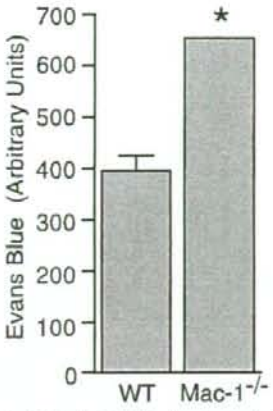

FIGURE 3. Role of Mac-1 in RPA-induced vascular leakage. WT and Mac-$1^{-/-}$ mice were subjected to the RPA model and 4 h later Evans blue was extracted from harvested skin to assess edema. *, $p < 0.05$; n = 8/group. Data are average ± SEM.

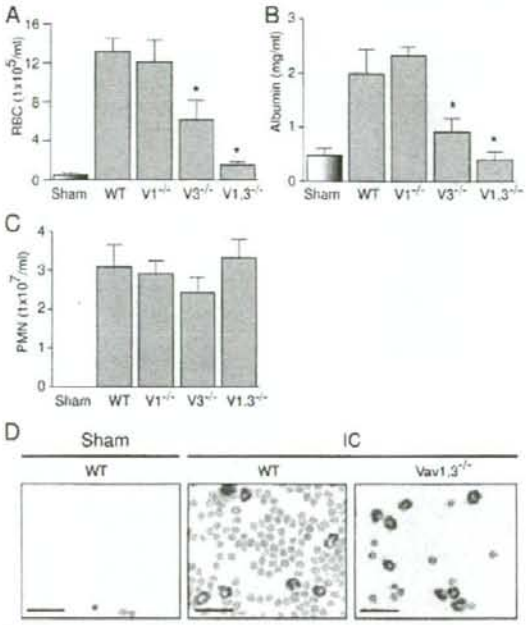

FIGURE 4. The effects of Vav deficiency in the lung RPA reaction. WT, Vav 1-deficient (V$1^{-/-}$), Vav 3 (V$3^{-/-}$)-deficient, and Vav 1,3 (V$1,3^{-/-}$)-deficient mice were given OVA injections i.v. and anti-OVA intratracheally. Sham-treated mice were injected intratracheally with buffer alone. Four hours later, the mice were killed and the BAL was collected to evaluate the number of RBC (*A*), the amount of albumin (*B*), and the number of emigrated neutrophils (*C*) in WT, V$1^{-/-}$, V$3^{-/-}$, and V$1,3^{-/-}$ mice. The content of RBC (*A*) and albumin (*B*) in BAL fluid of sham-treated mice of WT and Vav$^{-/-}$ mice were combined to generate the data presented since no statistical differences were detected among sham-treated animals. *, $p < 0.05$ between V$3^{-/-}$ or V$1,3^{-/-}$ mice and WT animals. n = 4/group. All data are average ± SEM. *D*, A representative image of Giemsa-stained cytospin preparations of BAL fluid retrieved after 4 h following sham injection in WT mice or following induction of the RPA reaction (IC) in WT and V$1,3^{-/-}$ mice. Bar, 25 μm.

$1,2^{-/-}$ mice were completely protected (Fig. 2, *B* and *C*). This suggests that Rac1 and Rac2 functionally overlap. We have previously shown that FcγR cross-linking induced Rac activation is significantly reduced in Vav $1,3^{-/-}$ neutrophils (22). Thus, our in vivo data infer that Vav regulation of Rac 1,2 activity is required for IC-induced tissue injury. A reduction in edema may be a consequence of diminished neutrophil accumulation in tissue and/or defective cytotoxic functions (37). Histological analysis revealed no significant difference in neutrophil accumulation in Vav $1,3^{-/-}$ mice compared with WT mice, and a partial reduction in neutrophil influx was observed in Rac $1,2^{-/-}$ mice (Fig. 2*D*). Thus, Vav is not required for neutrophil influx while Rac may contribute, but is not essential for neutrophil accumulation in this model.

