

Phospholipase C γ 2 is Essential for Experimental Models of Epidermolysis Bullosa Acquisita

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Phospholipase C γ 2 (PLC γ 2) mediates tyrosine kinase-coupled receptor signaling in various hematopoietic lineages. Although PLC γ 2 has been implicated in certain human and mouse inflammatory disorders, its contribution to autoimmune and inflammatory skin diseases is poorly understood. In this study, we tested the role of PLC γ 2 in a mouse model of epidermolysis bullosa acquisita triggered by antibodies against type VII collagen (C7), a component of the dermo-epidermal junction. PLC γ 2-deficient (*Plcg2*^{-/-}) mice and bone marrow chimeras with a *Plcg2*^{-/-} hematopoietic system were completely protected from signs of anti-C7-induced skin disease, including skin erosions, dermal-epidermal separation, and inflammation, despite normal circulating levels and skin deposition of anti-C7 antibodies. PLC γ 2 was required for the tissue infiltration of neutrophils, eosinophils, and monocytes/macrophages as well as for the accumulation of proinflammatory mediators (including IL-1 β , MIP-2, and LTB₄) and reactive oxygen species. Mechanistic experiments revealed a role for PLC γ 2 in the release of proinflammatory mediators and reactive oxygen species but not in the intrinsic migratory capacity of leukocytes. The phospholipase C inhibitor U73122 inhibited dermal-epidermal separation of human skin sections incubated with human neutrophils in the presence of anti-C7 antibodies. Taken together, our results suggest a critical role for PLC γ 2 in the pathogenesis of the inflammatory form of epidermolysis bullosa acquisita.

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INTRODUCTION

Epidermolysis bullosa acquisita (EBA) is an autoimmune subepidermal blistering skin disease caused by autoantibodies targeting the dermal anchoring fibril type VII collagen (C7), a component of the dermal-epidermal junction (Baum et al., 2014; Egami et al., 2020; Kridin et al., 2019; Schmidt and Zillikens, 2013; Turcan and Jonkman, 2015). In case of the inflammatory form of EBA, autoantibody deposition leads to local inflammation and disruption of the dermal-epidermal junction, mediated by complement activation and leukocyte (mainly eosinophil and neutrophil) infiltration. However, further details of its pathomechanism are poorly understood, limiting our options for therapeutic intervention (Kasperkiewicz and Schmidt, 2009; Maglie and Hertl, 2019).

Phospholipase C γ 2 (PLC γ 2) is primarily expressed in cells of hematopoietic origin and couples various tyrosine kinase-

mediated signal transduction pathways to downstream signaling events (Faccio and Cremasco, 2010; Jackson et al., 2021). PLC γ 2 plays important roles in B cells, neutrophils, macrophages, mast cells, NK cells, platelets, and osteoclasts and mediates signaling by B-cell receptors, Fc receptors, various integrins, C-type lectins, and the collagen receptor GpVI (Carau et al., 2006; Chen et al., 2008; Futosi et al., 2021; Graham et al., 2007; Hashimoto et al., 2000; Jakus et al., 2009; Kertész et al., 2012; Mao et al., 2006; Nonne et al., 2005; Tassi et al., 2005; Wang et al., 2000; Wen et al., 2002; Wonerow et al., 2003).

Several lines of evidence suggest a role for PLC γ 2 in autoimmune and inflammatory diseases. Gain-of-function mutations of *PLCG2* cause two human diseases: PLC γ 2-associated antibody deficiency and immune dysregulation (PLAID) (Ombrello et al., 2012) and autoinflammation and PLC γ 2-associated antibody deficiency and immune dysregulation (APLAID) (Zhou et al., 2012), characterized by immune dysregulation along with a high prevalence of autoimmune and autoinflammatory diseases. Gain-of-function mutations of *Plcg2* also trigger complex inflammatory diseases in experimental mice (Abe et al., 2011; Yu et al., 2005), whereas PLC γ 2-deficient (*Plcg2*^{-/-}) mice are protected from disease development in various arthritis models (Cremasco et al., 2010, 2008; Jakus et al., 2009). Although skin pathology emerged in some of those studies (Abe et al., 2011; Yu et al., 2005; Zhou et al., 2012), the role of PLC γ 2 in autoimmune and inflammatory skin diseases is poorly understood.

Tyrosine kinases are involved in diverse autoimmune and inflammatory skin diseases (Szilveszter et al., 2019). We have previously shown that myeloid Src family kinases and the Syk

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Abbreviations: C7, type VII collagen; EBA, epidermolysis bullosa acquisita; LTB₄, leukotriene B₄; PLC γ 2, phospholipase C γ 2; WT, wild type

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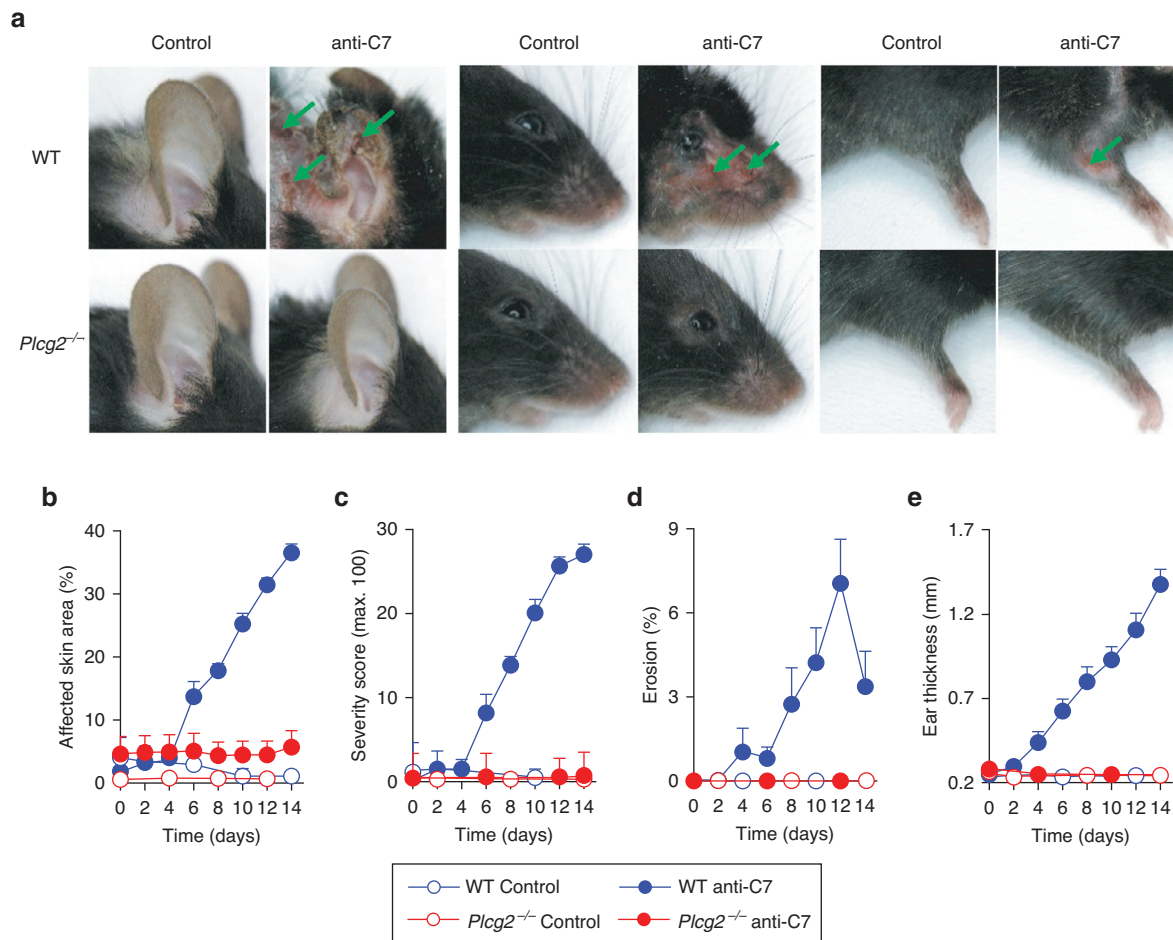


