

Disruption of the Complement Anaphylatoxin Receptor C5L2 Exacerbates Inflammation in Allergic Contact Dermatitis

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The complement anaphylatoxin C5a is a critical mediator of allergic contact dermatitis, bridging essential aspects of innate and adaptive immunity. This anaphylatoxin functions by interacting with two 7-transmembrane segment receptors, the C5aR and C5L2. The C5aR is a classical G protein coupled receptor, whereas C5L2 is deficient in coupling to G proteins because of variations in the sequence. Our previous work in human neutrophils revealed a unique role for C5L2 in negatively modulating anaphylatoxin receptor mediated cellular activation through interactions with β -arrestin. When C5L2 is deficient, C5aR-mediated β -arrestin signaling is greatly enhanced. The work described in this study was undertaken first to determine the effect of C5L2 deficiency in a murine model of contact sensitivity, and second to determine whether the resultant exacerbation of inflammatory parameters reflects a negative modulatory function of C5L2 on the C5aR. First, we find dramatic increases in inflammation in C5L2^{-/-} animals compared with wild type mice. Second, these increases are completely reversed following administration of mAb against the C5aR. Thus, in allergic contact sensitivity, as in isolated human neutrophils, C5L2 functions to suppress C5a-C5aR-mediated responses, further underscoring its role as a negative regulator of anaphylatoxin activity. *The Journal of Immunology*, 2013, 191: 000–000.

Allergic contact dermatitis is a common delayed type hypersensitivity reaction of the skin that results in more than 8 million outpatient visits per year in the United States (1, 2). The pathophysiology of this disease is examined primarily using animal models of contact sensitivity (CS) and involves induction of T-cell mediated skin inflammation resulting from exposure to a hapten in a sensitized animal (3). Current understanding describes at least two distinct phases of disease development: sensitization and elicitation. During the sensitization phase, the hapten painted on the animal's body penetrates into the epidermis and is taken up by resident dendritic cells. The haptenated dendritic cells induce rapid (within 1 d) production of IgM by B-1 cells (4). Subsequent topical application of the same hapten to a remote site of the body initiates the elicitation phase, triggering an inflammatory cascade of cytokine and chemokine production, neutrophil infiltration, and mast cell activation, leading to T cell recruitment. In mouse models, this process generally peaks at ~24 h (5).

Initial paradigms supported the concept of CS as mediated solely by T cell activation. Tsuji et al. (6–8), however, demonstrated a central role for complement activation by formation of IgM-antigen complexes. In mice deficient in C5 or the C5a receptor (C5aR), the inflammatory response fails to occur, indicating an

important linkage of C5a in early activation of innate immunity that is required for subsequent elicitation of the acquired T cell response.

Studies have also invoked a role for C3 or C3a in CS, although the mechanism is less clear. Purwar et al. (9) observed heightened contact sensitivity to multiple haptens in C3-deficient mice, as evidenced by increased swelling of challenged tissues, elevated tissue expression of IFN- γ , the chemokines CXCL-10, CCL-2, and CCL-17, as well as increased IFN- γ from splenocytes and draining lymph nodes. A subsequent study was undertaken to distinguish the individual roles of C3a and other C3 products using C3aR-deficient mice (10). This work recapitulated the increased cytokine response in the sensitization phase, but no differences were observed in the elicitation phase in terms of challenged tissue swelling and cytokine secretion. Thus, the apparent protective role of C3 was thought to result from a downstream cascade involving C3b or contributions of C5aR or C5L2.

The C5a anaphylatoxin is considered as one of the most potent proinflammatory components of the complement system, capable of activating neutrophils, monocytes, macrophages, and mast cells, among others, at nanomolar concentrations (11). It functions through two 7-transmembrane segment receptors, the C5aR and C5L2. The C5aR is a classical G protein-coupled receptor (12), whereas C5L2 fails to couple to G proteins because of an amino acid replacement in the second intracellular loop sequence (13, 14). C5L2 exhibits similar binding affinity for C5a and C5a_{desArg} as the C5aR and was initially described as a decoy receptor (15). In human polymorphonuclear leukocytes (PMNs), C5L2 has been shown to act as a negative modulator of C5a-mediated responses (16). In the presence of a blocking Ab against C5L2, certain C5a-mediated functions are markedly potentiated, including ERK1/2 activation and chemotaxis, resulting from increased C5aR-mediated β -arrestin signaling. Similar increases in inflammation and neutrophil activation have been observed in vivo in C5L2^{-/-} mice (14, 17, 18). Inflammatory indices were markedly increased in C5L2^{-/-} mice in a model of autoimmune glomerular nephritis induced by anti-neutrophil cytoplasmic Abs (19), as well as a model of diabetes when animals were placed on a high-fat, high-

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Abbreviations used in this article: CS, contact sensitivity; MPO, myeloperoxidase; OX, oxalozone; PMN, polymorphonuclear leukocyte.

