in control mice. Disease severity, as measured by percent lung water weight, was similar in the youngest (8 wk) and oldest (20 wk) mice used in our experiments (Fig. 1 J). Both the increase in lung water and Penh fully resolved by 24 h after mAb injection (Fig. 1, K and L). Thus, disease induced by anti–MHC class I mAb, mouse TRALI (mTRALI), shares many features with human TRALI and is likely caused by rapid damage to alveolar capillary walls, followed by leakage of fluid and protein into the lung parenchyma.

mTRALI has limited dependence on FcγRs

A previous study that was performed with BALB/c mice and 34-1-2s implicated FcγRs in mTRALI pathogenesis by demonstrating the failure of 34-1-2s–injected BALB/c background FcRγ-deficient mice (which lack all stimulatory FcRs; Takai et al., 1994) to develop disease (Looney et al., 2006). We found that BALB/c background FcRγ-deficient mice developed only slightly less severe disease than WT mice (Fig. 2 A, left). A greater defect was observed in FcRγ-deficient mice of a mixed C57BL/6 × BALB/c background (Fig. 2 A, right), suggesting a role for genetic background in FcRγ contribution to mTRALI susceptibility. Surprisingly, mice deficient in the stimulatory FcγRs FcγRIII (Fig. 2 B), FcγRI (Fig. 2 C), or both FcγRI and FcγRIII (Fig. 2 D) still developed severe mTRALI, as did FcγRI/FcγRIII double-deficient mice treated with a blocking anti-FcγRIV mAb (Fig. 2 E; Nimmerjahn et al., 2005). The trivial effect of deleting or blocking all stimulatory FcγRs on mTRALI, in fact, raises the possibility that the limited FcRγ participation in mTRALI reflects FcRγ association with an activating receptor other than an FcγR, such as the IL-3R (Hida et al., 2009), the leukocyte mono-Ig–like receptor family (Izawa et al., 2009), myeloid-associated Ig-like receptor II (Nakahashi et al., 2007), CMRF-35–like molecule-5 (Fujimoto et al., 2006), platelet glycoproteins VI (Gibbins et al., 1997; Takaya et al., 2005) and IIb (Wu et al., 2001), dendritic cell immunoactivating receptor (Kanazawa et al., 2003), natural killer receptor P1 (Arase et al., 1997), paired Ig-like receptor A (Maeda et al., 1998), Toll-like receptor 4 (Rittirsch et al., 2009), or dectin-2 (Sato et al., 2006).

While investigating the role of stimulatory FcγRs in mTRALI, we noted that treating mice with the mAb 2.4G2, which cross-links both the

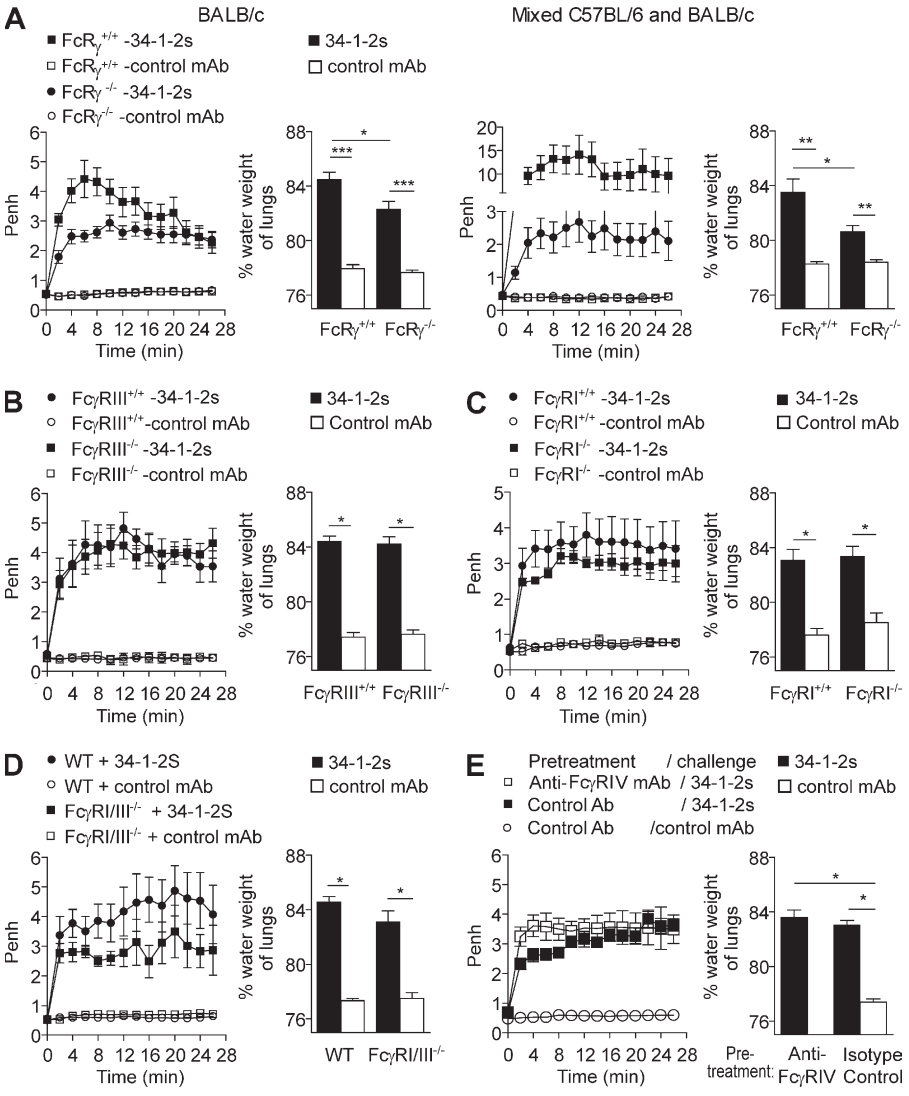

Figure 2. mTRALI is partially FcγR independent. (A) BALB/c male mice (14–24 mice per group, pooled from four experiments) or male mice with a mixed BALB/c and C57BL/6 background (8–9 mice per group, pooled from two experiments) that were FcRγ sufficient ($FcR\gamma^{+/+}$) or deficient ($FcR\gamma^{-/-}$) were injected i.v. with 34-1-2s or control mAb. Penh was evaluated for this and all other experiments in this figure. Lungs harvested 30 min after challenge were evaluated for water content in this and all other experiments in this figure. (B) $Fc\gamma RIII^{+/+}$ and $Fc\gamma RIII^{-/-}$ male mice (six mice per group) were injected i.v. with 34-1-2s or control mAb. Results are representative of at least two experiments for this and all subsequent panels in this figure. (C) $Fc\gamma RI^{+/+}$ and $Fc\gamma RI^{-/-}$ male mice (six mice per group) were injected i.v. with 34-1-2s or control mAb. (D) $Fc\gamma RI/III^{+/+}$ and $Fc\gamma RI/III^{-/-}$ male mice (four mice per group) were injected i.v. with 34-1-2s or control mAb. (E) $Fc\gamma RI/III^{-/-}$ male mice (four mice per group) were injected i.v. with anti-FcγRIV mAb or isotype control mAb and then challenged the next day with 34-1-2s or isotype control mAb. *, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$ using a nonparametric two-tailed *t* test. Mean ± SEM is shown.

stimulatory receptor FcγRIII and the inhibitory receptor FcγRIIb (Unkeless, 1979; Clynes et al., 1999; Strait et al., 2002; Hirano et al., 2007), completely prevented the development of mTRALI in WT mice (Fig 3 A). This result, taken together with our observations about the limited role of stimulatory FcγRs in mTRALI pathogenesis, suggests that 2.4G2 inhibits mTRALI by activating the inhibitor receptor, FcγRIIb, rather than by blocking FcγRIII. Consistent with this possibility, mTRALI was blocked in WT but not FcγRIIb-deficient mice treated with an FcγRIIb-specific mAb, anti-Ly17.2 (Fig. 3 B; Gessner et al., 1998; Schiller et al., 2000), and in FcγRI/RIII-deficient mice (Fig. 3 C) but not FcγRIIb-deficient mice (Fig. 3 D) treated with 2.4G2. However, WT and FcγRIIb-deficient mice developed mTRALI similarly if not treated with an anti-FcγRIIb mAb (Fig. 3, B and D). Collectively, these results demonstrate that stimulatory FcγRs have a limited role in mTRALI pathogenesis and that FcγRIIb does not typically regulate mTRALI development but can suppress this disorder if activated before anti–MHC class I mAb injection.

mTRALI pathogenesis is C5a and C5aR dependent

Our negative observations about stimulatory FcγRs' importance in mTRALI pathogenesis led us to investigate whether other Ig-related effector mechanisms might be involved. Because mouse IgG2a mAbs activate complement as well as FcγRs (Köhl and Gessner, 1999; Schmidt and Gessner, 2005), we evaluated whether the complement system is required for anti–MHC class I mAb–induced mTRALI. We observed C3 binding to lung cells soon after 34-1-2s injection (Fig. 4 A) and found an absolute requirement for C3 (Fig. 4 B), C5 (Fig. 4 C), and C5aR but not C3aR (Fig. 4 D) for mTRALI development. Deficiency of a second C5a receptor, C5L2, partially decreased mTRALI severity (Fig. 4 E). These results suggested an explanation for what appeared to us to be a limitation of the mTRALI model: mTRALI is induced by 34-1-2s injection in male but not female BALB/c mice (Fig. 5 A), whereas TRALI develops with similar frequency in men and women (Silliman et al., 2003b; Toy et al., 2004). Because C5 levels are considerably higher in mature male mice than female mice (Fig. 5 B; Churchill et al., 1967; Baba et al., 1984) or prepubertal males (Fig. 5 C; Cinader et al., 1964; Baba et al., 1984) and mature males have a molecular species of C5 that is absent in female or prepubertal male mice (differences not seen in humans; Baba et al., 1984), we evaluated whether transfer of plasma from male mice would make