Figure 1. *Plcg2*^{-/-} mice are protected from autoantibody-induced skin blistering. WT and *Plcg2*^{-/-} mice were injected with PBS (control) or polyclonal anti-C7 antibodies every second day until day 8. Disease development was followed by (a) photographing on days 12–15, (b) scoring the extent of total affected skin area, (c) scoring the severity of lesions, (d) scoring the percentage of erosions, and (e) ear thickness measurements. Green arrows in panel a indicate erosions. Photographs are representative of and quantitative data show the mean and SEM of 5–9 mice per group from four to five individual experiments except for control-treated *Plcg2*^{-/-} mice where data of three mice from two experiments are shown owing to the limited availability of *Plcg2*^{-/-} mice. C7, type VII collagen; max., maximum; WT, wild type.

tyrosine kinase are required for skin inflammation in a mouse model of EBA (Kovács et al., 2014; Németh et al., 2017). Because PLC γ 2 may lie downstream of Src family kinases and Syk (Jakus et al., 2009; Németh et al., 2016), those studies suggest a potential role for PLC γ 2 in the development of autoimmune blistering skin diseases.

The above findings prompted us to test the role of PLC γ 2 in an autoantibody-induced mouse model of the inflammatory form of EBA. Our experiments revealed that PLC γ 2 is indispensable for all tested aspects of disease development in this model, supposedly because of a role for PLC γ 2 in the release of leukocyte proinflammatory mediators. A phospholipase C inhibitor also blocked dermal–epidermal separation in a human ex vivo model, suggesting relevance to human EBA.

RESULTS

PLC γ 2 deficiency protects mice from autoantibody-induced skin blistering

To test the role of PLC γ 2 in autoantibody-induced skin blistering, we injected wild-type (WT) and PLC γ 2-deficient (*Plcg2*^{-/-}) mice with polyclonal rabbit IgG antibodies against mouse type VII collagen (C7). As shown in Figure 1a, repeated systemic injection triggered severe skin erosion,

crust formation, and cutaneous inflammation in WT mice, which was most prominent in the ears but was also seen around the nose and at various areas of the trunk. Importantly, no such changes could be observed on similarly treated *Plcg2*^{-/-} mice. The time course of the percentage of the total affected skin area (Figure 1b), an overall clinical score taking into account the size and severity of each lesion (Figure 1c), as well as the percentage of the skin area showing erosions as a sign of blister eruption without the masking effect of other more severe secondary elementary lesions (Figure 1d) showed a robust increase on anti-C7 antibody treatment in WT but not in *Plcg2*^{-/-} mice (Figure 1b–d; $P = 0.0011$, 1.2×10^{-8} , and 0.042 , respectively). *Plcg2*^{-/-} mice were also protected from anti-C7–induced increase of ear thickness as a more objective measure of disease severity (Figure 1e; $P = 5.2 \times 10^{-5}$).

Therefore, *Plcg2*^{-/-} mice are protected from clinical signs of anti-C7–induced skin disease.

Defective autoantibody-induced skin blistering in *Plcg2*^{-/-} bone marrow chimeras

Breeding of *Plcg2*^{-/-} mice is hindered by their male infertility (Ichise et al., 2016) and the lower-than-expected ratio of

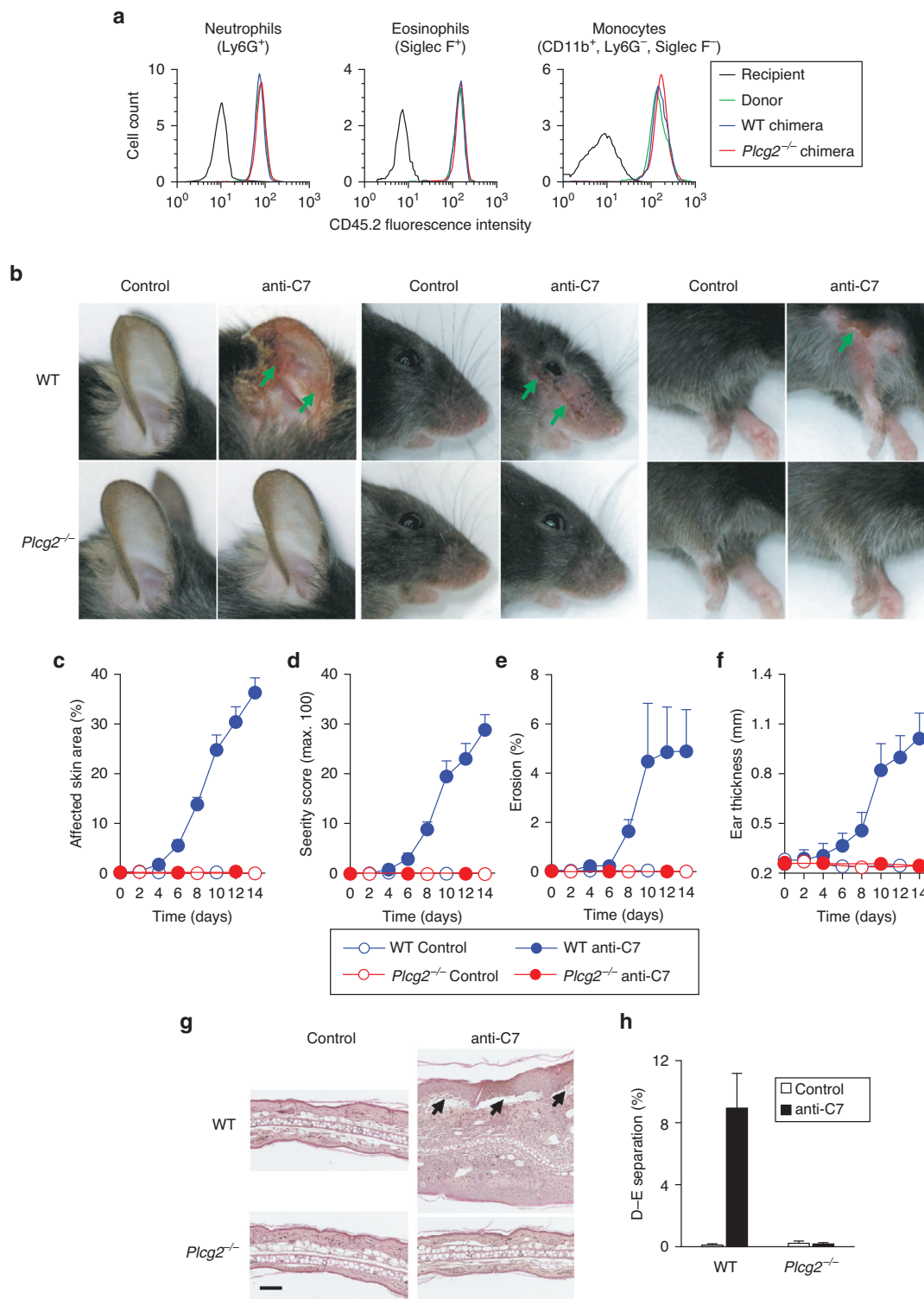
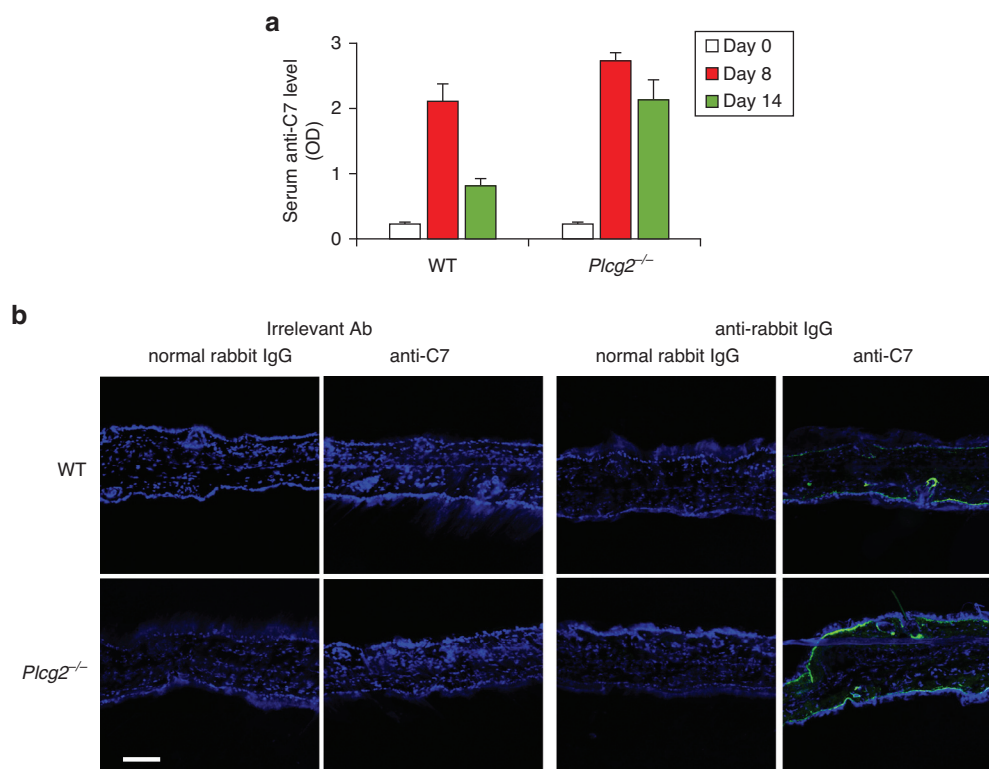