The skin RPA does not require Mac-1

FcγR engagement leads to Mac-1 activation and cooperation of these two receptors is required to sustain FcγR-mediated murine neutrophil adhesion (10) and other phagocytic functions (38). Given these reports and our previous data that Vav proteins are required for Mac-1-dependent functions in vitro (21), we examined the contribution of Mac-1 to the RPA reaction. Mac-1 deficiency resulted in an increase in Evans blue leakage compared with WT counterparts (Fig. 3). Although the mechanisms for the increase needs further investigation, these results demonstrate that Mac-1 is not required for the development of the RPA reaction and rules out the possibility that Vav proteins promote IgG-induced tissue injury by modulating Mac-1.

Contribution of Vav in the development of the RPA reaction in the lung

To examine Vav in a more clinically relevant model of IC disease, Vav$^{-/-}$ mice were subjected to the RPA reaction in the lung, which shares several features of acute lung injury in patients (39) and requires neutrophils and FcγRs (40, 41). The phenotype in Vav$^{-/-}$ mice was similar to that observed in the skin RPA model. Although mice with Vav 1 deficiency alone were similar in susceptibility to WT mice, Vav $1,3^{-/-}$ mice were protected from acute lung injury as assessed by a significant reduction in albumin and RBC content in the BAL fluid, which are readouts of edema and hemorrhage, respectively (Fig. 4, *A* and *B*). A partial reduction in edema and hemorrhage was observed in Vav $3^{-/-}$ mice. The number of infiltrated neutrophils in the BAL fluid of WT-, Vav 1,3-, Vav 1-, and Vav 3-deficient mice was equivalent (Fig. 4, *C* and *D*). Thus, similar to the cutaneous RPA reaction, Vav was not required for IgG-induced neutrophil accumulation but was critical for IgG/FcγR-induced tissue injury.

Vav- and Rac-deficient mice are susceptible to the LSR in the skin

Vav is required for Mac-1/complement-dependent functions in vitro including sustained adhesion to serum-coated plates and phagocytosis of serum-opsonized *E. coli* (21). To evaluate the physiological relevance of these findings in vivo, we examined the contribution of Vav and Rac on neutrophils to the development of the LSR, a response that is critically dependent on Mac-1 expressed on neutrophils, complement C3 deposition within the vessel wall, and neutrophil elastase release (12). The LSR does not require FcγRs since Fcγ chain-deficient mice display a normal LSR response (D. Mekala and T. N. Mayadas, unpublished data). The LSR is induced by the intradermal injection of LPS followed 24 h later by the delivery of TNF at the same site, which results in hemorrhage that is associated with