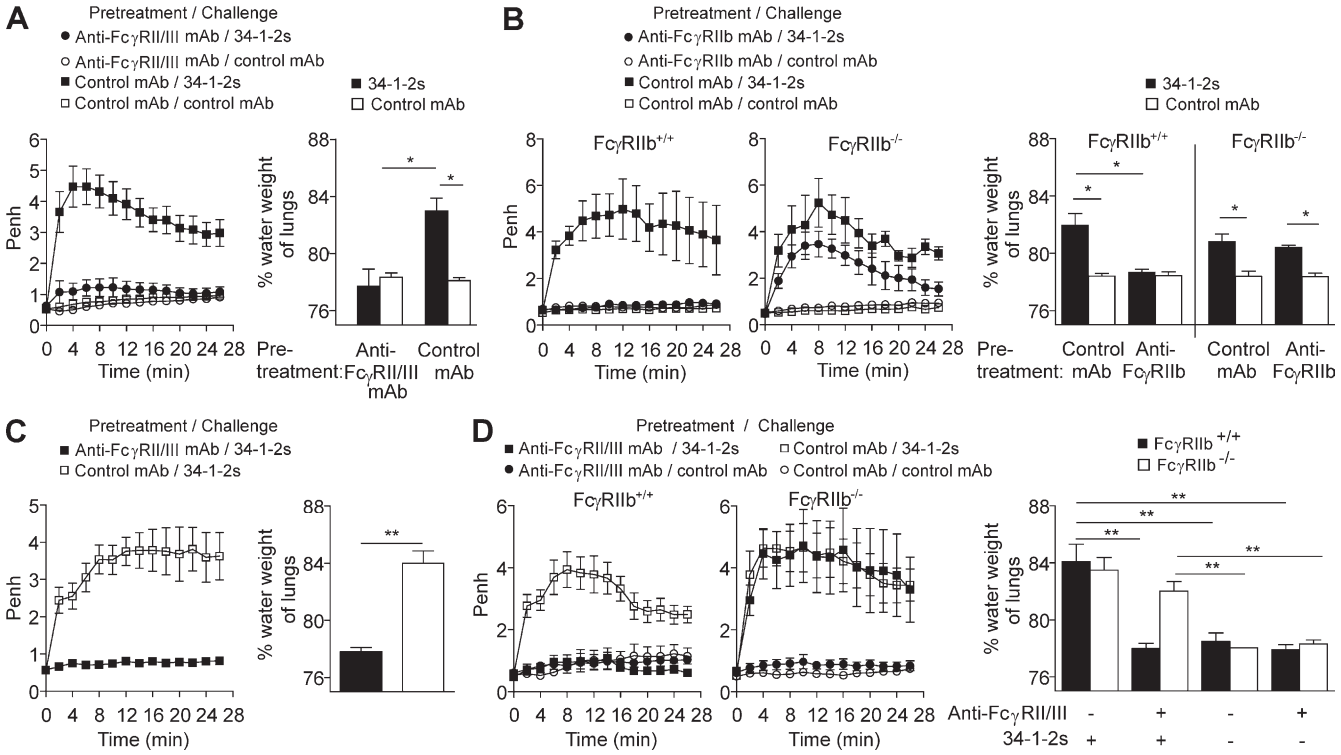

Figure 3. Suppression of mTRALI by FcγRIIb ligation. (A) BALB/c male mice (four to eight mice per group) were pretreated s.c. with 2.4G2 (rat IgG2b anti-FcγRII/III mAb) or control mAb and then injected i.v. the next day with 34-1-2s or control mAb. Penh and percent water weight of lungs were measured for this and all other experiments in this figure. Results are representative of at least two experiments for this and all subsequent panels in this figure. (B) BALB/c FcγRIIb$^{+/+}$ and FcγRIIb$^{-/-}$ male mice (four mice per group) were pretreated i.p. with mouse IgG2a anti-Ly17.2 mAb (anti-FcγRIIb mAb) or isotype control mAb and then injected i.v. the next day with 34-1-2s or isotype control mAb. (C) FcγRI/III$^{-/-}$ male mice (four mice per group) were pretreated s.c. with anti-FcγRII/III mAb or control mAb and then injected i.v. the next day with 34-1-2s. (D) BALB/c FcγRIIb$^{+/+}$ or FcγRIIb$^{-/-}$ male mice (six mice per group) were pretreated s.c. with anti-FcγRII/III mAb or control mAb and then injected i.v. the next day with 34-1-2s or control mAb. *, $P < 0.05$; and **, $P < 0.005$ using a nonparametric two-tailed *t* test. Mean ± SEM is shown.

females TRALI susceptible. Indeed, anti–H-2^d mAb–injected female BALB/c mice developed mTRALI if they were first administered plasma from WT or C3-deficient male mice but not plasma from WT female mice or C5-deficient males (Fig. 5 D). Prepubertal male mice, like female mice, failed to develop mTRALI in response to 34-1-2s (Fig. 5 E). Additional experiments demonstrated that FcRγ deficiency had no effect on C3 or C5 levels (Fig. 5 F), that male and female mice had similar plasma C3 levels (Fig. 5 G), that transfer of plasma from FcRγ-deficient male mice to WT males did not inhibit mTRALI (Fig. 5 H), that heat treatment, which destroys complement, prevented the ability of WT male plasma to reconstitute female mice for mTRALI development (Fig. 5 I), and that transfer of C5-sufficient plasma reconstituted the ability of C5-deficient male mice to develop mTRALI (Fig. 5 J). Further experiments demonstrated that the typical pulmonary vascular basement membrane lesions of mTRALI failed to develop in 34-1-2s–challenged female mice (Fig. 6 A), unless they were reconstituted with plasma from male mice (Fig. 6 B), that this lesion failed to develop in 34-1-2s–injected C5aR-deficient males (Fig. 6 C), and that LPS pretreatment did not restore the ability of C3-deficient mice to develop mTRALI (Fig. 6 D), even though it increased the sensitivity of WT male mice to 34-1-2s (Fig. 6 E). Collectively, these observations demonstrate a critical role for complement, including C5a and the C5aR, in mTRALI pathogenesis in both healthy mice and in mice with underlying lung injury.

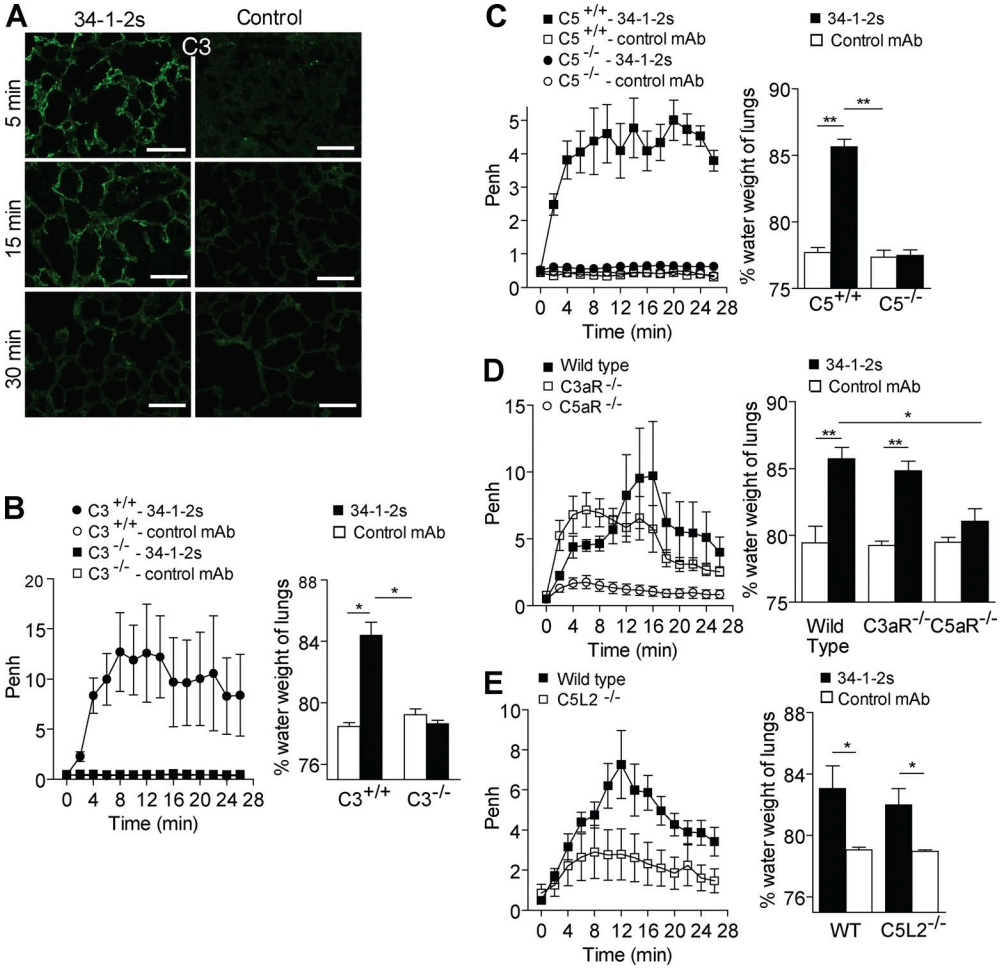

Figure 4. mTRALI is C5 and C5aR dependent. (A) BALB/c male mice (four mice per group) were injected i.v. with 34-1-2s or control mAb, and lungs were harvested 5, 15, and 30 min later. Deposition of C3 (bright green) in lung sections was evaluated by immunofluorescence staining. Representative slides are shown. Bars, 70 µm. (B) Mixed genetic background H-2^d C3$^{+/+}$ and C3$^{-/-}$ male mice (four mice per group) were injected i.v. with 34-1-2s or control mAb. Penh was evaluated in this and all subsequent panels in this figure. Lungs harvested 30 min after challenge were evaluated for percent water content in this and all subsequent panels in this figure. Results are representative of at least two experiments for all panels in this figure except as noted. (C) B10.D2 C5$^{+/+}$ and C5$^{-/-}$ male mice (four to eight mice per group) were injected i.v. with 34-1-2s or control mAb. (D) BALB/c WT, C5aR$^{-/-}$, and C3aR$^{-/-}$ male mice (four to eight mice per group) were injected i.v. with 34-1-2s or control mAb. Penh is only shown for 34-1-2s–injected mice in this and subsequent panels in this figure. (E) BALB/c male mice sufficient (WT) or deficient for the C5a receptor, C5L2 (C5L2$^{-/-}$; four mice per group) were injected i.v. with 34-1-2s or control mAb. This experiment was performed once. *, $P < 0.05$; and **, $P < 0.005$ using a nonparametric two-tailed *t* test. Mean ± SEM is shown.