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sucrose diet (20). In a mouse model of sepsis, blocking C5L2 resulted in increased IL-6 and TNF- α production (21). Similarly in human colonic epithelial cells C5a stimulation induced release of CXCL-8/IL-8, a response that was enhanced by blocking C5L2 (22).

This study was undertaken to elucidate the role of C5L2 as a modulator of C5a-C5aR-mediated inflammation. We tested *C5L2*^{-/-} mice in a model of oxalozone (OX) induced CS in comparison with wild type animals of the same genetic background. We demonstrate increased inflammation in the *C5L2*^{-/-} animals in support of the negative modulatory role of C5L2 toward the C5aR. Further supporting this role of C5L2, we show that CS inflammation in *C5L2*^{-/-} mice is reduced to the level of wild type animals by a blocking Ab against the C5aR.

Materials and Methods

Animals

The generation and initial characterization of *C5L2*-deficient mice have been described previously (17). Animals were backcrossed through at least 10 generations to the BALB/c background and maintained in the Children's Hospital of Boston Animal Facility. All experiments used female wild type and *C5L2*^{-/-} mice at 8–12 wk of age. All studies were conducted in accordance with the Institutional Animal Care and Use Committee of Children's Hospital.

Abs and reagents

Oxazolone, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (OX) was purchased from Sigma-Aldrich. Anti-C5aR (rat anti-mouse CD88 clone 20/70, low endotoxin) and rat isotype control IgG2b were obtained from AbD Serotec.

Contact sensitivity induction

On day 0, wild type and *C5L2*^{-/-} mice were sensitized by topical application of 50 μ l of 3% OX dissolved in a mixture of acetone and olive oil (4:1 v/v) to the shaved abdomen. On day 5, animals were challenged with topical application of 20 μ l of 0.5% OX to both sides of the experimental ear, whereas the contralateral ear received vehicle alone and served as the control. In experiments involving Ab blockade, anti-C5aR (35 μ g in 100 μ l PBS), isotype control IgG (35 μ g in 100 μ l PBS), or PBS (100 μ l) alone was injected retro-orbitally 1 h prior to challenge. Twenty-four hours later, mice were sacrificed and analyzed for CS reactions. Ear thickness was measured using a thickness gauge (Kafer J15, Long Island Indicator, Hauppauge, NY). Ears were harvested along the base of the lobes, and the wet weight difference between the control (vehicle only) and experimental (OX-challenged) ears was determined. In some experiments, the ears were subsequently dried under vacuum at 100°C overnight for determination of wet/dry weight ratios.

Myeloperoxidase assay

Ear samples (~30 mg) were homogenized in HBSS (pH 7.5). Homogenates were suspended in 1% hexadecyltrimethylammonium bromide (Sigma-Aldrich). Samples were subjected to multiple freeze-thaw cycles using liquid nitrogen, and insoluble material was removed by centrifugation. Supernatants were assessed for myeloperoxidase using 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) by determining the absorbance at 450 nm as described previously (23). Absorbance values were converted to neutrophil equivalents based on a standard curve generated with purified cells, and results are expressed as myeloperoxidase (MPO) equivalent to the neutrophil numbers.

Cytokine and chemokine analyses

For determination of the tissue content of cytokines, control and OX-challenged ears were homogenized with HBSS (pH 7.5), and the supernatants were used to assess levels of IFN- γ , IL-17A, IL-4, IL-5, IL-6, IL-10, and IL-12p40 using ELISA kits (OptEIA, BD Biosciences).

RNA extraction and quantitative real-time PCR

For RNA analyses, excised ears were stored in RNA-Later (Qiagen) at 4°C for up to 1 wk. RNA was extracted using a commercially available kit (RNeasy Mini Kit, Qiagen) and reverse transcribed (Superscript Vilo, Life Technologies) according to the manufacturer's protocols. Quantitative