Figure 2. Autoantibody-induced skin blistering is abrogated in *Plcg2*^{-/-} bone marrow chimeras. (a) Flow cytometric analysis of the donor-specific CD45.2 marker 4 weeks after bone marrow transplantation in major circulating myeloid cell types. Histograms are representative of 12–23 chimeras per group from two to three independent experiments. (b–f) Skin blistering was induced in WT and *Plcg2*^{-/-} chimeras by systemic injection of PBS (control) or anti-C7 antibodies. Disease development was followed by (b) photographing on days 12–14, (c) scoring the extent of total affected skin area, (d) scoring the severity of lesions, (e) scoring the percentage of erosions, and (f) ear thickness measurements. Green arrows in panel b indicate erosions. (g, h) Ear samples were obtained from WT and *Plcg2*^{-/-} bone marrow chimeras on day 8. (g) Paraffin-embedded sections were generated and stained with H&E, followed by (h) quantification of the extent of D–E separation. Black arrows in panel g indicate D–E separation. Bar = 100 μ m. (b, g) Photographs are representative of and (c–e, h) quantitative data show the mean and SEM from 5–12 mice per group from three to five independent experiments. Data in panel f show the mean and SEM of 3–4 chimeras per group from two independent experiments. C7, type VII collagen; D–E, dermal–epidermal; WT, wild type.

Figure 3. Serum anti-C7 antibody level and deposition along the dermal–epidermal junction. (a)

Serum levels of injected anti-C7 antibodies of WT and *Plcg2*^{−/−} chimeras were measured by indirect ELISA. Blood samples were obtained on days 0, 8, and 14. Bar graphs show the mean and SEM from eight mice per group from three independent experiments. (b) WT and *Plcg2*^{−/−} chimeras were injected with 4 mg anti-C7. After 24 hours, immunofluorescent staining of deposited rabbit anti-C7 antibodies was obtained on cryosections of ears using Alexa Fluor 488–labeled anti-rabbit IgG or irrelevant antibody. (b) Histological images are representative of three mice per group from three independent experiments. Bar = 60 μ m. Ab, antibody; C7, type VII collagen; OD, optical density; WT, wild type.



Plcg2^{−/−} offspring from heterozygous mating (data not shown). To overcome those difficulties, we generated bone marrow chimeras with a *Plcg2*^{−/−} hematopoietic system. WT and *Plcg2*^{−/−} bone marrow cells were transplanted into lethally irradiated WT recipients carrying the CD45.1 allele. Flow cytometric analysis of the donor-derived CD45.2 marker 4 weeks after transplantation revealed that practically all circulating neutrophils, eosinophils, and monocytes were of donor origin (Figure 2a).

Repeated injection of anti-C7 antibodies triggered severe skin erosion, crust formation, and cutaneous inflammation in wild-type bone marrow chimeras (Figure 2b–f). Importantly, similar to intact *Plcg2*^{−/−} mice (Figure 1a–e), *Plcg2*^{−/−} bone marrow chimeras were also completely protected from the clinical appearance of disease development (Figure 2b) as well as from all quantitative measures of skin disease, including skin erosions in our model (Figure 2c–f; $P = 6.9 \times 10^{-14}$, 9.3×10^{-11} , 0.0061, and 0.0063, respectively). Because such chimeras could be generated in significantly larger cohorts than in intact *Plcg2*^{−/−} mice, the following experiments were performed on such *Plcg2*^{−/−} bone marrow chimeras.

Histological analysis of wild-type and *Plcg2*^{−/−} bone marrow chimeras on day 8 revealed anti-C7–induced dermal–epidermal separation and thickening of the ear tissue (likely owing to both thickening of the epidermal layer and the infiltration of leukocytes) in WT but not in *Plcg2*^{−/−} chimeras (Figure 2g). Further quantification revealed a dramatic increase in dermal–epidermal separation upon anti-C7 treatment in WT chimeras, which was absent in *Plcg2*^{−/−} mutants (Figure 2h; $P = 0.012$).

Therefore, *Plcg2*^{−/−} bone marrow chimeras are protected from anti-C7–induced skin disease, including histological separation at the dermo–epidermal interface.

Antibody levels and deposition

We next tested whether the *Plcg2*^{−/−} mutation reduces circulating levels or skin deposition of anti-C7 antibodies. As shown in Figure 3a, PLC γ 2 deficiency even moderately increased circulating anti-C7 levels ($P = 9.0 \times 10^{-6}$), possibly reflecting reduced consumption of circulating anti-C7 antibodies in the skin. Anti-C7 deposition along the dermo–epidermal junction was comparable between WT and *Plcg2*^{−/−} mutant mice (Figure 3b). Therefore, the protection of *Plcg2*^{−/−} mice in our model is likely not due to reduced circulating levels or tissue deposition of anti-C7 antibodies.

Leukocyte accumulation and migration

Immunofluorescence staining on the ear tissue sections from day 8 showed abundant Ly6G-positive cells (supposedly neutrophils) in anti-C7–treated wild-type but not in *Plcg2*^{−/−} samples (Figure 4a). Flow cytometric analysis of digested ear tissue samples revealed robust accumulation of leukocytes (Figure 4b), including neutrophils (Figure 4c), eosinophils (Figure 4d), and monocytes/macrophages (Figure 4e), in anti-C7–treated WT chimeras. Importantly, no such infiltration could be observed in *Plcg2*^{−/−} chimeras (Figure 4b–e; $P = 7.7 \times 10^{-6}$, 9.1×10^{-4} , 0.0097, and 0.0013, respectively). Therefore, PLC γ 2 deficiency protects mice from leukocyte infiltration in our model.