Monocytes, not neutrophils or platelets, are required for mTRALI

Two publications suggest that neutrophils and platelets are required for 34-1-2s–induced mTRALI and cause disease by producing ROIs in response to FcγR stimulation (Looney et al., 2006, 2009). Our demonstrations that disease is mediated predominantly through C5a rather than any single FcγR and that FcγRIIb ligation can suppress mTRALI suggested a need to reexamine these conclusions with the view that Abs used to eliminate neutrophils or platelets might interfere with mTRALI by depleting complement or desensitizing effector cells through a stimulatory or inhibitory receptor. Injection of BALB/c mice with a single dose of a depleting rabbit anti–mouse platelet Ab 4 h before 34-1-2s injection confirmed the previous report that this treatment suppresses mTRALI (Fig. 7 A; Looney et al., 2009); however,

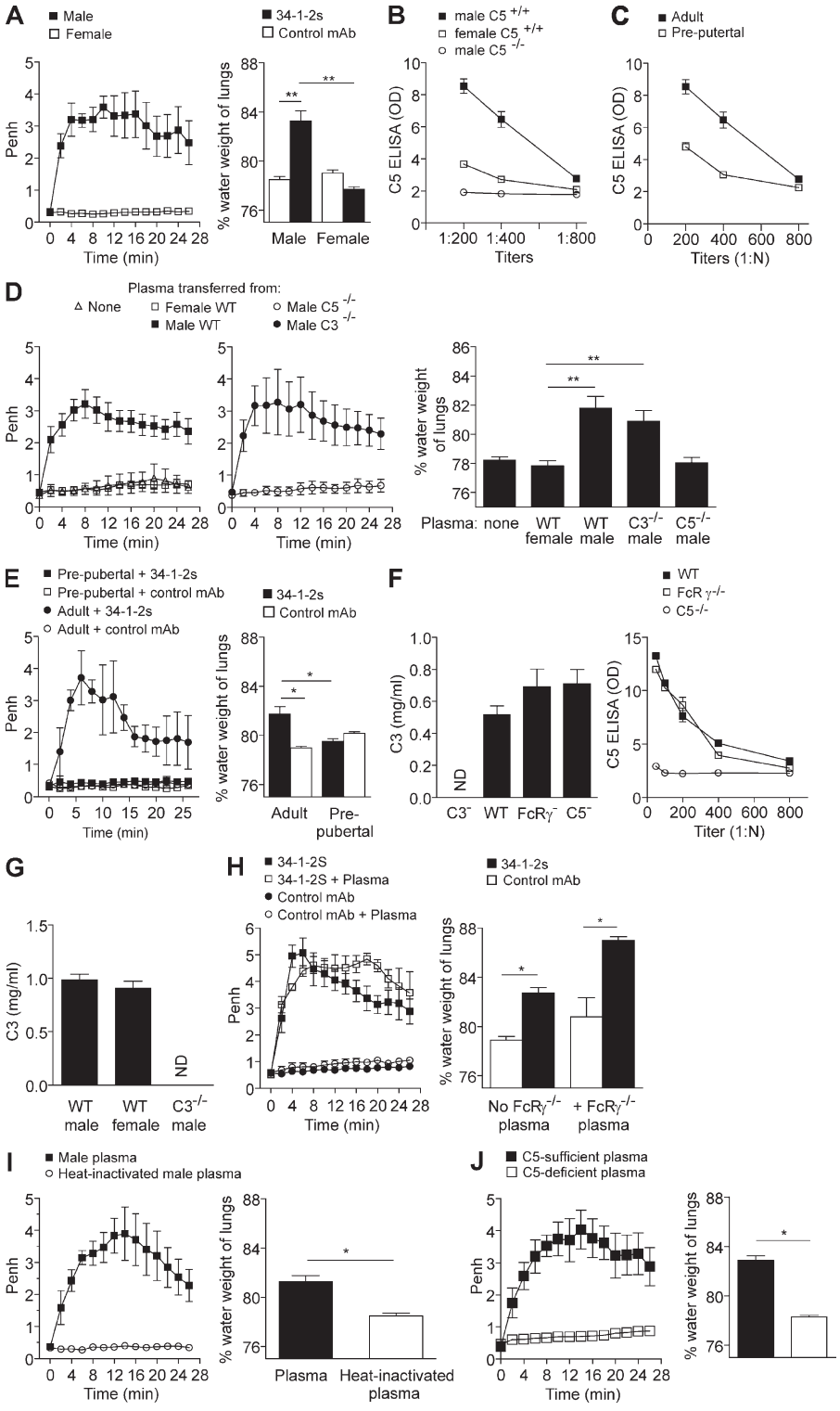

Figure 5. Low C5 levels prevent development of mTRALI in female mice. (A) BALB/c male and female mice (six mice per group) were injected i.v. with 34-1-2s or control mAb. For this and all subsequent experiments in this figure, except B and C, Penh was evaluated, and lungs harvested 30 min after challenge were evaluated for percent water content. Results are representative of at least two experiments for all panels in this figure. (B) Relative plasma C5 content was determined by ELISA for B10.D2 $C5^{+/+}$ male and female and $C5^{-/-}$ male mice (12 mice per group). (C) Relative plasma C5 content (12 mice per group) was determined by ELISA for 3.5-wk-old (prepubertal) or 8-wk-old (adult) WT male mice. (D) BALB/c female mice (four to eight mice per group) were pretreated i.v. with saline (none) or plasma from male or female WT mice, male $C5^{-/-}$ mice, or male $C3^{-/-}$ mice and then injected i.v. with 34-1-2s. (E) Adult and prepubertal male mice (four mice per group) were injected i.v. with 34-1-2s or control mAb. (F) BALB/c WT and FcRγ-deficient ($FcR\gamma^{-/-}$) and mixed background C3-deficient ($C3^{-/-}$) and B10.D2 C5-deficient ($C5^{-/-}$) male mice had their plasma C3 and C5 levels measured by ELISA ($n = 5$ mice per group for C3 and $n = 12$ mice per group for C5; ND = none detected). (G) BALB/c male and female and mixed background C3-deficient male mice had their plasma C3 levels measured by ELISA (five mice per group). (H) BALB/c male mice were pretreated with plasma from male $FcR\gamma^{-/-}$ mice before i.v. challenge with 34-1-2s or control mAb. (I) BALB/c female mice (four mice per group) pretreated with normal or heat-inactivated plasma from WT male before i.v. challenge with 34-1-2s or control mAb. (J) B10.D2 C5-deficient male mice (four mice per group) were pretreated with plasma from C5-deficent or -sufficient male mice before i.v. challenge with 34-1-2s or control mAb. *, $P < 0.05$; and **, $P < 0.005$ using a nonparametric two-tailed *t* test. Mean ± SEM is shown.

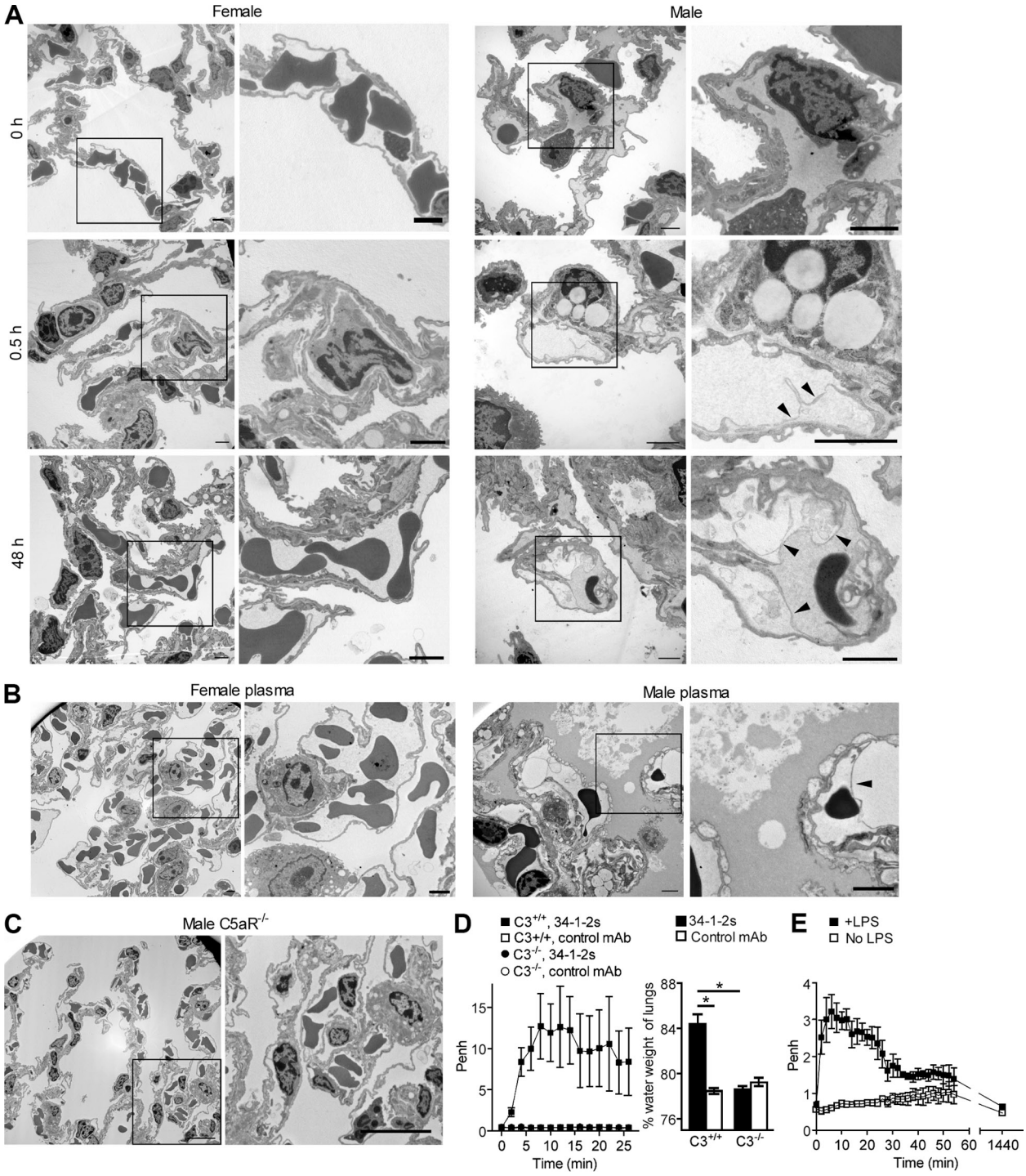

Figure 6. Association of mTRALI with complement in untreated and LPS-pretreated mice. (A) BALB/c male and female mice (four mice per group) were challenged i.v. with 34-1-2s; lungs were harvested 0, 0.5, or 48 h later. In this and all subsequent panels, black boxes in left photographs outline the enlarged area immediately to the right, and black arrowheads point to the region of separation of vascular endothelium from its basement membrane. (B) BALB/c female mice (four mice per group) were injected i.v. with female or male plasma and then injected i.v. with 34-1-2s. Lungs were harvested 30 min later. (C) BALB/c C5aR-deficient mice (four mice per group) were challenged i.v. with 34-1-2s; lungs were harvested 30 min later. (A–C) Representative electron micrographs of lungs are shown. Bars, 2 µm. (D) Mixed genetic background H-$2^{d/d}$ male $C3^{+/+}$ and $C3^{-/-}$ mice (four mice per group) were inoculated i.t. with 3 µg LPS and challenged i.v. 3 d later with 34-1-2s. Penh was evaluated for the next 26 min. Lungs were harvested 30 min after injection and evaluated for percent water content. (E) BALB/c male mice (four mice per group) were initially left untreated or inoculated i.t. with 2 µg LPS and challenged i.v. 3 d later with 8 µg 34-1-2s or control mAb. Penh was evaluated for the next 24 h. (D and E) A repeat experiment gave similar results. *, $P < 0.05$ using a nonparametric two-tailed *t* test. Mean ± SEM is shown.

injection of this Ab 24 and 48 h before 34-1-2s challenge still depleted platelets (Fig. 7 B) but no longer suppressed mTRALI (Fig. 7 A). This is consistent with the possibility that mTRALI is suppressed by the formation of platelet/antiplatelet Ab immune complexes that deplete complement, activate FcγRIIb, and/or desensitize effector cells by mechanisms other than platelet elimination. Presumably, immune complex effects dissipate by 2 d after the initial antiplatelet Ab injection, while slow regeneration of platelets limits new immune complex production. To confirm that platelets are not required for mTRALI, we evaluated whether platelet depletion by a nonimmune mechanism, injection of neuraminidase, could block mTRALI and again found that mTRALI developed despite platelet absence (Fig. 7, C and B, respectively). This is consistent with the ultrastructural finding that platelets were not observed in the alveolar capillary wall lesions (Fig. 1 I and Fig. 6, A and B).