PCR was performed using SYBR Green Supermix (BioRad). All primers were purchased from Integrated DNA Technologies and designed based on recommendations from Life Technologies Primer Design Tool. Amplified targets span at least one intron where possible, and the amplification efficiency was determined at 92%–100%. The primers used were as follows: IFN- γ (F5'-TGAACGCTACACTGCATCTTGG-3'; R5'-CGACTCCTTTTCCGCTT-CCTGAG-3'), CXCL-1 (F5'-GCTGGGATTACCTCAAGAA-3'; R5'-AGGTGCCATCAGAGCAGTCT-3'), CXCL-2 (F5'-TCCAGAGCTTGAGTGTGA-3'; R5'-AGGCACATCAGGTACGATCC-3'), CCL-2 (F5'-AGGTCCCTGTCTATGCTTCTG-3'; R5'-TCTGGACCCATTCCTTCTTG-3'), IL-17A (F5'-TCCAGAAGGCCCTCAGACTA-3'; R5'-AGCATCTTCTCGACCCCTGAA-3'), IL-6 (F5'-TCTGGGAAATCGTGGAAATGA-3'; R5'-TGGATGGTCTTGGTCTTTCAGC-3'), IL-10 (F5'-CACTGCTATGCTGCCTGCTCTTAC-3'; R5'-AAAATCACTCTTCACTGCTCCAC-3'), β -actin (F5'-TGTTACCACTGGGACGACA-3'; R5'-ACCAGAGGCATACAGGGACA-3'), and β -2-microglobulin (B2M; F5'-CTGACCGCCTGTATGCTAT-3'; R5'-CAGTCTCAGTGGGGTGAAT-3'). Results for cytokine and chemokine genes were determined by the $\Delta\Delta$ CT method, and normalized to both β -actin and B2M as reference housekeeping genes.

Lymph node cell cultures

For ex vivo stimulation experiments, bilateral inguinal and axillary lymph nodes were harvested from OX sensitized and challenged mice one day after challenge. Single-cell suspensions were prepared in complete Iscove's medium supplemented with 10% FCS, 100U/ml penicillin and 10 μ g/ml streptomycin. Cells were cultured at 1 $\times$ 10⁶/ml in the presence of 0 or 1 μ g/ml OX, and assessed for chemokine and cytokine production after 1 or 24h as described above.

Statistical analysis

Data are presented as the mean $\pm$ SEM. The data were analyzed using Prism for Windows software (GraphPad) and statistical analyses were performed using Student *t* test and two-way ANOVA. A *p* value $\leq$ 0.05 was considered statistically significant.

Results

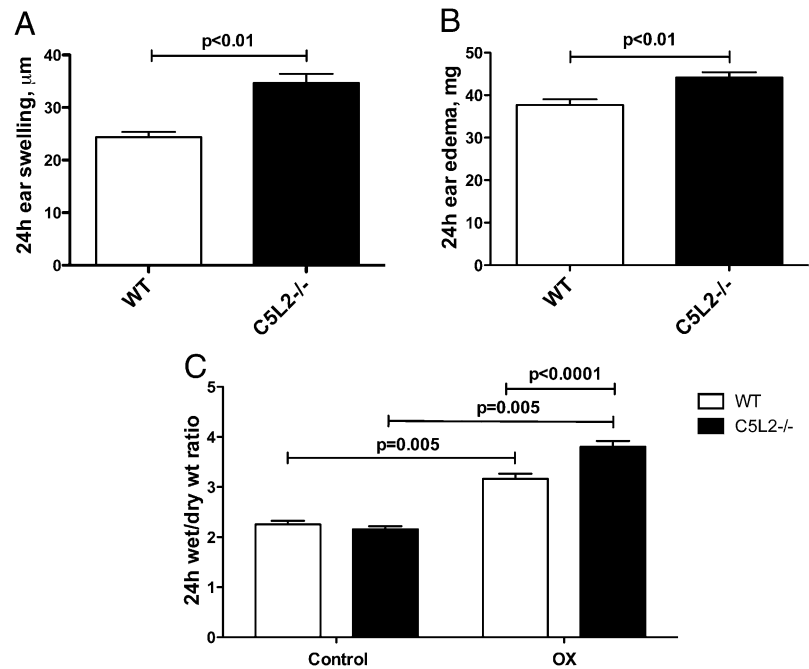
C5L2^{-/-} mice exhibit increased OX-induced contact sensitivity

The inflammation associated with allergic contact sensitivity in the mouse has been shown to be critically dependent on early generation of C5a (4, 7, 24). In animals deficient in C5 or the C5aR, inflammation was not observed following hapten challenge. Our work in isolated human neutrophils has revealed a role for the second C5a receptor, C5L2, in attenuating C5a-mediated responses (16). We therefore tested *C5L2*^{-/-} mice in the model of OX-induced contact sensitivity in comparison with wild type animals to test our hypothesis that *C5L2* deficiency would exacerbate this response. Five days after initiating OX sensitization, mice were challenged with the application of hapten to one ear, and the other received vehicle alone. Twenty-four hours later, animals were sacrificed and evaluated for inflammatory responses. The challenged ears from both *C5L2*^{-/-} and wild type mice exhibited significantly increased swelling after the application of the hapten compared with the control ears, assessed as ear thickness, wet weight, and the ratio of wet to dry weight (Fig. 1). Importantly, the response in *C5L2*^{-/-} mice was significantly greater than in wild type animals for all three parameters. The increase in thickness was ~40% greater in *C5L2*^{-/-} ears, and both wet weight and the wet/dry weight ratio were increased ~20%, compared with ears from wild type mice (*p* < 0.01 for each measurement; *n* = 9–10 mice/group).