To test whether the observed infiltration defect in *Plcg2*^{−/−} mice is due to an impaired intrinsic migratory capacity of leukocytes, we generated mixed bone marrow chimeras with

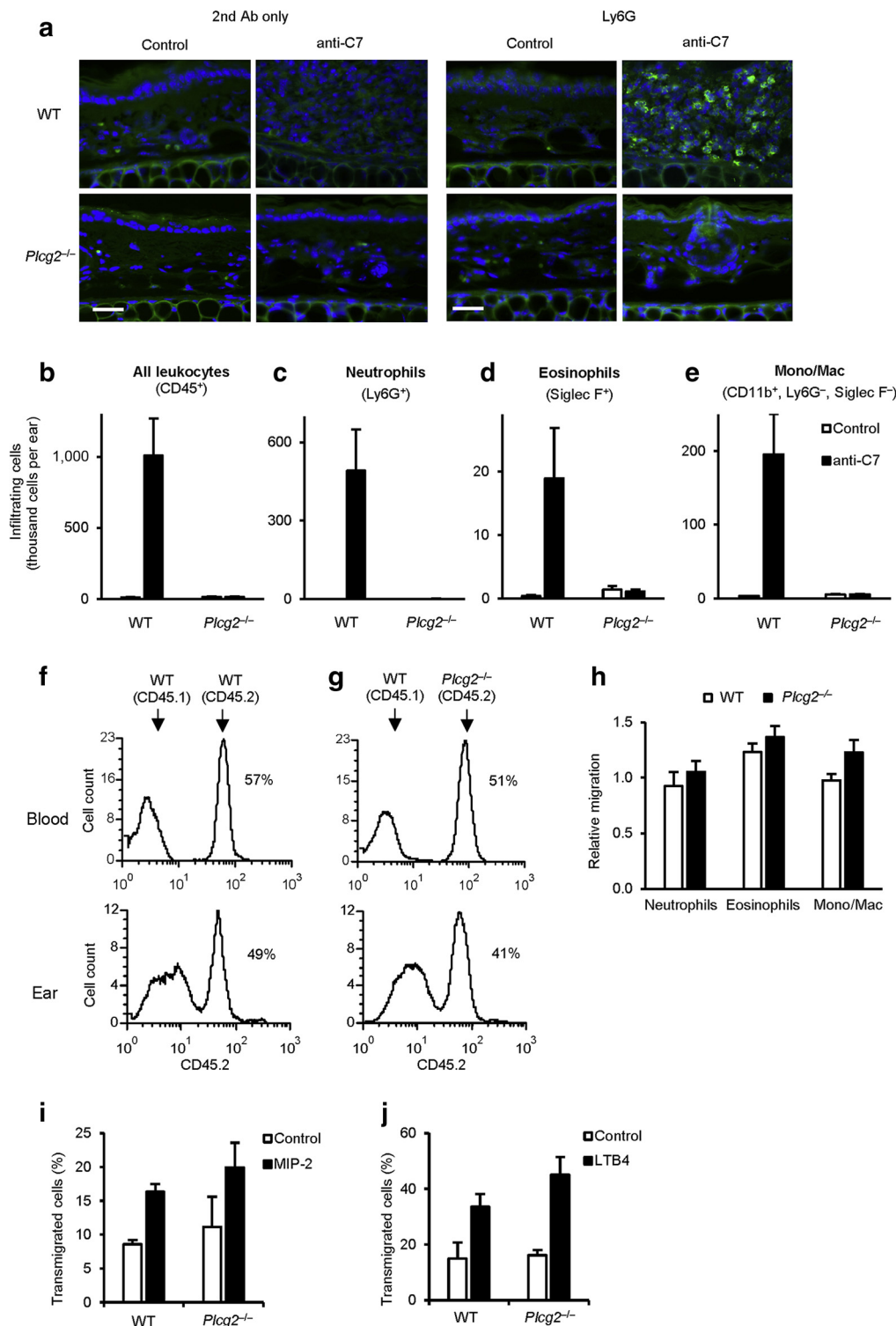


Figure 4. Defective leukocyte accumulation but normal cell-autonomous migration. (a–e) WT and *Plcg2*^{-/-} bone marrow chimeras were subjected to skin blistering, and ear tissues were obtained on day 8. (a) Dermal infiltration of neutrophils was examined in paraffin-embedded ear sections stained with anti-Ly6G, followed by Alexa Fluor 488-labeled secondary antibodies or secondary antibodies alone. (b–e) Quantitative analysis of different leukocyte populations was performed in digested ear samples using flow cytometry. (f–h) Mixed bone marrow chimeras containing CD45.1-expressing WT and CD45.2-expressing WT (WT:WT) or *Plcg2*^{-/-} (WT:*Plcg2*^{-/-}) bone marrow were subjected to skin blistering. Quantitative analysis of myeloid cells in the blood and in digested ear samples was performed by flow cytometry. The ratio of CD45.1- and CD45.2-expressing cells in the three major myeloid subtypes was determined in the blood and the ear. Panels f and g show the expression of CD45.2 in neutrophils in a WT:WT and a WT:*Plcg2*^{-/-} mixed bone marrow chimera, respectively. Panel h shows the relative migration of CD45.2-expressing cells compared with that of CD45.1-expressing WT cells within the same mouse. (i, j) In vitro migration of neutrophils isolated from the bone marrow of WT and *Plcg2*^{-/-} chimeras was tested in a transwell migration assay toward (i) 100 ng/ml MIP-2 or (j) 50 ng/ml LTB₄. (a) Histological images and (f, g) histograms are representative of 3–8 mice from two independent experiments. Bar = 30 μ m for a. (b–e, h–j) Bar graphs show the mean and SEM from 3–10 mice per group from two to four independent experiments. Ab, antibody; C7, type VII collagen; LTB₄, leukotriene B₄; Mono/Mac, monocyte/macrophage; WT, wild type.