Because neutrophils had also been implicated in mTRALI pathogenesis primarily through studies that used a relatively high dose of mAb to deplete these cells (Looney et al., 2006, 2009), it seemed possible that suppression of mTRALI was again caused by immune complex formation rather than by depletion of a cell required for disease pathogenesis. Consistent with published reports (Looney et al., 2006, 2009), we found that mTRALI was suppressed by injection of a high dose of anti-Ly6C/6G mAb (anti–Gr-1) before injection of 34-1-2s (Fig. 7 D, bottom). However, in contrast to our experiments with platelets, repeated injection of anti-Ly6C/6G mAb on successive d before 34-1-2s challenge still suppressed mTRALI (not depicted). Because Ly6C/6G mAb incompletely depletes splenic neutrophils (unpublished data), this did not completely disprove the possibility that Ly6C/6G mAb suppression of mTRALI was caused by immune complex generation rather than neutrophil depletion. To further study this possibility, we evaluated the ability of a combination of low-dose anti-Ly6C/6G mAb and the alkylating agent hydroxyurea to suppress both the blood neutrophil count and the migration of neutrophils to the lungs after 34-1-2s injection and mTRALI induction. Although the combination of low-dose anti-Ly6C/6G mAb and hydroxyurea was even more effective than high-dose anti-Ly6C/6G mAb at eliminating neutrophils in blood (Fig. 7 D, top left) and also blocked the 34-1-2s–induced increase in lung neutrophils (Fig. 7D, top right), this combination failed to suppress mTRALI (Fig. 7 D, bottom). To confirm that immune complex formation can suppress mTRALI, we injected mice with an anti-erythrocyte lineage mAb (Jordan et al., 2003) 1 d before 34-1-2s challenge and found total suppression of disease development (Fig. 7 E), even though it seems unlikely that erythrocytes or their precursors are involved in mTRALI pathogenesis.

Elimination of neutrophils and platelets as cells essential for mTRALI pathogenesis raised the question of what cells are involved. A previous study found that mTRALI pathogenesis involves generation of ROIs, as shown by suppression of mTRALI in mice pretreated with the reducing agent *N*-acetylcysteine (Hidalgo et al., 2009), a result which we confirmed (Fig. 8 A). Because monocytes and macrophages are capable of generating ROIs in response to C5a, we studied whether these cells are required for 34-1-2s induction of mTRALI. Depletion or inactivation of monocyte/macrophages was accomplished by i.v. injection of gadolinium or clodronate-loaded liposomes. Gadolinium aggregates in blood and is subsequently phagocytosed by monocytes and macrophages, inducing anergy and apoptosis (Singh and de la Concha-Bermejillo, 1998; Frid et al., 2006; Thenappan et al., 2011). Clodronate-loaded liposomes similarly kill monocytes and macrophages that ingest them (Thepen et al., 1989; Ajuebor et al., 1999; Popovich et al., 1999; Wang et al., 1999; Nikolic et al., 2005; Farley et al., 2006; Bhatia et al., 2011). Pretreatment with i.v. gadolinium or i.p. plus intratracheal (i.t.) clodronate-containing liposomes totally suppressed mTRALI (Fig. 8, B and C). To compare the roles of blood monocytes with resident lung macrophages in mTRALI pathogenesis, we selectively depleted either of the two populations by administering gadolinium i.v. or clodronate-containing liposomes i.p. (to deplete blood monocytes) or i.t. (to deplete resident lung macrophages) and found that mTRALI was suppressed by the i.v. and i.p. but not the i.t. treatments (Fig. 8, D and E). Thus, peripheral blood monocytes appear to be required for mTRALI and, most likely, are the source of ROIs that contribute to disease development.

Targeting of non-BM–derived cells by anti–MHC class I mAb is required for mTRALI induction

Identification of peripheral blood monocytes as the inflammatory cell type required for mTRALI induction left open the possibility that noninflammatory lung cells, such as vascular endothelial cells, had to be targeted by anti–MHC class I mAb to direct inflammatory cells to the lungs. After first demonstrating that 34-1-2s binds to lung vasculature by 1 min after i.v. injection (Fig. 8 F), we used H-2^d BM chimeric mice to directly evaluate whether 34-1-2s binding to non-BM–derived cells is essential for mTRALI pathogenesis in our model. mTRALI developed in mice that expressed H-2^d on all cell types and mice that expressed H-2^d predominantly on non-BM–derived cells (although they still had a small percentage of H-2^{d+} neutrophils) but failed to develop in mice that selectively lacked H-2^d on non-BM–derived cells and mice that totally lacked H-2^d (Fig. 8 G). Thus, anti–H-2^d mAb appears to initiate mTRALI predominantly by binding to non-BM–derived cells, such as vascular endothelial cells. In addition, the use of RAG2-deficient mice in this chimera experiment demonstrates that neither B nor T cells are required for the development of mTRALI.

mAb requirements for mTRALI induction

One remaining question about mTRALI induction by 34-1-2s is why this is the only anti–H-2^d mAb that induces mTRALI of eight IgG2a and IgM anti–H-2^d mAbs tested (Fig. 9 A). One obvious possibility was that 34-1-2s was the only mAb of our set that binds to both H-2D^d and H-2K^d.

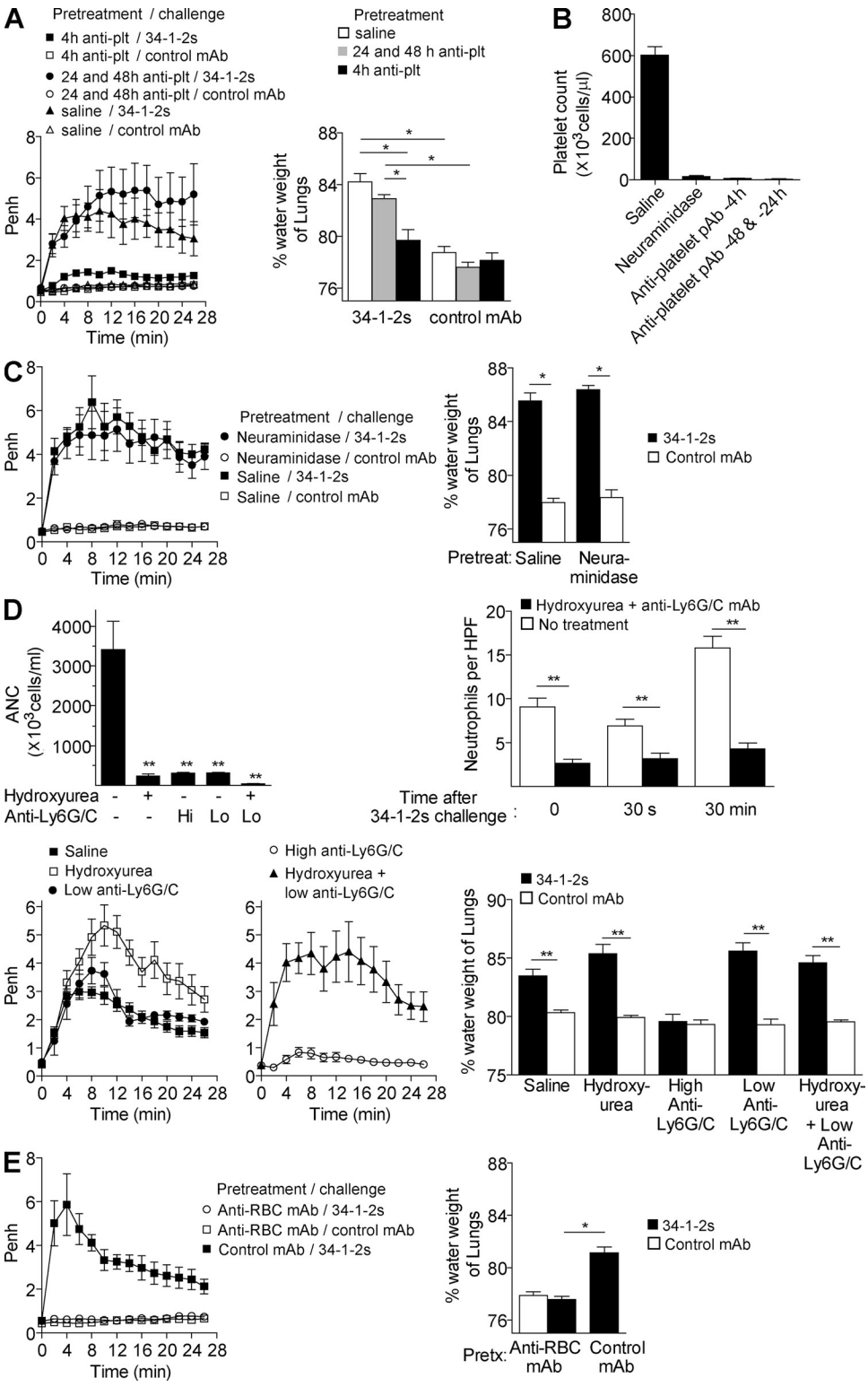

Figure 7. Neither platelets nor neutrophils are required for mTRALI pathogenesis. (A) BALB/c male mice (four mice per group) were pretreated i.v. with saline or rabbit antiplatelet pAb (anti-plt) both 24 and 48 h before or only 4 h before injection i.v. with 34-1-2s or control mAb. In all panels in this figure except B, Penh was evaluated, and then lungs were harvested 30 min after 34-1-2s injection and evaluated for percent water content. Results are representative of at least two experiments for all panels in this figure except as noted. (B) BALB/c male mice (4–10 mice per group) were injected i.v. with rabbit antiplatelet pAb 4 h or both 24 and 48 h before determination of peripheral blood platelet count or with neuraminidase or saline 24 h before determination of peripheral blood platelet count. (C) BALB/c male mice (four to eight mice per group) were pretreated with neuraminidase or saline i.p. 1 d

However, this possibility was eliminated by experiments that showed that 34-1-2s induced mTRALI in B10A-H2a mice, which express H-2D^d but not H-2K^d (Fig. 9 B). Another possibility was that 34-1-2s had a higher avidity for H-2^d than any of the other mAbs. This would be consistent with a recent human study that associates the development of TRALI with the presence of high-avidity anti-HLA Ab (Hashimoto et al., 2010). Although avidity is probably important in our mouse model, 34-5-8s and SF1-1.1.10 also bound with high avidity, based on the quantity of mAb required for half-maximal staining of BALB/c spleen cells (Fig. 9 C), yet they both failed to induce mTRALI by themselves (Fig. 9 A). A third possibility, supported by the data in Fig. 9 C, was that more 34-1-2s bound to BALB/c spleen cells than any of the other anti–H-2^d mAbs tested. Consistent with this possibility, 34-1-2s also activated complement to a greater extent than any of the other anti–H-2^d mAbs tested, as shown by C3d binding to nucleated blood cells (Fig. 9 D); however, we cannot prove that this is the critical feature that makes 34-1-2s pathogenic. Although we also tried to evaluate whether 34-1-2s could increase serum levels of C5a faster than any of the other anti–H-2^d mAbs, we have been unable to reproducibly quantitate C5a in plasma, possibly because of its short half-life.