C5L2^{-/-} mice exhibit significantly increased inflammatory cell influx relative to wild type animals

The CD8 T cell recruitment in contact sensitivity reactions has been shown to require neutrophil activation and infiltration resulting from generation of C5a (4, 7). We quantitated the tissue neutrophil content 24 h after OX challenge by measuring MPO levels in tissue extracts from *C5L2*^{-/-} and wild type mouse ears (Fig. 2A). Consistent with the increased tissue swelling, hapten challenged

FIGURE 1. *C5L2*^{-/-} deficiency results in exacerbation of tissue swelling and edema associated with OX-induced contact sensitivity. **(A)** Ear swelling determined as the thickness of OX-challenged ears minus the thickness of vehicle-treated control ears in *C5L2*^{-/-} and wild type mice. **(B)** Ear edema measured as the weight of OX-challenged ears minus vehicle-treated control ears in *C5L2*^{-/-} and wild type mice. **(C)** Wet/dry weight ratio for OX-challenged and vehicle-treated control ears from *C5L2*^{-/-} and wild type mice. Data are expressed as the mean $\pm$ SEM for each value, and the significance of differences is indicated; $n = 9$ –10 mice/group.



ears from both *C5L2*^{-/-} and wild type mice revealed significantly elevated levels of MPO at 24 h relative to vehicle-treated control ears. Sensitized and challenged *C5L2*^{-/-} animals exhibited $\sim 35\%$ greater MPO than wild type mice did (equivalent to 5.5×10^6 PMN/ml compared with 4×10^6 PMN/ml for wild type mice; $p < 0.01$; $n = 10$ –11 mice/group).

The generation of chemokines including CXCL-1, CXCL-2, and CCL-2 has also been shown to contribute to the recruitment

and activation of neutrophils and CD8 T cells and to amplify cell-mediated inflammation further in CS (7, 17, 25). We assessed the gene expression for these chemokines by quantitative PCR in ears of *C5L2*^{-/-} and wild type mice 24 h after elicitation (Fig. 2B–D). Consistent with previous studies, the challenged ears from both mouse strains exhibited dramatic increases in CXCL-1, CXCL-2, and CCL-2 mRNA compared with unchallenged control ears. For ears from *C5L2*^{-/-} mice, these values were further en-