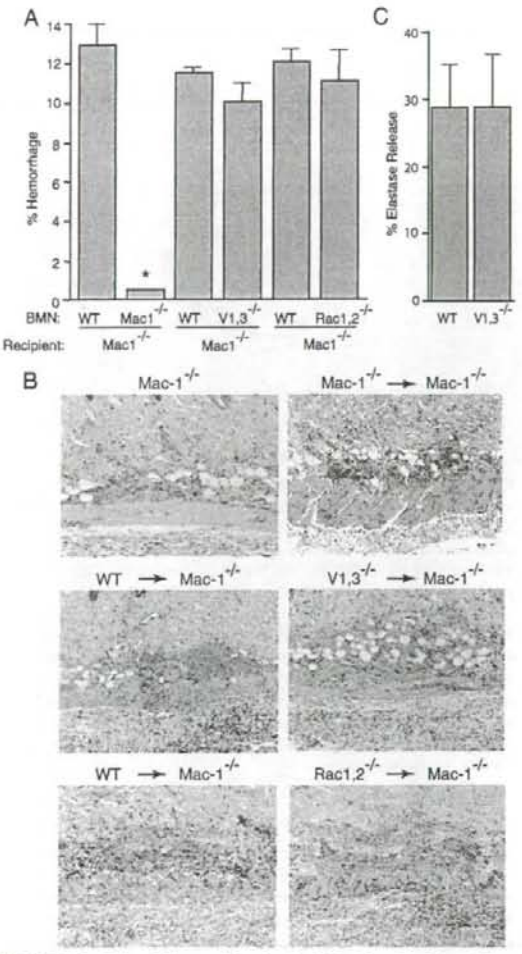

FIGURE 5. Role of Vav and Rac in the LSR. Mac-1-deficient (Mac-$1^{-/-}$) mice were i.v. reconstituted with BMN harvested from Mac-$1^{-/-}$, Vav$1,3^{-/-}$ (V$1,3^{-/-}$), Rac$1,2^{-/-}$ mice, or their respective WT counterparts and subjected to LSR. *A*, The percentage of tissue area with extravascular RBC (hemorrhage) was scored as described in *Materials and Methods*. *, $p < 0.05$. Data are average ± SEM. $n = 9$ recipient Mac$1^{-/-}$ mice for reconstitution with Vav$^{-/-}$ or WT BMN and $n = 4$ for reconstitution with Mac-$1^{-/-}$, Rac$^{-/-}$, or their WT counterparts. *B*, Representative H&E-stained slides of LSR in Mac-$1^{-/-}$ mice, and Mac-$1^{-/-}$ mice reconstituted with Vav neutrophils (V$1,3^{-/-}$ → Mac-$1^{-/-}$), Rac neutrophils (Rac$1,2^{-/-}$ → Mac-$1^{-/-}$), or their corresponding WT counterparts (WT → Mac-$1^{-/-}$). *C*, The percent elastase release in neutrophils and fluid retrieved from the air pouch of WT and V$1,3^{-/-}$ mice in which LSR was induced was evaluated by analyzing elastase substrate cleavage in vitro.

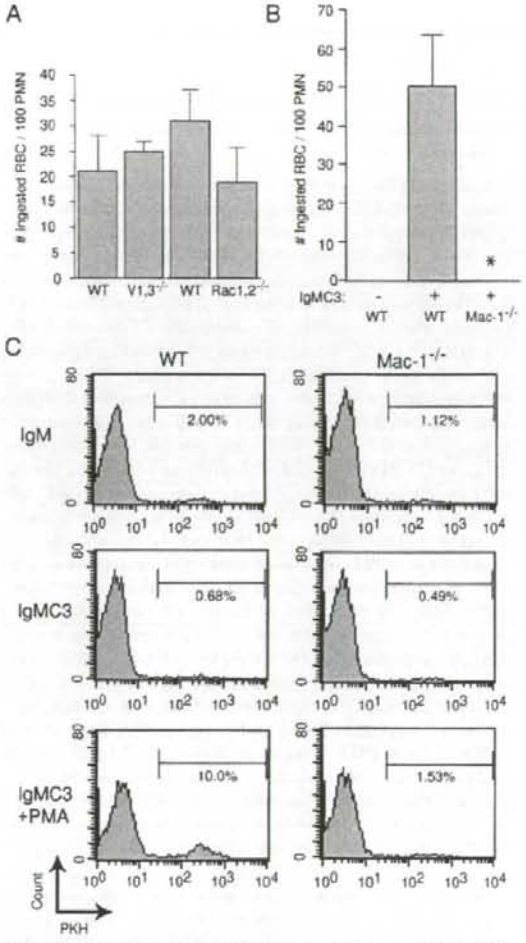

FIGURE 6. In vitro neutrophil binding to and phagocytosis of iC3b-coated SRBC. *A*, Vav- and Rac-deficient neutrophils and their corresponding WT counterparts were pretreated with PMA and incubated with IgM-C3-opsonized RBC. *B*, PMA-pretreated WT or Mac-1-deficient (Mac-$1^{-/-}$) neutrophils were incubated with nonopsonized (IgMC3, −) or opsonized (IgMC3, +) RBC. Cells in *A* and *B* were evaluated for RBC internalization following lysis of external RBC and the numbers of RBC internalized per 100 neutrophils were counted. No RBC internalization was observed without PMA pretreatment (data not shown). *, $p < 0.05$. $n = 6$ independent samples per group. *C*, RBC were PKH-fluorophore-labeled. WT or Mac-$1^{-/-}$ neutrophils were mixed with nonopsonized RBC (IgM) or IgM-C3-opsonized RBC (IgMC3) on ice with or without PMA as indicated, and RBC binding was analyzed by flow cytometry. One representative of two independent experiments are presented. The percentage of neutrophils that bound RBC are indicated in each panel.