However, the possibility that 34-1-2s pathogenicity is related to rapid complement activation predicted that injection of a nonpathogenic anti–H-2^d mAb before injection of 34-1-2s might suppress mTRALI development by depleting complement. Indeed, injection of either of the nonpathogenic anti–H-2^d mAbs, SF1-1.1.10 or 34-5-8s, which do not block 34-1-2s binding to H-2^d (Fig. 9 E, left), 2 h before 34-1-2s suppressed mTRALI (Fig. 9 E, middle and right). In contrast, simultaneous injection of a nonpathogenic anti–H-2^d mAb with 34-1-2s, which should increase total mAb binding and accelerate complement activation, increased mTRALI severity and complement binding (Fig. 9 F). Consistent with this, injection of BALB/c mice simultaneously with a cocktail of nonpathogenic anti–H-2^d mAbs that bind to different H-2^d epitopes increased the activation of complement as measured by C3d binding to peripheral nucleated blood cells (not depicted) and induced mTRALI (Fig. 9 G). Collectively, these observations suggest that the rapid focusing of a relatively large quantity of IgG mAb onto specific epitopes of vascular endothelial H-2^d molecules is required to induce C5a production with sufficient rapidity to stimulate macrophage/monocyte migration to the lungs and ROI secretion and, consequently, sufficient damage to vascular endothelial cells to cause clinically apparent disease (diagramed in Fig. 10).

DISCUSSION

Although TRALI can be induced by at least two different mechanisms, both of which increase pulmonary vascular permeability, we have confined our study to a mouse model of alloantibody-induced TRALI. Our studies with this model confirm and extend a previously described observation (Looney et al., 2006, 2009): a disorder very similar to human TRALI can be induced by injecting mice with an mAb against MHC class I, with rapid disruption of the alveolar capillary wall, fluid and protein leak into the lungs, a change in breathing mechanics, and a decrease in blood neutrophil count. However, although our experiments use the same mAb and mouse strain as other publications, they reveal a very different pathogenic mechanism than has been proposed. First, we find that almost complete depletion of neutrophils or platelets from BALB/c mice has no effect on the ability of 34-1-2s to induce mTRALI, conclusions which are consistent with our ultrastructural findings that neither cell type is observed in the vicinity of the alveolar capillary wall lesions. We agree with published reports that injection of mice with a rabbit antiplatelet pAb or a high dose of a rat mAb to Ly6C and Ly6G (anti–Gr-1) acutely prevents mTRALI (Looney et al., 2006, 2009). However, injection of either Ab forms immune complexes with platelets or neutrophils that may inhibit mTRALI by consuming complement or binding to inhibitory or stimulatory FcγRs. We hypothesized that this process, rather than loss of platelets or neutrophils, was responsible for mTRALI inhibition. Indeed, formation of mAb complexes with erythrocytes and erythrocyte precursors had the same effect, even though it is unlikely that erythrocyte lineage cells participate directly in mTRALI pathogenesis. Furthermore, repeated injection of rabbit antiplatelet Ab, which maintains thrombocytopenia in the absence of further immune complex production (because of the time required to produce more platelets), does not inhibit mTRALI. Neither does platelet depletion by a nonimmune mechanism (injection of neuraminidase) inhibit mTRALI.

Testing the hypothesis that neutrophils are not required for mTRALI proved more difficult; the incomplete depletion of splenic neutrophils by anti–Gr-1 mAb did not allow this same strategy to be used to deplete blood neutrophils without maintaining immune complex production. Instead, we used a combination of low-dose anti–Gr-1 mAb and the

before i.v. injection with 34-1-2s or control mAb. (D) BALB/c male mice (four to eight mice per group) were pretreated with saline or hydroxyurea i.p. daily on day 0–6 and/or anti-Ly6G/C mAb i.v. at a dose of either 0.1 mg (Lo) on days 5 and 6 or 1 mg (Hi) on day 6. On day 7, mice were bled for determination of absolute neutrophil count (ANC; top left) or injected i.v. with 34-1-2s or control mAb (bottom). Penh values for control mAb-injected mice were all <1 (not depicted). In addition, some mice (10 mice per group; top right) were left untreated or were treated i.p. daily with hydroxyurea from day 0 to 6 and with low-dose anti-Ly6G/C mAb on days 5 and 6. On day 7, all mice were challenged i.v. with 34-1-2s, and lungs were harvested before or 30 s or 30 min after i.v. 34-1-2s injection. The number of neutrophils per high power field was determined for 10 fields of H&E-stained lung sections from each mouse. The quantitation of lung neutrophils part of the experiment was performed once. (E) BALB/c male mice (four to five mice per group) were pretreated i.p. with TER119 (rat IgG2b anti-RBC mAb) or isotype control mAb and injected i.v. 24 h later with 34-1-2s or control mAb. *, $P < 0.05$; and **, $P < 0.005$ using a nonparametric two-tailed *t* test. Mean ± SEM is shown.

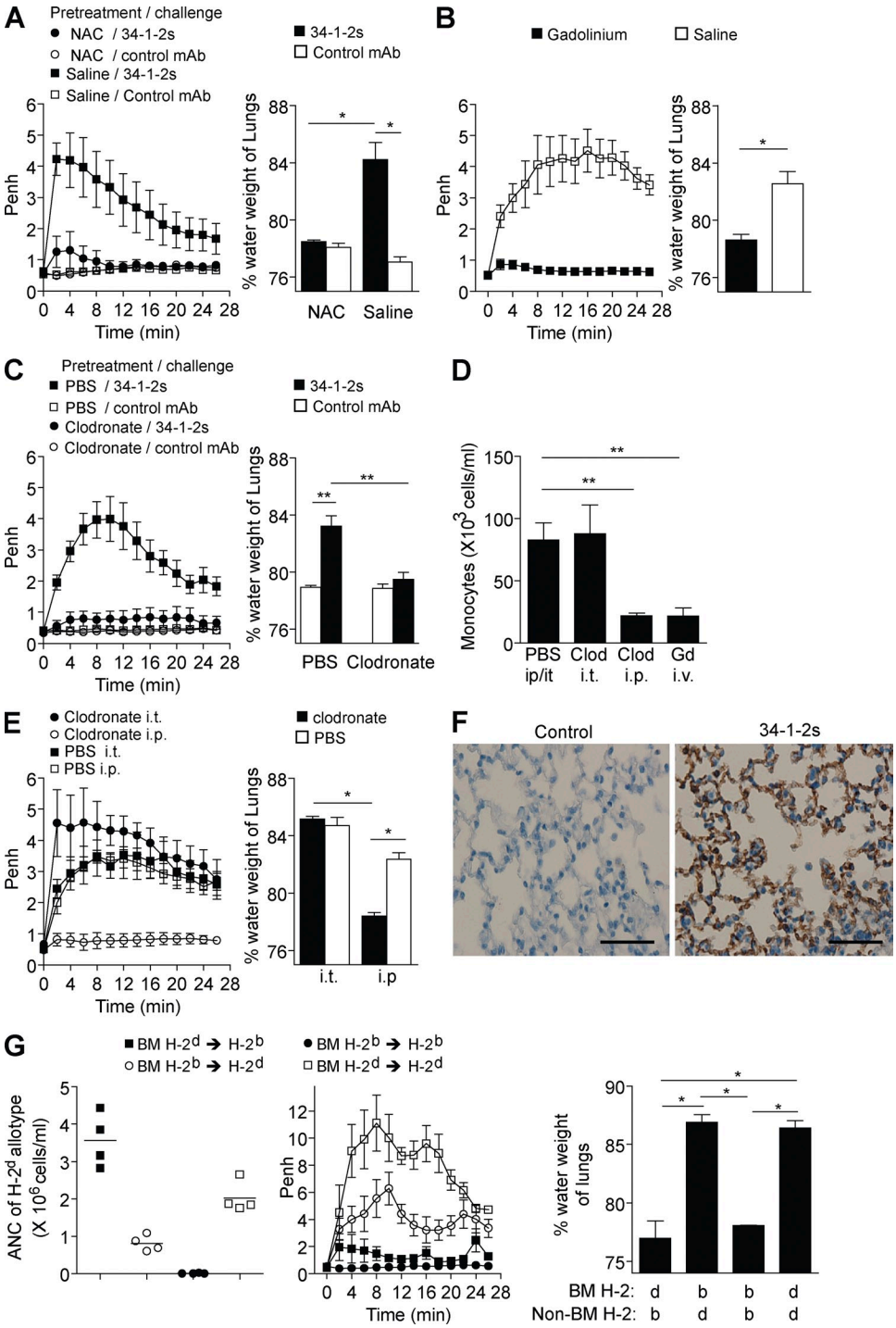

Figure 8. Involvement of ROIs, monocytes, and non-BM–derived cells in mTRALI pathogenesis. (A) BALB/c male mice (four mice per group) were pretreated i.v. with *N*-acetylcysteine (NAC) or saline and injected i.v. 5 min later with 34-1-2s or control mAb. In all panels, except for D and F, Penh was evaluated and then lungs were harvested 30 min after 34-1-2s injection and evaluated for percent water content. Results are representative of at least two experiments for this and all subsequent experiments in this figure. (B) BALB/c male mice (five to eight mice per group) were pretreated i.v. with gadolinium or saline and injected i.v. the next day with 34-1-2s. (C) BALB/c male mice (four mice per group) were pretreated i.p. and i.t. with liposomes containing clodronate or PBS and injected i.v. 2 d later with 34-1-2s or control mAb. (D) BALB/c male mice (four mice per group) were injected with liposome-encapsulated clodronate (Clod) or PBS i.p. or i.t. Absolute monocyte count was determined for blood obtained 2 d later. Another group was injected i.v. with gadolinium (Gd). Blood was drawn the next day, and absolute monocyte count was determined. (E) BALB/c male mice (four mice per group) were injected with liposome-encapsulated clodronate or PBS either i.p. or i.t. and challenged i.v. 2 d later with 34-1-2s. (F) BALB/c male mice (four mice per group) were injected i.v. with biotinylated 34-1-2s or control mAb. Lungs were obtained 60 s later and stained for biotin (brown). Representative

alkylating agent hydroxyurea to fully deplete blood neutrophils and prevent their appearance in the lungs without generating sufficient immune complex to suppress TRALI.