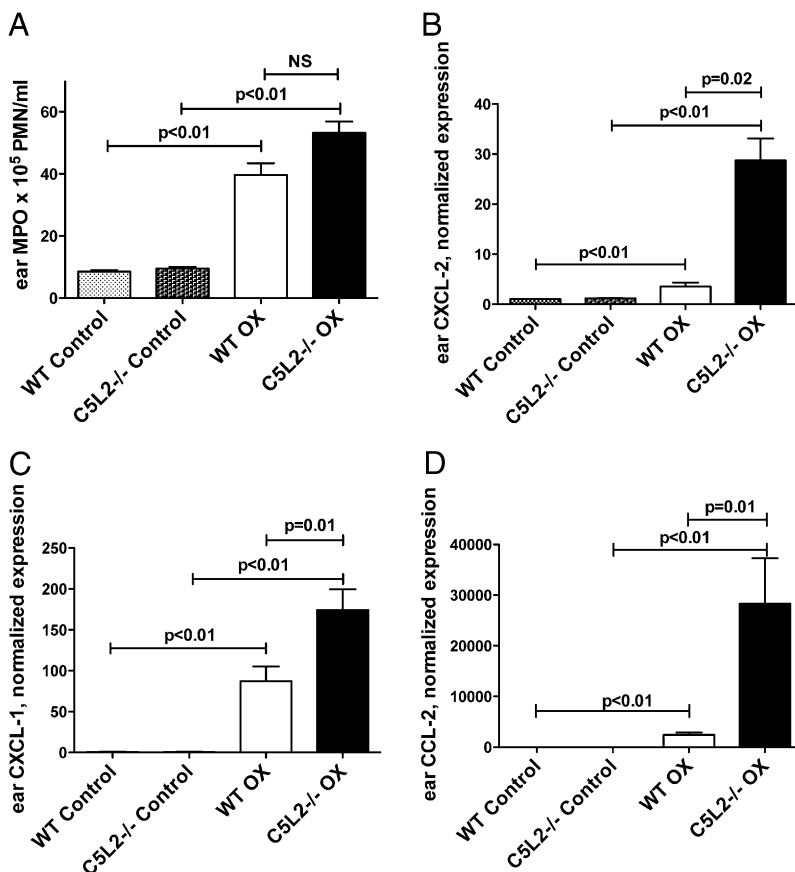


FIGURE 2. *C5L2*^{-/-} mouse ears challenged with hapten exhibit increased inflammatory cell influx and proinflammatory chemokines relative to wild type animals. **(A)** MPO content of ears from *C5L2*^{-/-} and wild type mice sensitized to OX, determined 24 h after challenge with OX or vehicle (control). Data are expressed as the mean $\pm$ SEM MPO value, and significance of differences is indicated; $n = 10$ –11 mice/group. **(B–D)** Gene expression of CXCL-2 (B), CXCL-1 (C), and CCL-2 (D) in the ears of OX-sensitized *C5L2*^{-/-} and wild type mice 24 h after challenge with OX or vehicle (control). Results are normalized to β -actin, mean $\pm$ SEM for 8–9 mice/group. Significance of differences is indicated.