neutrophil accumulation and neutrophil elastase release. Neutrophils isolated from either Vav $1,3^{-/-}$, Rac $1,2^{-/-}$ mice, or their respective WT counterparts were i.v. injected into Mac-1-deficient (Mac-$1^{-/-}$) mice that are resistant to developing the LSR. The reconstituted mice were then subjected to LSR. This approach allowed us to evaluate the contribution specifically of the Vav/Rac pathway in neutrophils in Mac-1/complement-dependent neutrophil cytotoxicity in vivo. Mac-$1^{-/-}$ mice injected with Vav $1,3^{-/-}$ or Rac $1,2^{-/-}$ developed hemorrhage to a similar extent as mice reconstituted with WT neutrophils (Fig. 5, *A* and *B*), whereas Mac-$1^{-/-}$ mice given Mac-1-deficient neutrophils failed to develop disease (Fig. 5, *A* and *B*) as previously described (12). Extracellular elastase activity was quantitated in reconstituted Mac-$1^{-/-}$ mice by real-time analysis of elastase substrate cleavage in neutrophils and fluid retrieved from air pouches in which the LSR was induced. Elastase activity was comparable in Mac-$1^{-/-}$ mice reconstituted with Vav $1,3^{-/-}$ or

WT neutrophils (Fig. 5*C*). Thus, Vav and Rac proteins on neutrophils are dispensable for the development of hemorrhage, a major aspect of disease in the LSR as well as elastase release, which contributes to tissue injury in this model (12).

Mac-1-dependent phagocytosis of C3-opsonized RBC does not require Vav or Rac proteins

In light of our data that Vav- and Rac-deficient neutrophils were able to promote LSR, we revisited complement-mediated phagocytosis in vitro to reassess the role of these proteins in this process. Vav was required for neutrophil phagocytosis of complement-opsonized *E. coli* in vitro (data not shown), as published previously (21). However, Vav and Rac proteins were dispensable for PMA-mediated neutrophil uptake of complement C3-opsonized RBC (C3-RBC). C3-RBC were generated by incubating IgM-coated RBC with fresh serum deficient in complement C5 (Fig. 6*A*), which results in complement activation and subsequent iC3b deposition but avoids RBC lysis (42, 43). Unlike the phagocytosis of serum-opsonized *E. coli*, which is only partially Mac-1 dependent, uptake of C3-RBC depended exclusively on Mac-1 and complement opsonization. That is, Mac-$1^{-/-}$ neutrophils or WT cells incubated with unopsonized RBC exhibited no detectable phagocytosis (Fig. 6*B*). Furthermore, phagocytosis was only detected when cells were PMA stimulated (data not shown). Previous studies in human monocytes and neutrophils indicate that C3-RBC bind to Mac-1 on the surface of resting cells but that subsequent phagocytosis requires PMA (44–46). To examine whether this also applies to murine BMN, we exploited FACS analysis to examine the binding of the fluorophore-conjugated C3-RBC to the surface of neutrophils in the absence and presence of PMA. Resting WT neutrophils exhibited no binding to either RBC alone or C3-RBC. Upon PMA stimulation, binding of C3-RBC significantly increased. This was Mac-1 dependent, because Mac-$1^{-/-}$ BMN exhibited no significant binding to C3-RBC under these conditions (Fig. 6*C*). Thus, PMA stimulation in murine neutrophils promotes Mac-1 binding and subsequent phagocytosis of C3-RBC. Together our studies suggest Vav and Rac are not required for PMA-induced binding and phagocytosis of C3-RBC, which are strictly Mac-1-dependent processes. The results of this in vitro assay correlated well with the lack of effect of Vav and Rac deficiency in the development of the LSR, which is dependent on Mac-1 and complement C3 (12).