In contrast to the lack of a requirement for either platelets or neutrophils in TRALI pathogenesis, development of this disorder required functional peripheral blood monocytes, as demonstrated by studies that prevented TRALI by these cells with clodronate-containing liposomes (Thepen et al., 1989; Popovich et al., 1999; Wang et al., 1999; Jordan et al., 2003; Murphy et al., 2004; Nikolic et al., 2005; Farley et al., 2006; Zhao et al., 2006; Bhatia et al., 2011) or inactivating and depleting them with gadolinium (Singh and de la Concha-Bermejillo, 1998; Strait et al., 2002; Frid et al., 2006; Thenappan et al., 2011). The requirement for monocytes is consistent with an essential role in mTRALI for ROIs, which are produced by activated monocytes/macrophages.

Our experiments also differ from published results by questioning the importance of FcγRs in mTRALI pathogenesis. The previously reported conclusion that FcγRs are required for TRALI pathogenesis (Looney et al., 2006) was based on the observation that mTRALI is not induced by 34-1-2s injection in FcγR-deficient mice, which lack all stimulatory FcRs. Studies with FcRγ-deficient mice have the advantage that this polypeptide is an essential part of all stimulatory FcRs in the mouse, so that FcRγ deficiency simultaneously prevents FcγRI, FcγRIII, FcγRIV, and FcεRI function. However, we found that mTRALI still occurs with only a minor decrease in severity in BALB/c FcRγ-deficient mice and with a greater decrease in severity in FcRγ-deficient mice that have a mixed BALB/c – C57BL/6 genetic background. More importantly, because FcRγ also associates with many other receptors and influences or is required for their signaling, the absence of mTRALI in FcRγ-deficient mice does not necessarily indicate FcγR involvement. The development of mTRALI to nearly the same degree in B10.D2 FcγRI/III double-deficient mice pretreated with a blocking anti-FcγRIV mAb as in untreated WT mice supports the conclusion that stimulatory FcγRs are not essential in mTRALI pathogenesis.

Our observation that mTRALI can be blocked by pretreating mice with 2.4G2, which activates and then blocks FcγRIIb, FcγRIII, and possibly FcγRIV (Unkeless, 1979; Clynes et al., 1999; Strait et al., 2002; Hirano et al., 2007), initially seemed to support a role for stimulatory FcγRs in mTRALI pathogenesis. However, our subsequent experiments revealed that 2.4G2 blocks mTRALI in WT and FcγRI/RIII-deficient mice but not in mice deficient in the inhibitory receptor, FcγRIIb. Thus, 2.4G2 inhibits mTRALI by giving a negative signal to cells via FcγRIIb rather than by blocking the stimulatory receptors FcγRI, FcγRIII, or FcγRIV. Inhibition of mTRALI by FcγRIIb activation was confirmed by demonstrating that this disorder could also be suppressed in WT but not FcγRIIb-deficient mice by treatment with an FcγRIIb-specific anti-Ly17.2 mAb.

In contrast to a previous study (Looney et al., 2006), our observations establish a critical role for complement, particularly C5a, in this disorder. mTRALI fails, in our hands, to develop in mice deficient in C3, C5, or the C5aR, although Looney et al. (2006) described normal disease development in C5aR-deficient mice. It is possible that differences in animal husbandry and/or bacterial flora account for our different results. Additionally, the use of general anesthesia for anti–H-2^d mAb–treated mice in the study by Looney et al. (2006), but not in our experiments, could influence lung physiology and possibly disease development. More importantly, the requirement for C5 in our experiments explains why 34-1-2s injection induces mTRALI in adult male but not female BALB/c mice, which have only ∼25% as much plasma C5 as males. In addition, male mice have a form of C5 that is totally lacking in females (Baba et al., 1984). This quantitative and qualitative gender dimorphism for C5 explains the male/female difference in mTRALI susceptibility, as shown by our experiments in which female mice became susceptible to mTRALI induction when first infused with plasma from WT or C3-deficient male mice but not when infused with plasma from WT females or C5-deficient males. These observations are consistent with the lack of a gender difference in human TRALI susceptibility, inasmuch as there are no known gender differences in human plasma C5 levels.

Although our disease pathogenesis experiments have been restricted to a mouse model of TRALI, the immune complex–related artifacts that we have uncovered are probably not specific to studies of this disorder. In fact, we have previously shown that IgG–platelet complexes caused secondary effects that mistakenly suggested that platelets are essential for IgG-mediated murine anaphylaxis (Strait et al., 2002). We suspect that immune complex–mediated complications may confuse investigation of several disease models, as can the mistaken assumption that abnormalities in FcRγ-deficient mice necessarily reflect a requirement for IgG or IgE receptors (FcγRI, FcγRIII, FcγRIV, and/or FcεRI).

In addition to establishing critical roles for monocytes and C5a and confirming a role for ROIs in mTRALI pathogenesis, we show that MHC class I must be expressed on non-BM–derived cells (most likely pulmonary vascular endothelium) for mTRALI induction by 34-1-2s. This vascular endothelial location is particularly interesting in view of a report of TRALI induced solely in the transplanted lung of a posttransplant

sections are shown. Bars, 70 µm. (G) BALB/c (H-2^d) and C57BL/6 (H-2^b) RAG2-deficient male mice (four mice per group) were lethally irradiated and injected i.v. the next day with BM cells from RAG2-deficient H-2^d or H-2^b mice. 6 wk later, H-2^{d+} blood neutrophil counts were determined. 1 wk after that, mice were injected i.v. with 34-1-2s. An experiment in which BM chimeras were produced by reconstituting irradiated BALB/c or C57BL/6 mice with BM from (BALB/c × C57BL/6) F1 mice and challenged with 34-1-2s gave nearly identical results. Horizontal bars indicate the mean. *, $P < 0.05$; and **, $P < 0.005$ using a nonparametric two-tailed *t* test. Mean ± SEM is shown.

patient by donor plasma that contained Abs reactive with MHC antigens expressed by the lung donor but not by the recipient's native lung (Dykes et al., 2000). Collectively with our other observations, this suggests a relatively complex mechanism for mTRALI induction (diagramed in Fig. 10): Abs binding to MHC class I on pulmonary vascular endothelial cells activate complement with the production of C5a, which is chemotactic for monocytes/macrophages and additionally activates them to release ROIs, which rapidly damage the endothelial cells. It is additionally possible that complement activation also directly damages endothelial cells (although not enough to induce TRALI in the absence of macrophages or ROIs), that the small size of pulmonary

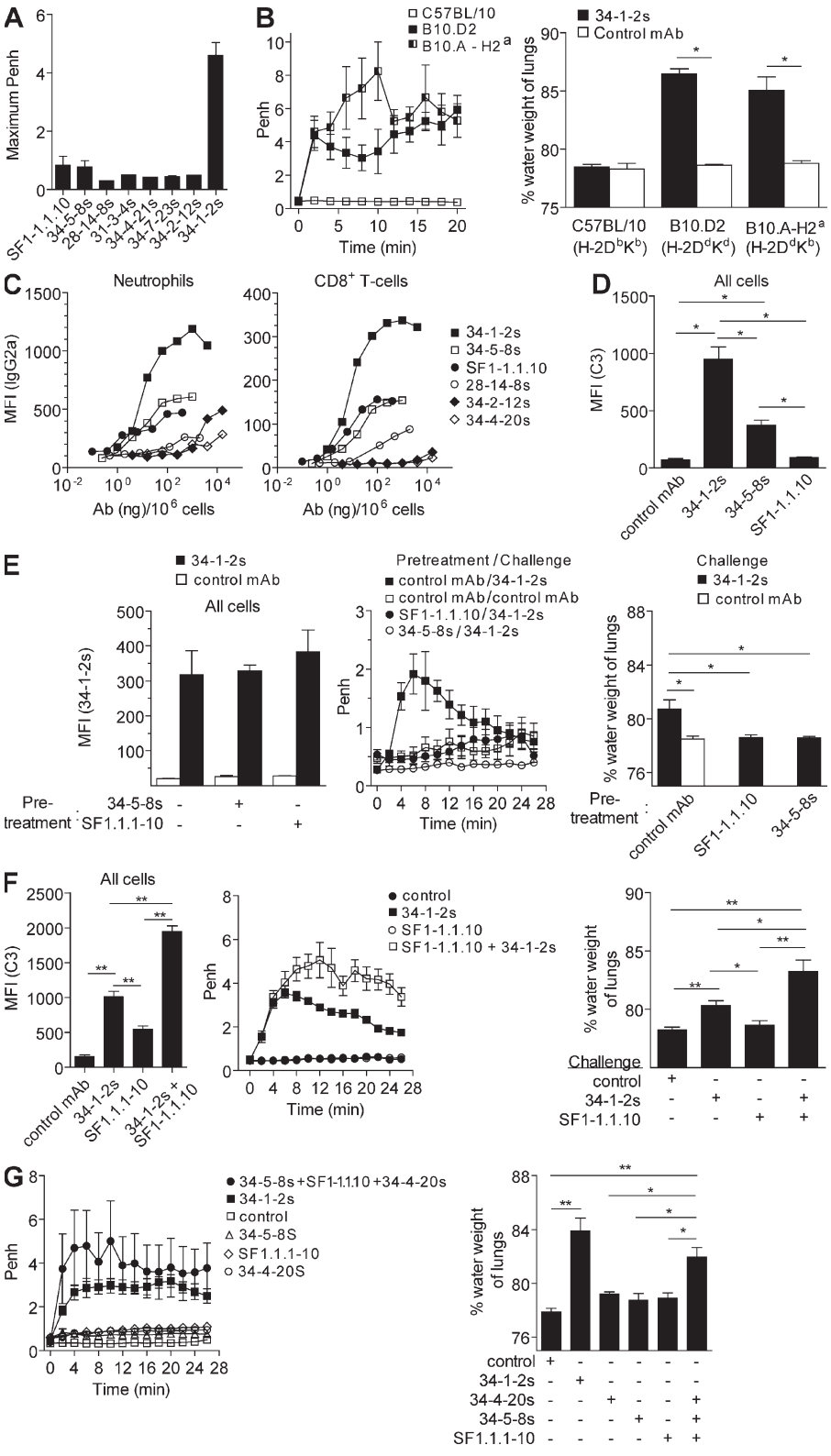

Figure 9. mAb properties required to induce mTRALI. (A) BALB/c male mice (four mice per group) were challenged i.v. with eight different mouse IgG2a anti–H-2^d mAbs. Mean maximum Penh during the next 26 min is shown. Results are representative of at least two experiments for all panels in this figure. (B) C57BL/10, B10.D2, and B10A-H2^a male mice (four mice per group) were injected i.v. with 34-1-2s or control mAb. For all panels in this figure except C and D, Penh was evaluated, and lungs harvested 30 min after injection were evaluated for percent water content. In this panel, Penh values for control mAb-injected mice were all <1 (not depicted). (C) Spleen cells harvested from BALB/c male mice were evaluated in vitro by flow cytometry for neutrophil and CD8$^+$ T cell binding of six different mouse IgG2a anti–mouse H-2^d mAbs to neutrophils (MFI, mean fluorescence intensity). (D) BALB/c male mice (four mice per group) were challenged i.v. with 34-1-2s, 34-5-8s, SF1-1.1.10, or control mAb. Blood drawn 3 min later was evaluated by flow cytometry for C3 binding to nucleated cells. (E) BALB/c male mice (four mice per group) were pretreated i.v. with SF1-1.1.10, 34-5-8s, or control mAb and injected i.v. 2 h later with 34-1-2s or control mAb. Blood drawn 3 min later from some mice was evaluated for 34-1-2s binding to nucleated cells. Penh and percent water content of lungs were determined for the other mice. (F) BALB/c male mice (four mice per group) were injected i.v. with 34-1-2s, SF1-1.1.10, 34-1-2s + SF1-1.1.10, or control mAb. Some mice were evaluated for abnormal breathing (Penh; middle) and percent lung water content (right). Mononuclear cells in blood drawn from other mice 3 min after mAb injection were evaluated by flow cytometry for C3 binding (left). (G) BALB/c male mice (four mice per group) were injected i.v. with control mAbs, 34-1-2s, SF1-1.1.10, 34-5-8s, or 34-4-20s, or with the combinations of these mAbs shown. *, $P < 0.05$; and **, $P < 0.005$ using a nonparametric two-tailed *t* test. Mean ± SEM is shown.

capillaries relative to systemic capillaries facilitates macrophage binding and activation, and that the relatively high oxygen tension in the lung makes pulmonary capillaries more susceptible than systemic capillaries to oxidative damage.