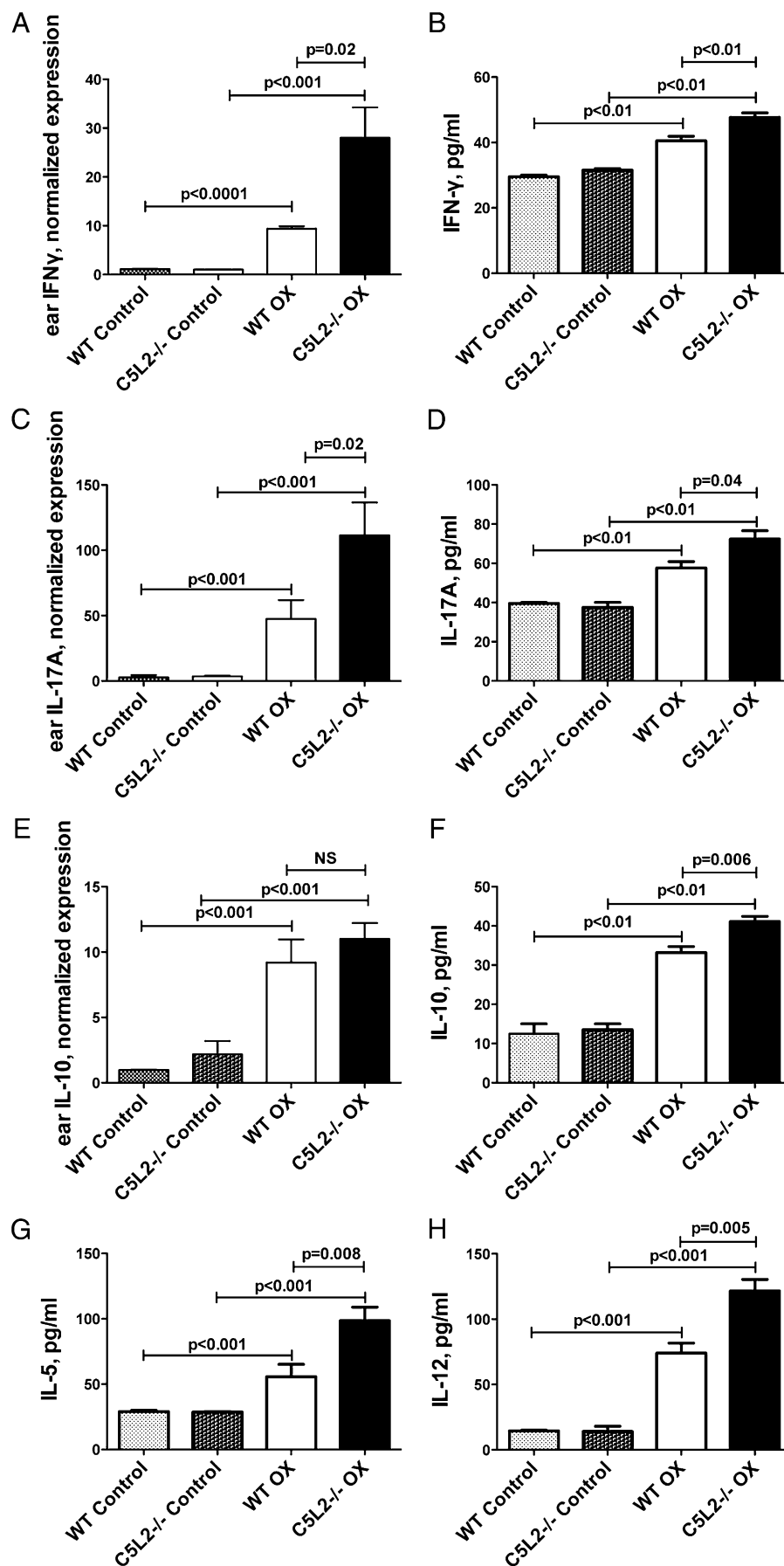


FIGURE 3. $C5L2^{-/-}$ mouse ears challenged with hapten exhibit increased Th1/Th17 cytokines relative to wild type animals. (**A, C, E**) Expression analysis of $IFN-\gamma$, $IL-17A$, and $IL-10$ in $C5L2^{-/-}$ and wild type mouse ears following sensitization and challenge with OX or vehicle (control) by quantitative PCR. Results are normalized to β -actin, mean $\pm$ SEM for 6–9 mice/group. Significance of differences is indicated. (**B, D, F–H**) Cytokine levels for $IFN-\gamma$, $IL-17A$, $IL-10$, $IL-5$, and $IL-12$ in $C5L2^{-/-}$ and wild type mouse ears following sensitization and challenge with OX or vehicle (control) determined by ELISA. Results are the mean $\pm$ SEM for 9–10 mice/group. Significance of differences is indicated.

hanced compared with wild type animals. *CXCL-1* was increased ~2-fold compared with wild type ($p < 0.01$), *CXCL-2* was increased by >7-fold ($p = 0.02$), and *CCL-2* was increased by >10-fold ($p = 0.01$) compared with ears from OX-challenged wild type animals ($n = 8-9$ mice/group). These increases in chemokine levels in *C5L2*^{-/-} mice compared with wild type animals also support a sequence in which complement activation and C5a generation precede chemokine production.

We also assessed the levels of a number of cytokines reported to participate in CS in the mouse ears 24 h after OX challenge (Fig. 3), and found increases in both *C5L2*^{-/-} and wild type animals compared with unchallenged control tissues for IFN- γ , IL-5, IL-10, IL-12p40, and IL-17A. Importantly, *C5L2*^{-/-} mouse ears exhibited significantly increased levels of all cytokines tested at this time

point relative to wild type mice, with the exception of the gene expression for IL-10. The IL-10 protein level was increased in *C5L2*^{-/-} mice, and other cytokines for which we evaluated levels of protein and gene expression revealed good correlation between the two. These findings are consistent with the Th1/Th17 nature of contact dermatitis (10, 17, 26) and are supportive of the negative modulatory role of C5L2 on C5a-C5aR-mediated responses.

*C5aR blockade ameliorates the increases in inflammation associated with contact sensitivity in *C5L2*^{-/-} mice*

To test our hypothesis that the enhanced contact sensitivity in *C5L2*^{-/-} mice compared with wild type animals is the result of deficient negative regulation of C5a-C5aR signaling, we treated OX-sensitized *C5L2*^{-/-} and wild type mice with rat monoclonal