Discussion

A deficiency in activating FcγRs, by virtue of deletion of the common γ-chain subunit, results in protection from several IgG-mediated, neutrophil-dependent diseases, including acute glomerulonephritis, arthritis, acute lung injury, and autoimmune skin disorders (3, 4, 7, 8, 17, 18). In all of these models, a reduction in neutrophil influx was observed in mice deficient for FcγRs, thus precluding conclusions about the role of these receptors in subsequent steps of neutrophil activation that lead to tissue injury. Our data using mice with defects in neutrophil FcγR signaling preserve the ability of FcγRs to bind ICs, thus allowing us to distinguish these different roles for the receptors in inflammatory disease. Thus, our work suggest that FcγR binding of tissue-deposited IC is required for neutrophil migration, but the signaling reactions from the FcγRs is critical in cellular activation leading to tissue injury (Fig. 7). The restoration of disease in Vav $1,3^{-/-}$ recipients reconstituted with WT but not Fcγ chain$^{-/-}$ neutrophils provides compelling evidence that FcγR signaling through Vav proteins in neutrophils is responsible for IC-induced tissue injury (Fig. 7). However, we cannot rule out the possibility that the two mutations, Vav and FcγR, disrupt two entirely separate parallel pathways.

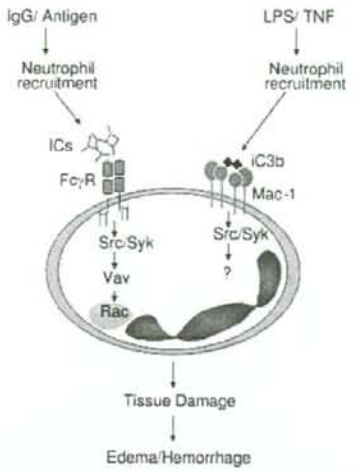

FIGURE 7. Model of Vav-mediated inflammation-induced tissue injury in the RPA reaction. Ab interaction with Ag results in the generation of ICs. IC deposited within vessels and perivascular sites trigger neutrophil recruitment that is dependent on FcγRs on mast cells and/or neutrophils. This step does not require Vav and is partially dependent on Rac. Neutrophil FcγR engagement of ICs leads to recruitment of Src and Syk kinases that promotes Vav activation and subsequent GTP loading of Rac GTPases. Vav/Rac trigger neutrophil cytotoxicity that leads to tissue damage and subsequent edema and hemorrhage through mechanisms that remain to be determined. Sequential injections of LPS and TNF result in neutrophil recruitment and C3bi deposition within the vasculature (12). Mac-1 interaction with C3bi triggers the activation of Src and Syk kinases. The signaling cascade downstream of these tyrosine kinases that promote tissue damage is currently unclear.

Previous characterizations of gene knockout mice including Fcγ chain (18), C5a (47), urokinase-type plasminogen activator receptor (48), ICAM-1 (49), L-selectin (31), Syk (50), CD18 (51), and elastase-cathepsin-G double knockout (52) in the skin and/or lung RPA reaction have shown that reductions in edema and hemorrhage correlate closely with reductions in the numbers of transmigrated neutrophils in tissue. However, this is not the case when a signaling deficiency involves the Vav proteins, because neutrophils lacking these proteins are able to migrate normally into the tissue but fail to become activated. The uncoupling of FcγR-mediated neutrophil transmigration from cytotoxicity at the level of Vav provides the first genetic evidence that these two processes can be dissociated. In contrast, Vav/Rac proteins were not required for complement C3 and complement receptor Mac-1-mediated inflammation, which demonstrates selectivity of the Vav/Rac axis for IgG-mediated cytotoxicity in vivo.