Our data provide further information about mTRALI pathogenesis by evaluating the relative ability of different IgG2a anti–H-2^d mAbs to induce mTRALI. Surprisingly, only 34-1-2s, by itself, induced mTRALI out of eight mAbs tested. Although 34-1-2s is the only member of our set of anti–H-2^d mAbs that binds to both H-$2D^d$ and H-$2K^d$ (Ozato and Sachs, 1981; Ozato et al., 1982; Noun et al., 1996), it still induced mTRALI in mice that expressed only one of these MHC class I molecules (Fig. 9 B). Although 34-1-2s binds to MHC class I with higher affinity than some other members of our mAb panel, it was not the only mAb that bound with high affinity. These observations suggest that it is necessary for Ab to bind to MHC class I in sufficient quantity to activate enough complement with sufficient rapidity to attract and activate monocytes/macrophages. Consistent with this possibility, we found that mTRALI was induced when mice were injected with a combination of anti–H-2^d mAbs that, by themselves, did not induce mTRALI. We hypothesize that the combined binding of these mAbs to different H-2^d epitopes creates sufficient IgG density to meet the rapid complement-activating requirements for mTRALI induction. These observations would be consistent with the threshold model suggested by some (Bux and Sachs, 2007; Vlaar et al., 2009) and supported by the finding of a correlation between strength of donor anti-HLA Ab and induction of TRALI (Hashimoto et al., 2010). These findings may also provide some explanation for why there is no simple relationship between anti-MHC Ab titers in human plasma and the likelihood of inducing TRALI when infused into a susceptible recipient. In addition, they suggest that the anti–MHC class I pAbs generated in multiparous women, which should bind to multiple epitopes on an MHC class I molecule, should be more effective than a single mAb at inducing TRALI.

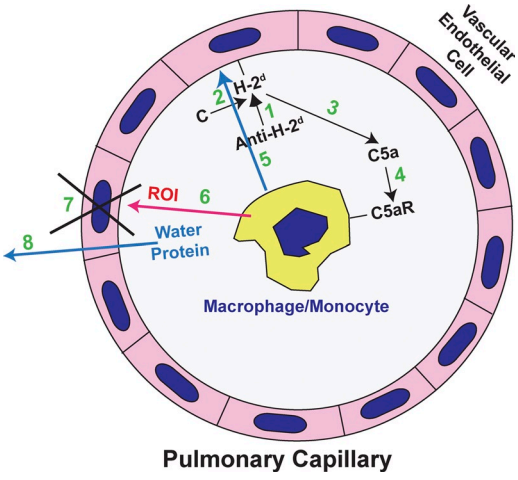

Figure 10. Induction of mTRALI by anti–MHC class I mAb. The simplest mechanism of mTRALI induction consistent with our data is shown. Anti–H-2^d mAb (1) binds to H-2^d on vascular endothelial cells, activating complement (2) with production of C5a (3), which binds to the C5aR on monocyte/macrophages (4), attracting them to lung vasculature (5) and inducing them to secrete ROIs (6), which disrupts pulmonary vascular endothelial basement membranes (7), allowing fluid and protein to leak into the lung parenchyma (8), causing noncardiogenic pulmonary edema.

Although mTRALI could be induced by the simultaneous injection of anti–H-2^d mAbs that were individually incapable of inducing mTRALI, mTRALI induction by 34-1-2s could be prevented by first injecting mice with an anti–H-2^d mAb that was incapable of inducing mTRALI (Fig. 9 E). Although we have not yet identified the mechanism responsible for mTRALI prevention by the initial injection of a nonpathogenic mAb (it could involve complement consumption, MHC class I antigen modulation, FcγRIIb activation, FcγRIII-dependent macrophage desensitization, an adaptive response by pulmonary vascular endothelium, or a combination of these), this phenomenon suggests approaches that might be used to reduce the risk of TRALI development in susceptible individuals.

Finally, it is necessary to point out two limitations of our study. First, clinical epidemiology and animal model studies suggest that TRALI can be induced by more than one mechanism, with the common feature being induction of a sufficient increase in pulmonary vascular permeability and fluid leak into the lung parenchyma to compromise breathing and gas exchange (Silliman et al., 1997, 1998, 2009; Kopko et al., 2003; Looney et al., 2006, 2009; Gajic et al., 2007; Chapman et al., 2009; Fung and Silliman, 2009). Epidemiologic features that favor an Ab-mediated pathogenesis include higher incidence of TRALI after transfusion of blood from multiparous female than male donors, by the considerably decreased incidence of TRALI in the USA and UK after reduction in the use of female donors for plasma (Chapman et al., 2009; Eder et al., 2010; Stafford-Smith et al., 2010), and the high frequency of anti–MHC class I, anti–MHC class II, and antileukocyte Abs that react with recipient cells in TRALI-associated donor blood (Popovsky and Moore, 1985; Kopko et al., 2003; Kopko, 2004; Toy et al., 2004; Silliman et al., 2005; Bux and Sachs, 2007). Ab-mediated pathogenesis is also supported by individual case reports, including one in which different units of blood from the same multiparous donor caused TRALI in multiple recipients (Kopko et al., 2002). In contrast, other studies have shown a relationship between the period of blood storage and the likelihood of TRALI development, with increased storage causing the production of lipid mediators that can increase pulmonary vascular permeability (Silliman et al., 1998, 2003a,b). This model is compatible with the development of TRALI in the absence of detectable alloantibodies in transfused blood and the development of TRALI after transfusion of an individual's own stored blood. In our view, both the Ab- and the vasoactive mediator–dependent mechanisms are likely to contribute to human disease, and disease may be most likely to develop when both etiologies are present.

Second, although we restricted most of our experiments to mice that initially had no lung or vascular abnormalities, TRALI is most likely to develop in individuals who have preexisting inflammatory conditions. These conditions can activate macrophages, making them more likely to secrete large amounts of ROIs and cause production of vasoactive mediators and cytokines that may act synergistically with ROIs (Gilliss and Looney, 2011). These conditions may also promote direct Ab-induced, complement-mediated endothelial cell damage that can rapidly increase vascular leak to levels that result in noncardiogenic pulmonary edema (Gilliss and Looney, 2011). Consistent with this, inoculation of mice with LPS has been shown to considerably increase sensitivity to induction of mTRALI by anti–H-2^d and other mAbs that react with leukocytes (Fig. 6 E; Khan et al., 2006; Looney et al., 2009). Interestingly and relevant to our model, sepsis and associated LPS have also been shown to up-regulate the expression of C5aRs in lung cells (Riedemann et al., 2002), macrophages, and endothelial cells (Hunt et al., 2005). Also consistent with this observation are reports that mice kept in an especially clean environment lack mTRALI susceptibility, which is restored by treatment with LPS or housing in an environment where they are exposed to a broad bacterial spectrum (Lögdberg et al., 2009; Looney et al., 2009). We agree with suggestions already in the literature that human TRALI is most likely to occur when the effects of preexisting inflammation are exacerbated by infusion of vascular endothelial cell– and/or leukocyte-reactive Abs and vasoactive mediators to simultaneously cause multiple insults to vascular integrity (Shaz et al., 2011).

MATERIALS AND METHODS

Mice. All mice were bred and maintained in a specific pathogen-free colony at the Cincinnati Children's Research Foundation (CCRF) except where otherwise stated, and all experiments were approved by the Institutional Animal Care and Use Committee. Mice were used between age 8 and 20 wk except in one series of experiments that used prepubertal males age 3.5 wk (Fig. 5, C and E). BALB/c and FcRγ-deficient mice were purchased from Taconic. C57BL/6, B10.D2-*Hc*^{1} *H2*^{d} *H2-T18*^{c}/nSnJ (B10.D2), B10.D2-*Hc*^{0} *H2*^{d} *H2-T18*^{c}/o2SnJ (C5$^{-/-}$), B10.A-H2a, BALB/c, BALB/k, CB6, and BALB/c FcγRIIb-deficient and both C57BL/6 and BALB/c background RAG2-deficient mice were purchased from the Jackson Laboratory. C57BL/6 background C3-deficient (a gift of M. Wills-Karp, CCRF) and FcγRI- and FcγRIII-deficient mice (gifts from J. Ravetch, The Rockefeller University, New York, NY) were bred with BALB/c (C3 deficient) and B10.D2 (FcγRI and FcγRIII) background mice to produce C3-deficient, FcγRI-deficient, FcγRIII-deficient, and FcγRI/III-deficient mice on an H-$2^{d/d}$ background and typed by PCR for the presence of C3, FcγRI, and FcγRIII and by flow cytometry for H-$2^{d\ and\ b}$. Littermates of similar matched mixed lineage, but C3, FcγRI, and FcγRIII sufficient, that were also on a H-$2^{d/d}$ background and bred as controls along with B10.D2. BALB/c background C3aR-, C5L2-, and C5aR-deficient mice (a gift from C. Gerard, Harvard University, Cambridge, MA) were used.

H-2 typing. Peripheral blood leukocyte H-2 haplotyping was performed by flow cytometry after staining with AF6-88.53 (H-2^b specific) conjugated to Alexa Fluor 647 and SF1-1.1.10 (H-2^d specific) conjugated to FITC (BioLegend).