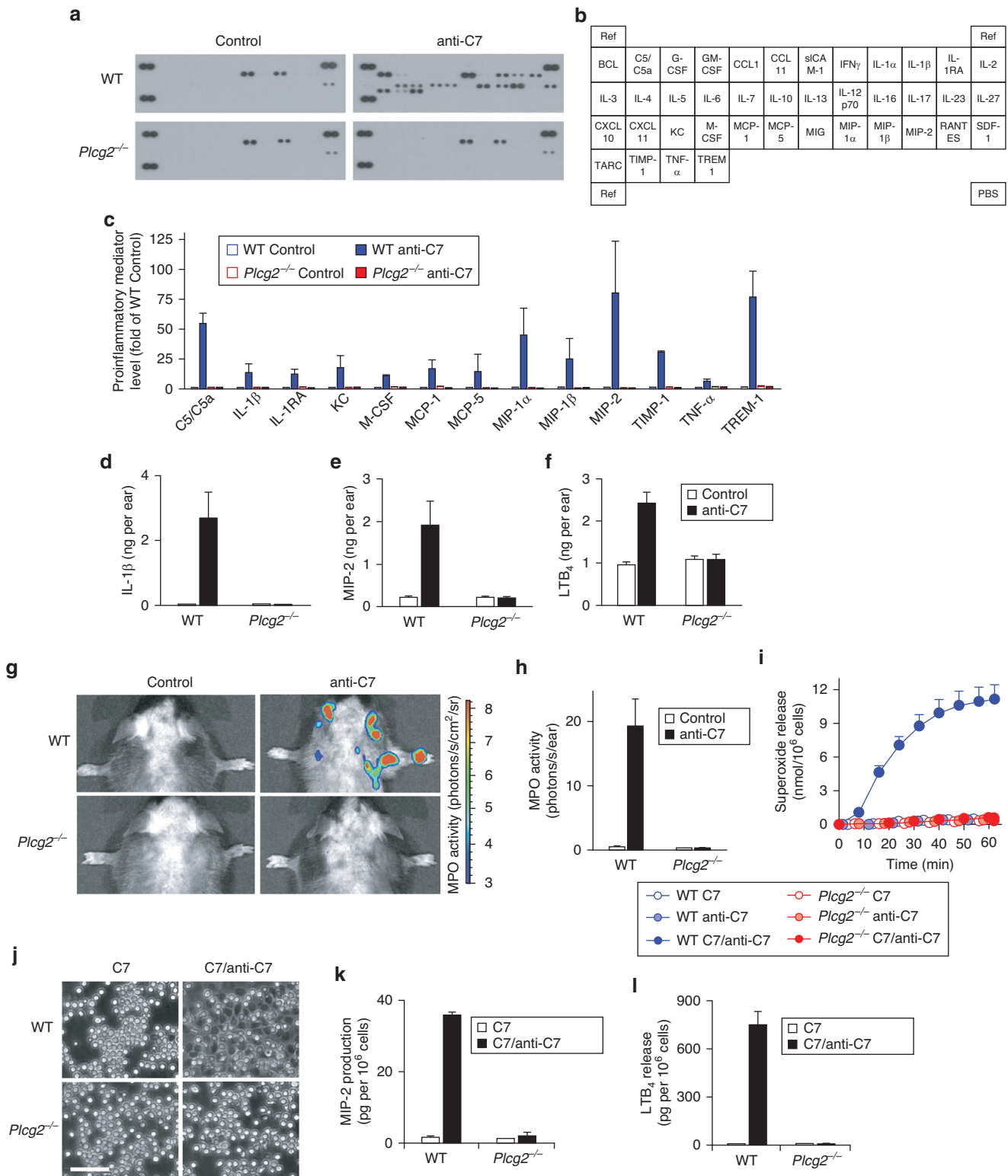


Figure 5. PLC γ 2 deficiency abrogated the generation of inflammatory microenvironment. (a–f) Cell-free supernatants were obtained from the ear tissue of WT and *Plcg2*^{-/-} bone marrow chimeras on day 8. The presence of several chemokines and cytokines was tested using (a–c) a semiquantitative cytokine array and (d) quantitative ELISA for IL-1 β , (e) MIP-2, and (f) LTB $_4$. (a) Representative images and (b) the layout of analytes in the cytokine array are presented together with integrated densitometry values normalized to those of (c) WT control samples from two independent experiments. (g) In vivo MPO activity was tested by chemiluminescence imaging after intraperitoneal injection of luminol on day 8. (h) Photon flux intensity was quantified in defined regions of the ears. Chemiluminescence images are representative of and quantitative data show the mean and SEM from 7–8 mice per group from two independent experiments. (i–l) In vitro activation of WT and *Plcg2*^{-/-} bone marrow-derived neutrophils. (i) Superoxide release, (j) cell spreading, (k) MIP-2 production, and (l) LTB $_4$ release of neutrophils were measured upon plating the cells onto C7/anti-C7 immune complex-coated or antigen (C7)-coated control surfaces. Images in panel j are representative of and (i, k, l) quantitative data show the mean and SEM from at least three independent experiments. Bar = 50 μ m. C7, type VII collagen; LTB $_4$, leukotriene B $_4$; MPO, myeloperoxidase; PLC γ 2, phospholipase C γ 2; WT, wild type.

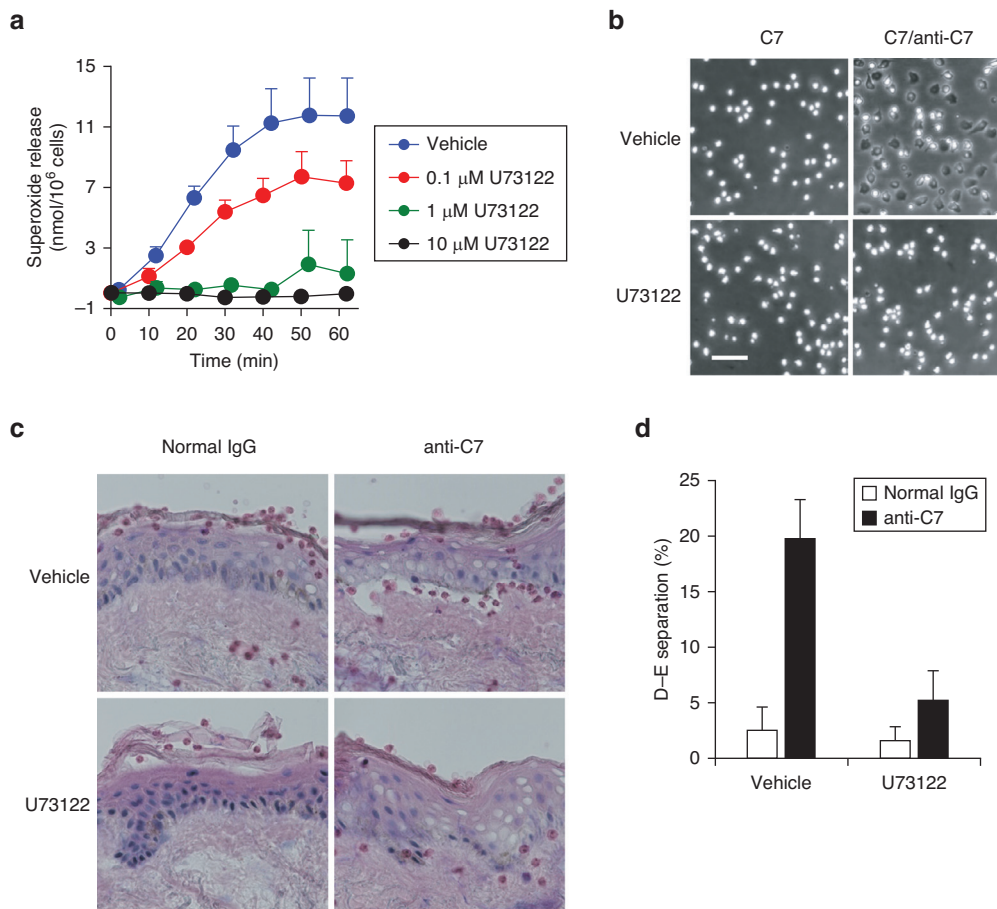


Figure 6. U73122 blocks immune complex-mediated activation of human neutrophils and ex vivo dermal-epidermal separation. (a, b) Human neutrophils were activated by immobilized C7/anti-C7 immune complexes in vitro. (a) Superoxide release was measured in neutrophils treated by the indicated concentrations of the PLC inhibitor U73122. (b) Cell spreading was tested in neutrophils treated with either 0.05% DMF (vehicle) or 1 μ M U73122. (c, d) Ex vivo D–E separation was evaluated in cryosections of normal IgG- or anti-C7-treated healthy human skin. (c) Sections were incubated with 0.05% DMF (vehicle)- or 10 μ M U73122-treated neutrophils for 1.5 hours, followed by staining with H&E. The percentage of D–E separation is shown in panel d. Microscopic images are representative of and quantitative data show the mean and SEM from at least three independent experiments. Bar = 50 μ m. C7, type VII collagen; D–E, dermal–epidermal; DMF, dimethylformamide; min, minute; PLC, phospholipase.