Neutrophil diapedesis to sites of IgG-mediated tissue injury in the skin and lung has been associated with vessel injury (8, 51). Neutrophil accumulation in the BAL is likely dependent on macrophage and partially on mast cell functions since elimination of these cells reduced neutrophil accumulation in the alveolar space (53). In the skin, FcγRIII and C5aR on mast cells and leukotrienes have been primarily implicated in promoting neutrophil recruitment (16, 54, 55). However, a variable role for mast cells in neutrophil recruitment has been reported in the skin RPA. Some studies demonstrate a significant reduction in neutrophil accumulation in mast cell-deficient mice (55), while others including our own data not shown suggest that mast cell-deficient mice exhibit only a partial reduction in neutrophil accumulation and edema compared with their WT counterparts in the skin RPA (16, 54). This indicates

that other mast cell-independent mechanisms contribute to neutrophil accumulation. Indeed, we and others have shown that neutrophils may be recruited via their own FcγRs by directly tethering to deposited ICs (3, 13, 56). In summary, FcγRs on both mast cells and neutrophils may contribute to neutrophil recruitment. A possible model is that FcγRs induced release of mast cell mediators such as TNF may prime FcγRs on circulating neutrophils for enhanced binding to ICs (57). The phenotype in Vav-deficient mice suggest that the process of neutrophil diapedesis alone is not sufficient for causing tissue injury during IgG-mediated inflammation as previously suggested. Rather, the interaction of transmigrated neutrophils with perivenular and/or tissue ICs are likely primarily responsible for the ensuing changes in vascular integrity. The requirement for Vav 3 alone in neutrophil cytotoxicity suggests that although certain functions of Vav proteins are redundant; individual Vav proteins in neutrophils also serve specialized functions as shown in lymphocytes and osteoclasts (28, 58). Previous work has not established which specific Rho family GTPases serves as a direct substrate for each Vav family member to mediate a specific function. Our studies suggest that Vav links to Rac proteins in neutrophils to mediate neutrophil cytotoxicity in vivo (Fig. 7). It is noteworthy that unlike Vav-deficient mice, Rac deficiency results in a 50% reduction in neutrophil accumulation at the site of RPA induction. This reduction alone is likely not the primary explanation for the observed decrease in edema in these animals, which is far greater than would be expected from a partial loss of neutrophil recruitment. Indeed, mice lacking the neutrophil recruitment receptors L-selectin (31) or ICAM-1 (49) exhibit a 50% reduction in neutrophil accumulation that is associated with only a 40% decline in edema in the skin RPA. Thus, we deduce that Rac plays an additional role downstream of neutrophil recruitment in promoting IgG-mediated neutrophil cytotoxicity in our studies.