PCR genotyping. FcRγ-deficient and -sufficient mice were genotyped using a protocol and primer sequences supplied by Taconic. C3-sufficient and -deficient mice were genotyped using a protocol from the Jackson Laboratory. FcγRI-sufficient and -deficient mice were genotyped using the following primers: 5′-CTTCCTCTGTGCTCTTCC-3′, 5′-ATTACATGATTCTTACCAGCTTTG-3′, and 5′-AAGGATGGAAAGGGAGCCTCT-3′. FcγRIII-sufficient and -deficient mice were genotyped using the following primers: 5′-CTCGTGCTTTACGGTATCGCC-3′, 5′-TCCGAAGGCTGTGGTGAAACTG-3′, and 5′-ACTCCCAGATCTACAGGGTCG-3′.

mAbs. Hybridomas that produce the mouse anti–H-2^d mAbs 34-1-2s, SF1-1.1.10, 34-5-8s, 28-14-8s, 31-3-42, 34-7-23s, 34-7-23s, 34-4-20s, and 34-2-12s and the rat IgG2a anti-FcγRII/III mAb 2.4G2 were purchased from the American Type Culture Collection. The rat IgG2b anti–Gr-1 mAb (anti-Ly6G/C), RB68c5, and its isotype control, J1.2, were gifts of R. Coffman and J. Abrams, respectively (DNAX Corp, Palo Alto, CA). The isotype control mouse IgG2a, δAI6.2 (anti–human IgD mAb), was a gift of J. Kearney (University of Alabama, Birmingham, AL). Hybridomas were grown i.p. in pristine primed nude or BALB/c mice. Ascites was collected, and IgG fractions were purified by ammonium sulfate precipitation (25–50% saturated fraction), followed by either affinity purification on protein A–Sepharose with $MgCl_2$ elution or cation exchange column chromatography (DE-52; GE Healthcare). Fractions that contained the desired mAbs were identified by Ouchterlony analysis with isotype-specific pAbs. The rat anti–mouse RBC mAb (TER119) was a gift from M. Jordan (CCRF). The purified hamster anti–mouse FcγRIV mAb 9E9 was a gift of J. Ravetch, whereas an isotype control hamster IgG was purchased from eBioscience.

TRALI induction and characterization. All experiments were repeated at least once unless otherwise specifically stated. Mice were injected i.v. through the tail vein with 125 µg anti–H-2^d mAb in 200 µl saline unless otherwise specified. Development of a breathing pattern characteristic of dyspnea was evaluated with whole body barometric plethysmography (Buxco Research Systems) as described in Results. The alteration in breathing pattern is characterized by an increase in an index known as Penh, which reflects prolongation of the late phase of expiration and an increased rate of pressure change during expiration. An increase in Penh does not precisely identify the mechanism responsible for respiratory distress.

Determination of lung water. Mice were anesthetized by i.p. injection of sodium pentobarbital and rapidly exsanguinated by complete transection of their abdominal aorta and vena cava. Lungs were removed intact, frozen in liquid nitrogen, weighed, lyophilized for 24 h, and reweighed. The total weight of lung water was determined as the difference between pre- and postlyophilization weight. The percentage of lung weight accounted for by water was determined by dividing the difference between the pre- and postlyophilization weights by the prelyophilization weight and multiplying the result by 100%.

Bronchoalveolar lavage (BAL) and protein content. Mice were anesthetized by i.p. injection of sodium pentobarbital followed by dissection of the neck with isolation of the trachea. Polyethylene catheters (OD = 0.97 mm; BD) were inserted into their tracheas through a small slit made with iris scissors. Lungs were lavaged three times with 1 ml Ca^{2+}- and Mg^{2+}-free HBSS (Lonza) that contained 0.02% EDTA and EGTA. The BAL fluid was centrifuged at 1,000 rpm for 5 min at 4°C, and the cell pellets were removed. The cell-free BAL fluid was aliquoted and stored at −80°C. The protein content of the BAL fluid was measured according to the manufacturer's instructions using the Pierce Protein Assay (Thermo Fisher Scientific) with BSA as standard.

Evaluation of hypoxemia. This was performed by measuring the pO_2 of blood obtained by intracardiac puncture 15 min after anti–H-2 mAb challenge with an IRMA blood gas analyzer (RNA Medical).

Histology. After exsanguination under pentobarbital anesthesia, lungs were gently inflated in situ with formalin and then excised and fixed in formalin

for a minimum of 24 h before paraffin embedding and staining with hematoxylin and eosin (H&E). To evaluate 34-1-2s binding to lungs, mice were injected with biotinylated 34-1-2s or isotype control mAb (biotinylated using EZ-link protocol by Thermo Fisher Scientific), lungs were gently inflated in situ with OCT, harvested, and snap-frozen in liquid nitrogen. Frozen sections were cut and affixed to microscope slides. Rabbit antibiotin Ab (HRP conjugated; Bethyl Laboratories, Inc.) was incubated (1:100; 32 min) at room temperature. After washing, sections were incubated with biotinylated anti–rabbit Ig secondary Abs. After subsequent washing, staining with streptavidin-peroxidase, peroxidase development, and coverslipping, sections were photographed with a digital camera (E5000; Nikon).

Electron microscopy. Lungs excised after exsanguination under pentobarbital anesthesia were cut into 1-mm^3 cubes and fixed in phosphate-buffered 3% glutaraldehyde at 4°C for 24 h. Samples were washed three times in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide buffered with cacodylate, pH 7.2, at 4°C for 1 h. After dehydration in serial alcohol and propylene oxide, samples were infiltrated with and embedded in LX112. Thin sections were stained with uranyl acetate and lead citrate. Imaging was performed on a transmission electron microscope (7600; Hitachi).

Immunofluorescence microscopy. Lungs from mice anesthetized and exsanguinated by abdominal aorta incision were inflated with a 50:50 mixture of OCT and saline and then excised. After placement in OCT, the lungs were immediately frozen in liquid nitrogen. Frozen tissue sections were cut, mounted on glass slides, fixed in acetone, and air dried. After rehydration and blocking, immunofluorescence microscopy was performed with FITC-labeled anti-C3 mAb (ICN Biomedicals Inc./Cappel). After washing, slides were coverslipped using antifade medium containing DAPI (Prolong Gold; Invitrogen) and assessed and photographed at an original magnification of 400 using a digital camera (RT Slider; Diagnostic Instruments Inc.) mounted on a fluorescent microscope (E600; Nikon).

Macrophage depletion. Mice were depleted of macrophages by injecting 1 mg gadolinium (Sigma-Aldrich) in 200 µl saline i.v. 24 h before challenge (Strait et al., 2002) or i.t. with 100 µl and/or i.p. with 200 µl clodronate encapsulated in liposomes (a gift of Roche) as described previously (Thepen et al., 1989; Van Rooijen and Sanders, 1994, 1997) 48 h before anti–H-2^d mAb challenge. Macrophage, neutrophil, and lymphocyte absolute numbers were measured by a Beckman Coulter counter and confirmed by flow cytometry.

Neutrophil depletion. Neutrophils were depleted by injecting mice i.p. daily for 7 d before challenge with 20 mg hydroxyurea (Sigma-Aldrich). In a second protocol, mice were injected i.p. once daily for 7 d with 20 mg hydroxyurea before challenge and i.p. with 100 µg Rb68c5 (anti–Gr-1/Ly6G/C mAb) 24 and 48 h before challenge.

Evaluation of neutropenia. Blood was collected from an incised tail vein into an EDTA-containing tube (BD), and neutrophil counts were performed with a Hemavet 850FS apparatus (Drew Scientific). Counts were confirmed manually by counting total nucleated cells microscopically with a hemocytometer and determining the percentage of nucleated cells that were neutrophils by microscopic exam of a Giemsa stain of a peripheral blood smear.

Platelet depletion. Mice were depleted of platelets by injecting them i.v. with either 200 µl of a 1:40 vol/vol dilution in saline of rabbit anti–mouse platelet antiserum (Accurate Chemical) once daily for 2 d before challenge (Strait et al., 2002) or with 0.1 U neuraminidase type IV (Sigma-Aldrich) i.p. 24 h before challenge (Stenberg et al., 1991; Strait et al., 2002). Peripheral blood platelet counts were performed as previously described (Strait et al., 2002).

ROI inhibition. ROI inhibition was obtained by following the published method of Hidalgo et al. (2009). In brief, *N*-acetylcysteine (Sigma-Aldrich) was freshly diluted to a concentration of 3.5 mg in 200 µl saline and then injected i.v. into mice 5 min before challenge with 34-1-2s.

Quantitation of C3 and IgG2a binding to targeted blood cells. Blood was collected from an incised tail vein into a heparin-containing tube on ice. Erythrocytes were lysed with ammonium chloride lysis solution at 4°C, and nucleated cells were washed twice by centrifugation with cold HBSS. Cells were stained with biotinylated anti-C3 pAb (ICN Biomedicals Inc./Cappel) and anti–mouse IgG2a fluorochrome-labeled mAbs (BD) and then analyzed by flow cytometry with a FACSCalibur (BD).

C3 and C5 ELISAs. Blood from tail vein was drawn into K2-EDTA–containing tubes (BD) on ice and centrifuged for separation of plasma. Plasma was aliquoted and stored at −80°C. Plasma C3 concentration was measured according to the manufacturer's instructions using a mouse C3 ELISA kit (Immunology Consultants Laboratory). Plasma C5 concentration was detected by sandwich ELISA, using rabbit anti–mouse C5 pAb (Cedarlane Laboratories) to capture C5, a biotin-labeled preparation of the same Ab to detect bound C5 and streptavidin followed by the luminogenic substrate, Supersignal (Thermo Fisher Scientific). An ELISA plate reader from Fluoroskan Ascent FL was used to measure luminescence.

Plasma transfer. Blood was drawn from the tail veins of genetic background– and gender-matched mice into 3.2% sodium citrate–containing tubes (1:10 [vol/vol]) on ice and centrifuged at 4°C, and plasma was separated and pooled, aliquoted, and stored at −80°C. 400 µl of the pooled plasma was injected i.v. 60 and 30 min before challenge with 34-1-2s or control mAb, which were both diluted in 200 µl of the same pooled plasma.

Generation of BM chimeric mice. RAG2-deficient or -sufficient C57BL/6 and BALB/c background male mice were injected i.p. with 50 µl anti-asialo GM1 antiserum, (Wako Chemicals USA) and then exposed to 350 rads of gamma radiation. The next day, the irradiated mice were injected i.v. with 15–24 million BM cells from donor C57BL/6 or BALB/c background RAG2-deficient or CB6 mice. The mice were fed doxycycline-containing food for 2 wk followed by conventional chow thereafter. Peripheral blood obtained 6 wk after reconstitution was analyzed by flow cytometry for percentage of H-2^d and H-2^b cells.

LPS treatment. Mice were anesthetized with AnaSed and ketamine i.p. and then suspended by their incisors in the upright position followed by instillation of 2 µg LPS i.t. (*Salmonella enterica*; Sigma-Aldrich). Mice were allowed to recover and then challenged i.v. 3 d later with 8 (Fig. 6 E) or 125 µg (Fig. 6 D) 34-1-2s and followed for the development of symptoms of mTRALI.

Statistics. A two-tailed Mann-Whitney *t* test (Prism; GraphPad Software) was used to assess differences between treatment groups. A Pearson correlation (PrismA) was used to evaluate the coefficient of determination (r^2) of the two variables. Linear regression was used to plot the best-fit line with 95% confidence bands. A p-value <0.05 was used as the demarcation of significance.

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