CD45.1-expressing WT and CD45.2-expressing WT or *Plcg2*^{−/−} hematopoietic cells (giving rise to WT:WT and WT:*Plcg2*^{−/−} mixed bone marrow chimeras, respectively) and subjected them to our skin disease model. All mice developed signs of skin disease, likely caused by the presence of wild-type cells (data not shown). On day 8, we determined the ratio of CD45.1- and CD45.2-expressing cells in the blood and in the digested ear samples, allowing us to compare the percentage of CD45.2-positive cells in the blood and in the tissue within the same mouse. Representative histograms of CD45.2 expression within a neutrophil gate are shown in Figure 4f–g. As expected, the percentage of CD45.2-expressing wild-type neutrophils in the WT:WT chimeras was comparable in the blood and the ear tissue (Figure 4f), although a modest reduction in the ear tissue (likely due to some technical issue) could be observed. Surprisingly and in sharp contrast to the complete defect of neutrophil infiltration in *Plcg2*^{−/−} chimeras (i.e., when all hematopoietic cells were of *Plcg2*^{−/−} genotype; see Figure 4a and c), the percentage of CD45.2-expressing *Plcg2*^{−/−} neutrophils in the WT:*Plcg2*^{−/−} chimeras was also comparable between the blood and the ear tissue (Figure 4g; although a moderate reduction in the ear tissue could again be seen). In other words, *Plcg2*^{−/−} neutrophils were recruited to the ear tissue from the circulation as efficiently as wild-type cells. This was also confirmed by calculation of the recruitment efficiency of WT and *Plcg2*^{−/−} neutrophils to the ear tissue

(Figure 4h), and similar findings were observed for eosinophils and monocytes/macrophages tested in the same mice, as well (Figure 4h). Therefore, PLC γ 2 is likely not required for the intrinsic ability of neutrophils, eosinophils, and monocytes/macrophages to accumulate at the skin lesion site in our model.

Additional in vitro transwell assays also revealed normal migration of *Plcg2*^{−/−} neutrophils toward MIP-2 or LTB₄ (Figure 4i and j; *P* = 0.87 and 0.33, respectively).

Taken together, the protection of *Plcg2*^{−/−} mutant mice from skin lesions are likely not due to a cell-autonomous migration defect of *Plcg2*^{−/−} neutrophils, eosinophils, or monocytes/macrophages.

PLC γ 2 is required for the generation of an inflammatory microenvironment

Since defective leukocyte infiltration may also be secondary to the inappropriate development of the inflammatory microenvironment, we tested the presence of proinflammatory mediators in the ear tissue of WT and *Plcg2*^{−/−} bone marrow chimeras. As shown in the cytokine array in Figure 5a (see array map in Figure 5b), anti-C7 treatment triggered a robust accumulation of a number of chemokines and cytokines in the ear of WT but not in those of *Plcg2*^{−/−} chimeras. This was confirmed by densitometric quantification of selected chemokines/cytokines, with the most robust (>5-fold) increase in WT samples (Figure 5c). In addition, anti-C7

treatment strongly increased the concentration of IL-1 β , MIP-2, and the lipid chemoattractant LTB $_4$ measured by ELISA in the ears of WT but not in *Plcg2*^{-/-} chimeras (Figure 5d–f; $P = 0.0044$, 0.0046 , and 1.1×10^{-4} , respectively).

Because reactive oxygen species are involved in autoantibody-induced skin blistering (Chiriac et al., 2007), we tested in vivo reactive oxygen species production using a chemiluminescence-based in vivo imaging of myeloperoxidase activity. As shown in Figure 5g and h, anti-C7 treatment triggered a robust chemiluminescence signal in WT but not in *Plcg2*^{-/-} chimeras ($P = 3.3 \times 10^{-7}$).

Since neutrophils may contribute to the development of the inflammatory microenvironment (Mócsai, 2013; Németh and Mócsai, 2016) and likely mediate anti-C7–induced in vivo pathology (Chiriac et al., 2007; Csepregi et al., 2018), we have also tested the in vitro responsiveness of neutrophils to C7/anti-C7 immune complexes. As shown in Figure 5i, plating isolated neutrophils on C7/anti-C7 immune complex–coated surfaces triggered robust superoxide production from WT but not from *Plcg2*^{-/-} cells (Figure 5i; $P = 3.5 \times 10^{-9}$). Similar results were seen when testing the spreading of the cells as a more general measure of cellular activation (Figure 5j). The *Plcg2*^{-/-} mutation also blocked the immune complex–induced release of MIP-2 and LTB $_4$ from neutrophils (Figure 5k and l; $P = 0.027$ and 1.3×10^{-7} , respectively).

Taken together, PLC γ 2 is required for the generation of various aspects of the inflammatory microenvironment upon anti-C7 treatment, which may explain the defective recruitment of leukocytes to the skin tissue. A role for PLC γ 2 within neutrophils is likely at least partially responsible for those findings.

Human in vitro studies

To extend the findings mentioned earlier to human pathology, we tested the effect of the phospholipase C inhibitor U73122 on human neutrophils and in a human ex vivo skin separation assay.

Plating human neutrophils on C7/anti-C7 immune complex–coated surfaces triggered a robust respiratory burst response, which was inhibited by U73122 in a concentration-dependent manner, reaching complete inhibition at 10 μ M concentration (Figure 6a). U73122 also blocked the spreading of human neutrophils on a C7/anti-C7 immune complex–coated surface (Figure 6b). Those results mirror the phenotype of *Plcg2*^{-/-} mouse neutrophils (Figure 5i and j).

Finally, we set up a human ex vivo dermal–epidermal separation assay by preincubating frozen healthy human skin sections with the anti-C7 antibodies mentioned earlier, followed by further incubation with healthy human neutrophils and normal human plasma as a source of complement components. As shown in Figure 6c, anti-C7 treatment led to the accumulation of neutrophils at the dermo–epidermal junction and skin separation between the dermal and epidermal layers in vehicle-treated samples. Importantly, U73122 added during the incubation of the skin sections with neutrophils strongly inhibited dermal–epidermal separation in this assay (Figure 6c). This was confirmed by quantification of skin separation along the dermal–epidermal

interface, showing robust anti-C7–induced dermal–epidermal separation in vehicle-treated but not in U73122-treated samples (Figure 6d; $P = 0.020$).

Taken together, the phospholipase C inhibitor U73122 strongly inhibits C7/anti-C7 immune complex–induced activation of human neutrophils and anti-C7–induced ex vivo dermal–epidermal separation of human skin sections.

DISCUSSION

We show that PLC γ 2 is critical for the development of a widely used mouse model of the inflammatory form of EBA. Our results improve our understanding of the pathomechanism and identify a possible therapeutic target of this rare autoimmune blistering skin disease.

The inflammatory form of EBA and its anti-C7–induced mouse model are characterized by signs of dermal–epidermal separation (including blistering and skin erosions) along with inflammatory features such as leukocyte infiltration and accumulation of proinflammatory mediators. The fact that the *Plcg2*^{-/-} mutation abrogated both dermal–epidermal separation (Figures 1a and d and 2b, e, g, and h) and inflammation (Figures 4a–e and 5a–h) suggests that PLC γ 2 plays an important role in a common step of disease development. Since PLC γ 2 is an intracellular signaling molecule and because complete protection from disease development could be observed in chimeric mice with a *Plcg2*^{-/-} hematopoietic system (Figure 2), this common step is likely mediated by cells of hematopoietic origin (rather than cell-free processes such as the classical pathway of complement activation). Although our experiments do not allow us to identify the cell type(s) requiring PLC γ 2 expression during disease development, preliminary lineage-specific deletion experiments point to a role for PLC γ 2 within neutrophils (unpublished observations), which would also be in line with the critical role of neutrophils in this model (Chiriac et al., 2007; Csepregi et al., 2018). Nevertheless, we cannot exclude the role of PLC γ 2 within eosinophils, monocytes/macrophages, or other nonlymphoid cells, whereas our experimental design (passive transfer of anti-C7 antibodies) and the absence of PLC γ 2 in circulating T cells (unpublished observation) argue against a critical role for PLC γ 2 in B or T cells.