Our previous work has shown that hemorrhage in the LSR relies on Mac-1 interaction with complement deposited in the vessel wall and the coupling of Mac-1 to Syk and Src family kinases to trigger elastase release (12). The normal LSR response in both the Vav- and Rac-deficient mice demonstrates that the Vav/Rac axis is not required for Mac-1/complement C3-mediated tissue injury. The dispensability of Vav and Rac in the LSR was surprising given that these proteins are required for neutrophil phagocytosis of complement-opsonized *E. coli* (21). However, PMA-induced phagocytosis of complement-opsonized RBC, which is strictly dependent on Mac-1 and complement fixation, did not rely on Vav and Rac. *E. coli* is a complex target that engages neutrophil LPS receptors such as CD14 and TLR2, which activate Mac-1 (59). The observed defect in engulfment of serum-opsonized *E. coli* in Vav- and Rac-deficient neutrophils may be the result of ineffective Mac-1 activation. The emerging model of integrin activation is that agonist-induced conformational changes in the integrin increases affinity for ligand. Integrin clustering induced by multivalent ligand binding is coupled with agonist-induced intracellular signals that contribute to integrin redistribution. These steps, modulated by the actin cytoskeleton, are responsible for adhesion strengthening (60). Vav $1,3^{-/-}$ neutrophils are able to bind serum-opsonized *E. coli* to their surface (21). This indicates that Vav is not required for integrin affinity changes, thus leaving a possible role for Vav in integrin lateral mobility and subsequent productive adhesion required for C3-*E. coli* phagocytosis. The fact that C3-RBC phagocytosis in vitro assay correlated better with the development of tissue injury in the LSR than the complement-opsonized *E. coli* uptake assay suggests that the C3-RBC phagocytosis is a more reliable predictor of in vivo cytotoxicity. We propose that PMA, used to promote C3-RBC phagocytosis, increases β_2 integrin lateral diffusion (61, 62) likely through protein kinase C-dependent effects on the cytoskeleton (45, 63), thus bypassing any role for Vav in Mac-1 integrin activation. Indeed, in agreement with the results in C3-RBC, PMA treatment rescued the defect in phagocytosis of C3-opsonized *E. coli* in Vav $1,3^{-/-}$ neutrophils (21). Unlike human neutrophils, PMA is essential for target binding in murine neutrophils. This difference may be attributed to species-specific differences in the activation state of integrin. Our observed correlation of results from phagocytosis of C3-RBC in vitro and outcomes in the LSR in vivo provides support for the idea that PMA-stimulated C3-RBC phagocytosis may serve as a reliable predictor for complement-mediated vasculitis in vivo. This hypothesis is also supported by our finding that PMA-induced neutrophil phagocytosis of C3-RBC requires Src family and Syk kinases (A. Utomo and T. N. Mayadas, unpublished data), which are essential for LSR development (12). How can PMA-induced C3-RBC phagocytosis be a predictor of a physiological process in vivo? We suggest that PMA-induced activation of Mac-1 is served by multiple stimuli (e.g., TLR and G protein-coupled receptor agonists) encountered in vivo during the inflammatory response. However, the subsequent phagocytic step in vitro may be akin to Mac-1-mediated adhesion to complement C3 leading to vessel injury. Thus, signaling pathways identified in the in vitro assay may predict their contribution to vessel injury in vivo. Spreading or sustained adhesion on opsonized surfaces has been coined as "frustrated" phagocytosis, because the surface of the coated plates mimics a topology of an infinitely large target particle (64). Although Vav-deficient neutrophils cannot sustain adhesion/spreading on IgG- or iC3b-coated surfaces (21, 22), they can phagocytose iC3b-coated SRBC. Thus, the requirements for neutrophil phagocytosis rather than spreading correlate with those needed for mediating tissue injury in vivo. In contrast to the results in neutrophils, Vav and Rac proteins were recently shown to be essential for phagocytosis of complement-coated RBC by murine macrophages (Ref. 35 and A. Utomo and T. N. Mayadas, unpublished data). These differences in intracellular signaling pathways may be used as explanations for why neutrophil phagocytosis is more proinflammatory (i.e., results in greater reactive oxygen species generation) than macrophages. This dichotomy in use of the Vav/Rac axis by the same receptor family in two different phagocyte populations may also provide an opportunity to differentially target specific functions in one or the other phagocytic cell type.

There are likely differences in the mechanisms of tissue injury induced by the LSR and the RPA reaction. In the LSR, neutrophil interaction with complement within the vessel leading to elastase release is responsible for the observed edema and hemorrhage (12). Conversely, in the Arthus-type lesion, extravascular PMN stimulated by perivascular or tissue ICs are likely responsible for impairment of vascular integrity (65). FcγR engagement by ICs initiates the production of cytokines and lipid mediators. Of these, leukotrienes, platelet-activating factor, and bradykinin likely promote plasma extravasation in the RPA reaction as inhibitors or knockouts of these known vascular permeability factors or their corresponding receptors significantly blunts the RPA reaction (65). Of these factors, we speculate that bradykinin is a plausible candidate for Vav-dependent edema because tissue kallikreins, which generate kinins from low- and high-molecular mass kininogens, are present in neutrophils as are the kininogens themselves (66, 67).

In conclusion, by separating neutrophil recruitment and activation at the level of signaling molecules downstream of FcγRs, we demonstrate that effector functions triggered by FcγR engagement are central to disease pathology in IC-mediated processes. Furthermore, we have shown that Vav selectively links one of the two

major neutrophil opsonic receptors to specific cytotoxic functions in vivo. There has been a recent focus on targeting signaling molecules as an approach to reduce inflammation in vivo (68). Targeting Vav in neutrophils has the potential of specifically reducing IgG-mediated tissue injury in immune-mediated diseases while retaining neutrophil recruitment, a strategy that would minimize overall immune suppression.

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Disclosures

The authors have no financial conflict of interest.

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