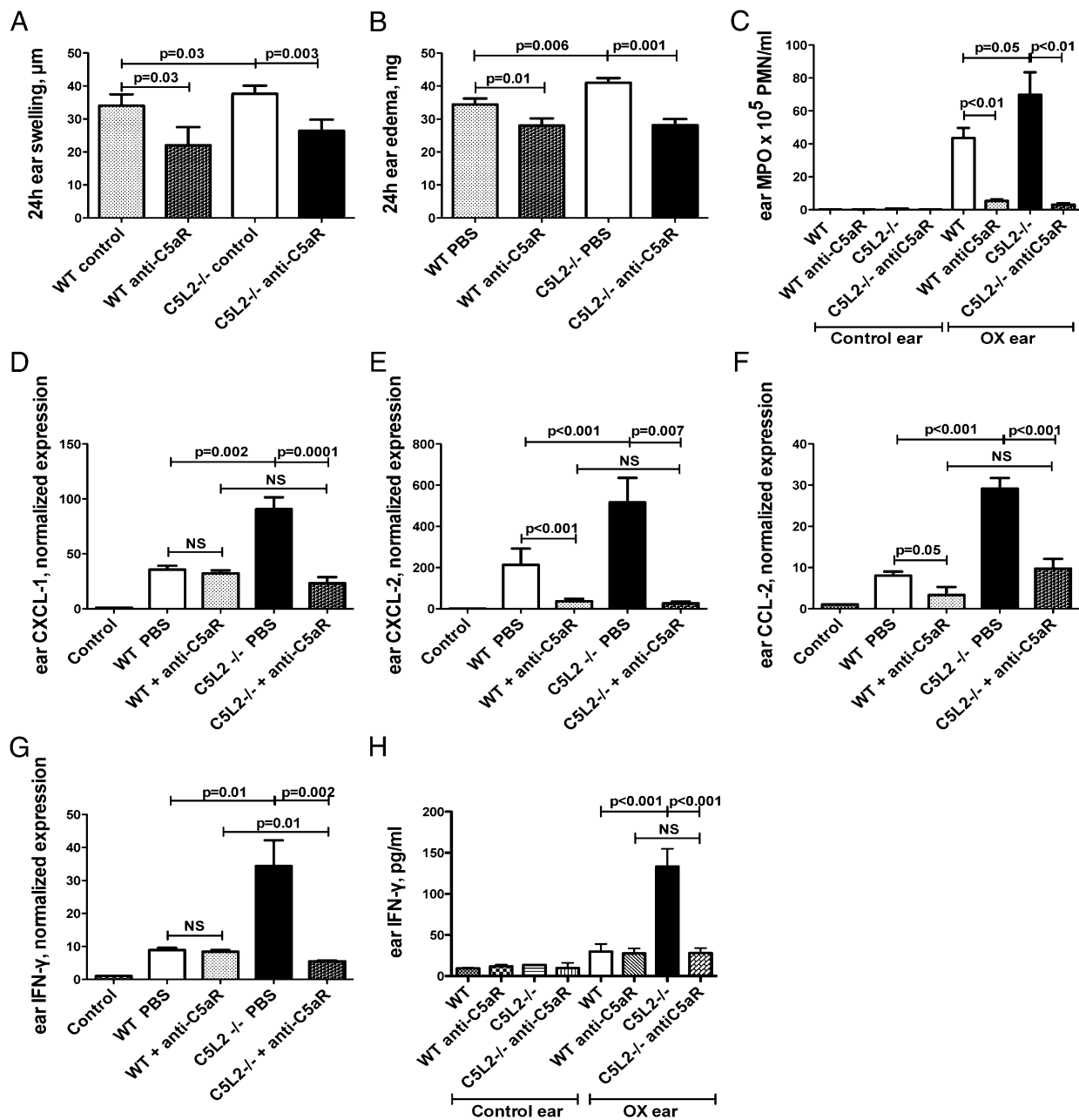


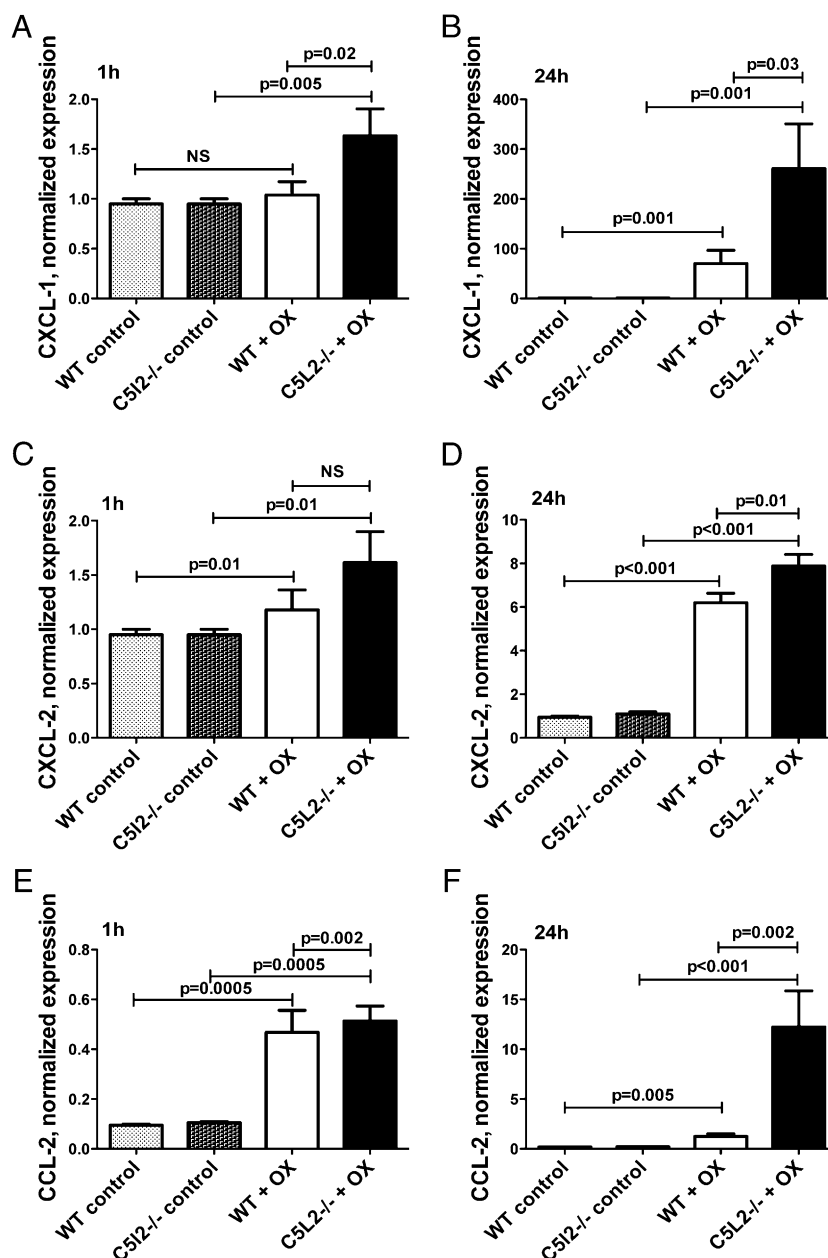
FIGURE 4. Ab blockade of the C5aR reverses the increases in inflammatory parameters in *C5L2*^{-/-} mice. OX-sensitized *C5L2*^{-/-} and wild type mice were treated with anti-C5aR mAb or isotype control 1 h prior to OX challenge on one ear with vehicle alone on the other. After 24 h, animals were sacrificed and ears were assessed for inflammatory changes as in Figs. 1–3. (A) Ear swelling, (B) Ear edema determined as in Fig. 1. (C) Ear MPO content, (D) CXCL-1, (E) CXCL-2, (F) CCL-2 determined as in Fig. 2. (G) IFN- γ gene and (H) protein expression as in Fig. 3. Control in panels (D)–(G) represents the response of the vehicle-treated ears from mice of either strain. Data are the mean $\pm$ SEM for $n = 3-8$ mice/group. Significance of differences is indicated.