We have performed a large number of experiments to reveal how PLC γ 2 mediates anti-C7–induced disease development. It is unlikely that PLC γ 2 plays a major role in myeloid cell development because the *Plcg2*^{-/-} mutation did not reduce the circulating number or the critical cell surface marker expression (including various β_2 -integrin chains and Fc γ receptors) of major myeloid-lineage cells (Jakus et al. [2009] and unpublished observations). PLC γ 2 was not required for the maintenance of circulating anti-C7 antibody levels or antibody deposition at the dermal–epidermal interface (Figure 3). Although PLC γ 2 deficiency abrogated the accumulation of all tested myeloid cell types in the skin of anti-C7–treated mice (Figure 4a–e), this was likely not due to a cell-intrinsic migration defect because *Plcg2*^{-/-} neutrophils, eosinophils, and monocytes/macrophages were recruited normally to the skin when wild type cells were also present (Figure 4f–h), and *Plcg2*^{-/-} neutrophils showed normal migration in in vitro transwell assays (Figure 4i and j).

In contrast, PLC γ 2 deficiency abrogated various aspects of the generation of an inflammatory microenvironment such as tissue accumulation of proinflammatory mediators (Figure 5a–f) or in vivo reactive oxygen species production (Figure 5g and h). *Plcg2*^{−/−} neutrophils also failed to respond to C7/anti-C7 immune complexes (Figure 5i–l). The most plausible explanation of all those findings is that anti-C7 antibody deposition at the dermal–epidermal junction activates neutrophils (and possibly other myeloid-lineage cells), which release proinflammatory mediators, thus recruiting further leukocytes and amplifying the inflammatory response. This would be in line with the feedback amplification of neutrophil function in other disease processes (Németh and Mócsai, 2016) and the biphasic nature of neutrophil recruitment on anti-C7 administration (Hundt et al., 2020). PLC γ 2 deficiency likely blocks the release of proinflammatory mediators and therefore abrogates the entire feedback amplification loop, including recruitment of additional leukocytes. It should be noted that LTB₄ has been proposed to be a critical driver of neutrophil recruitment in a model similar to ours (Sezin et al., 2017). The role of PLC γ 2 in the tissue accumulation of LTB₄ (Figure 5f) and the in vitro release of LTB₄ from immune complex–stimulated neutrophils (Figure 5l) in our hands suggest that blocking an LTB₄-mediated feedback amplification loop is at least partially responsible for the defective skin disease in *Plcg2*^{−/−} mutants. Besides the role of PLC γ 2 in this inflammatory amplification loop, it likely also plays a direct role in the degradation of the dermal–epidermal junction, as suggested by the inhibitory effect of U73122 on ex vivo dermal–epidermal separation (Figure 6c and d).

Besides detailed genetic experiments in mice, we have also performed experiments on human cells and tissues using a pharmacological approach. The inhibitory effect of U73122 on the responses of human neutrophils to C7/anti-C7 immune complexes (Figure 6a and b) and on the ex vivo dermal–epidermal separation of human skin sections in the presence of human neutrophils and anti-C7 antibodies (Figure 6c and d) are in line with a critical role for PLC γ 2 in the inflammatory form of EBA.

It is interesting to note that rare *PLCG2* gain-of-function mutations also caused manifestations resembling those of human EBA and its mouse model. Both originally described patients with APLAID had EBA-like eruptions, skin lesions infiltrated by neutrophils and eosinophils, as well as corneal blistering that progressed to corneal erosions and ulceration (Zhou et al., 2012). Bullous skin eruptions and corneal lesions were also prominent findings in other patients with APLAID–like diseases caused by other gain-of-function missense mutations of *PLCG2* (Martín-Nalda et al., 2020; Morán-Villaseñor et al., 2019; Neves et al., 2018; Novice et al., 2020). In addition, both the *Ali5* and *Ali14* gain-of-function mutations of mouse *Plcg2* led to severe skin inflammation with granulocytic and eosinophilic infiltrates (Abe et al., 2011; Yu et al., 2005). Those reports further support a role for PLC γ 2 in the pathogenesis of the inflammatory form of EBA.

A few limitations of our study should also be mentioned. Autoantibodies in the noninflammatory form of EB acquisita likely directly block the molecular interactions of C7,

weakening the dermo–epidermal junction without a significant inflammatory component. Given the very different molecular pathomechanism, the results of our experiments should not be directly extrapolated to that other disease processes. In addition, we cannot exclude the role of PLC γ 2 expressed within cell types of nonhematopoietic origin (e.g., fibroblasts or keratinocytes) in the pathogenesis of the inflammatory form of EBA. However, the dominantly hematopoietic expression of PLC γ 2 (Faccio and Cremasco, 2010; Jackson et al., 2021) and the mostly normal development of a mechanistically similar autoantibody-induced arthritis model in *Plcg2*^{−/−} recipients transplanted with wild type bone marrow cells (Futosi et al., 2021) make that possibility rather unlikely.

Taken together, our studies identify an important component of the development of the inflammatory form of EBA and point to a potential molecular target of future pharmacological intervention.

MATERIALS AND METHODS

Mice

Mice carrying the *Plcg2*^{tm1Jni} mutation (referred to as *Plcg2*^{−/−}) inactivating the PLC γ 2-encoding gene (Wang et al., 2000) were obtained from James Ihle (St. Jude Children's Research Hospital, Memphis, TN) and were maintained by heterozygous breeding to obtain *Plcg2*^{−/−} mice. Genotyping was performed by allele-specific PCR from tail DNA. *Plcg2*^{+/+} littermates or wild type C57BL/6 mice from our colony were used as WT control mice. For bone marrow transplantation, recipients carrying the CD45.1 allele on the C57BL/6 genetic background (B6.SJL-*Ptprca*^a; purchased from the Jackson Laboratory, Bar Harbor, ME) were lethally irradiated by 11 Gy from a ¹³⁷Cs source using a Gamma-Service Medical D1 irradiator, followed by intravenous injection of unfractionated donor bone marrow cells. Repopulation of the hematopoietic system was tested by flow cytometry 4 weeks after transplantation (Jakus et al., 2010). The mice were kept in individually sterile ventilated cages (Tecniplast, Buguggiate, Italy) under specific pathogen-free conditions and were transferred to a conventional facility for experiments. All animal experiments were approved by the Animal Experimentation Review Board of Semmelweis University (Budapest, Hungary) or of the University of Pécs (Pécs, Hungary).

Autoantibody-induced skin blistering

The murine model of the inflammatory form of human EBA was induced by systemic injection of antibodies against C7 (Sitaru et al., 2005). GST-tagged (GST–C7) and His-tagged (His–C7) fusion proteins containing a fragment of the immunodominant NC1 domain of mouse C7 were expressed in *E. coli* BL21 using plasmids obtained from Cassian Sitaru (University of Freiburg, Freiburg im Breisgau, Germany) and purified using glutathione- or Ni²⁺-based affinity chromatography, respectively (Csorba et al., 2010; Sitaru et al., 2005). C7 fragment was obtained by cleavage with PreScission protease (GE Healthcare, Chicago, IL). Polyclonal anti-C7 antibodies were produced by immunizing rabbits at the Hungarian National Agricultural Innovation Center with GST–C7, followed by total IgG preparation using protein G–based affinity chromatography (rProtein G agarose, Thermo Fisher Scientific, Waltham, MA). Reactivity of rabbit sera was tested by ELISA using His–C7 as the capturing antigen to exclude anti-GST signals (Csorba et al., 2010).

For the induction of skin blistering, 12 mg anti-C7 or PBS (as control) was injected subcutaneously under isoflurane anesthesia on days 0, 2, 4, 6, and 8 (60 mg total IgG/mouse) (Németh et al., 2017; Sitaru et al., 2005). Disease onset and progression was followed by evaluation of the size of total affected skin area, the size of erosions alone, and a severity score reflecting both lesion size and severity (alopecia, scaling, induration, erosion, and crust formation in increasing order) added together into a single score. Ear thickness was measured by a spring-loaded caliper (Kroeplin, Schlüchtern, Germany). Serum anti-C7 levels were determined on days 0, 8, and 14 by ELISA using His-C7 as the capturing antigen.

Histological analyses in mice

Control and anti-C7–treated chimeras were sacrificed on day 8. Ear tissue was removed, fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections were stained with H&E or immunostained using antibodies against Ly6G (clone 1A8, BD Biosciences, San Jose, CA) followed by fluorescently labeled secondary antibodies.

For anti-C7 deposition assays, chimeras were injected once with 4 mg anti-C7 or normal rabbit IgG. After 24 hours, the mice were sacrificed, and ear cryosections were stained with Alexa Fluor 488–labeled anti-rabbit IgG or irrelevant anti-rat IgG as control.

Analysis of leukocyte infiltration and inflammatory mediators in the ear

Control and anti-C7–treated chimeras were sacrificed on day 8. Ear tissue was removed and digested with Liberase TM (Roche, Basel, Switzerland) to obtain single-cell suspensions for flow cytometry. Cell-free supernatants of the digested samples were used to evaluate inflammation-related chemokines and cytokines using a mouse cytokine antibody array kit (panel A) or commercial IL-1β, MIP-2, or LTB₄ ELISA kits (all from R&D Systems, Minneapolis, MN) as described (Kovács et al., 2014).

Flow cytometry

Blood samples or single-cell suspensions of digested ear tissues were washed; stained with antibodies against CD11b (M1/70), CD45.2 (104), Ly6G (1A8), and Siglec F (E50-2440); and then resuspended in FACS lysing solution (all reagents from BD Biosciences). Specified volumes were used throughout, allowing a precise determination of absolute cell counts. Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences) and analyzed by FCS Express 6 (De Novo Software, Pasadena, CA). Neutrophils were identified as CD11b⁺Ly6G⁺Siglec-F[−], eosinophils were identified as CD11b⁺Ly6G[−]Siglec-F⁺, and monocytes/macrophages were identified as CD11b⁺Ly6G[−]Siglec-F[−] cells within their typical forward scatter/side scatter gates.

Competitive in vivo migration

In vivo migration of neutrophils, eosinophils, and monocytes/macrophages was investigated in mixed bone marrow chimeras (Jakus et al., 2009; Kovács et al., 2014; Mócsai et al., 2002). Briefly, CD45.1-expressing WT recipients were transplanted with CD45.1-expressing WT and CD45.2-expressing WT or *Plcγ2*^{−/−} bone marrow at varying ratios. After bone marrow repopulation, skin blistering was induced by anti-COL7 antibodies. On day 8, single-cell suspensions were obtained from blood and from ear tissues and the percentage of CD45.2-expressing cells was assessed by flow cytometry. Relative migration was calculated as described (Jakus et al., 2009).

In vivo chemiluminescence assay

In vivo myeloperoxidase activity was detected using a chemiluminescence assay (Gross et al., 2009; Kovács et al., 2014). Control and anti-C7–treated chimeras were injected intraperitoneally with 150 mg/kg luminol sodium salt (Gold Biotechnology, St. Louis, MO) on day 8. Luminescence images were captured by IVIS Lumina II imaging system (PerkinElmer, Waltham, MA; 120 s acquisition, Binning = 8, F/Stop = 1) under 120/6 mg/kg intraperitoneal ketamine–xylazine anesthesia. For quantitative analysis, total radiance values (total photon flux/s) were used from standardized regions of interest of the ears.

Isolation and in vitro activation of mouse neutrophils

Mouse neutrophils were isolated from the bone marrow by Percoll (GE Healthcare) gradient centrifugation (Mócsai et al., 2003). Neutrophil assays were performed at 37 °C in Hank's balanced salt solution (Lonza, Basel, Switzerland) supplemented with 20 mM HEPES, pH 7.4, or in DMEM (Sigma-Aldrich, St. Louis, MO; for measurement of LTB₄ release).

In vitro migration was assessed by a transwell assay system (Jakus et al., 2009; Mócsai et al., 2002). Transwell inserts with 5 μm pore size (Corning, Acton, MA) were precoated with fibrinogen, filled with neutrophil suspension, and placed into media containing 100 ng/ml MIP-2 (PeproTech GmbH, Hamburg, Germany) or 50 ng/ml LTB₄ (Santa Cruz Biotechnology, Dallas, TX). After 1 hour of incubation, the ratio of transmigrated neutrophils into the lower compartment was determined by an acid phosphatase assay (Mócsai et al., 2002).

Immobilized immune complex–coated surfaces were obtained by binding C7 at 20 μg/ml to Nunc MaxiSorp F96 plates (Thermo Fisher Scientific), blocking, and then treating with anti-C7 at 300 μg/ml. C7 or anti-C7 treatment alone served as controls. Superoxide release was followed by a cytochrome c reduction test (Németh et al., 2010). Neutrophil spreading was examined using phase-contrast microscopy (DMI600 B fluorescence microscope, Leica, Wetzlar, Germany) after 30 minutes of stimulation. For analysis of mediator release, neutrophils were stimulated for 1 hour (LTB₄) or 4 hours (MIP-2). Supernatants were collected and measured by commercial ELISA kits described earlier.

Isolation and in vitro activation of human neutrophils

Human neutrophils were isolated from the venous blood of healthy volunteers by Ficoll (GE Healthcare) gradient centrifugation (Futosi et al., 2012). All experiments on human samples were approved by the Scientific and Research Ethics Committee of the Medical Research Council of Hungary.

The phospholipase C inhibitor U73122 (Selleck Chemicals, Houston, TX) was dissolved in dimethylformamide (DMF). Isolated neutrophils were pretreated at 37 °C for 20 minutes with vehicle (DMF) or U73122 at the indicated concentrations. The final vehicle- and inhibitor-treated samples contained 0.05% dimethylformamide. In vitro neutrophil assays were performed at 37 °C in Hank's balanced salt solution. Superoxide release and cell spreading on immobilized C7/anti-C7 immune complex surfaces were measured as described earlier.

Ex vivo human skin separation assay

Neutrophil-mediated dermal–epidermal separation in cryosections of healthy human skin was performed essentially as described (Gammon et al., 1982). Experiments involving human skin samples were approved by the Scientific and Research Ethics Committee of the Medical Research Council of Hungary. Healthy human skin samples were obtained from surgical interventions, with written

informed consent of donors prior to surgery. Cryosections were made, incubated with 6 mg/ml anti-C7 or normal rabbit IgG for 1.5 hours, and washed. Neutrophils isolated from healthy donors were diluted in RPMI (Biosera, Marikina, Philippines) to 10^7 per ml in the presence of human plasma, treated with vehicle (0.05% DMF) or 10 μ M U73122, preincubated at 37 °C, and then plated over the antibody-treated cryosections. After 1.5 hours of incubation at 37 °C, sections were washed, fixed, and stained with H&E.

Presentation of the data and statistical analysis

Experiments were performed at the indicated number of times. Quantitative graphs and kinetic curves show the mean and SEM from all individual mice or samples from the indicated number of experiments. Statistical analyses were carried out by the STATISTICA software (Tibco Software, Palo Alto, CA) using two-way (factorial) ANOVA, with treatment and genotype being the two independent variables. One-way ANOVA was used to evaluate in vivo migration experiments. The area under the curve was used for statistical analysis in kinetic measurements. *P*-values below 0.05 were considered statistically significant.

Ethics statement

All animal experiments were approved by the Animal Experimentation Review Board of Semmelweis University or of the University of Pécs. Human experiments were approved by the Scientific and Research Ethics Committee of the Medical Research Council of Hungary. Human subjects provided written informed consent before blood sampling.

Data availability statement

No datasets were generated or analyzed during this study.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: KPS, AM; Investigation: KPS, SV, ÁIH; Resources: MS, ZH; Supervision: AM; Writing - Original Draft Preparation: KPS, AM; Writing - Review and Editing: KPS, AM

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