anti-mouse C5aR or isotype control Ab 1 h prior to OX challenge. We chose this Ab because it has been used effectively to block C5a-C5aR interactions by other investigators with no apparent untoward effects (27, 28). *C5L2*^{-/-} mice treated with anti-C5aR mAb exhibited significant reductions in ear swelling associated with OX challenge assessed as increases in both thickness and weight (Fig. 4). In the absence of mAb, the swelling of the challenged ears was increased by ~30% in *C5L2*^{-/-} mice compared with wild type animals ($p = 0.03$; $n = 6-8$ mice/group), and this was completely reversed by treatment with the anti-C5aR mAb ($p = 0.03$). Swelling was also reduced by anti-C5aR mAb in OX-challenged ears from wild type animals (25% reduction; $p = 0.03$). Ear edema was similarly increased 20% in *C5L2*^{-/-} mice compared with wild type animals ($p = 0.006$; $n = 7-8$ mice/group) and treatment with the anti-C5aR mAb reduced this parameter ~45% in *C5L2*^{-/-} mice ($p = 0.001$) and 20% in wild type animals ($p = 0.01$). These changes were mirrored by the changes in ear MPO content. In the absence of Ab blockade, the MPO content of *C5L2*^{-/-} ears was elevated by 60% compared with wild

type mice ($p = 0.05$; $n = 4-15$ mice/group). Anti-C5aR mAb reduced this level ~20-fold for *C5L2*^{-/-} ears and 8-fold for wild type ears ($p < 0.01$ for both *C5L2*^{-/-} and wild type ears). These data are consistent with the critical role of C5a in CS-associated inflammation (7) and further support the role of C5L2 in the negative regulation of C5a-C5aR-mediated responses (16).

We then used quantitative PCR to evaluate expression of the chemoattractants *CXCL-1*, *CXCL-2*, and *CCL-2* in the ears of *C5L2*^{-/-} and wild type mice pretreated with anti-C5aR mAb or isotype control prior to OX challenge. As we observed previously, ears from OX-sensitized and -challenged *C5L2*^{-/-} mice exhibit ~2.5-fold more *CXCL-1* and *CXCL-2*, and almost 4-fold more *CCL-2* compared with wild type ($p < 0.01$; $n = 3-7$ mice/group). Pretreatment with the anti-C5aR mAb resulted in 4-fold, 20-fold, and ~3-fold reductions for *CXCL-1*, *CXCL-2*, and *CCL-2*, respectively ($p < 0.01$ for all three). The chemokine content of wild type ears was also reduced by blocking the C5aR, but to a lesser extent (NS for *CXCL-1*, ~6-fold for *CXCL-2* and 2.4-fold for *CCL-2*). Analysis of IFN- γ expression by both quantitative PCR

FIGURE 5. Lymph node cells from sensitized *C5L2*^{-/-} mice incubated with OX exhibit increased gene expression for proinflammatory chemokines compared with wild type mice. Cells were incubated with 0 or 1 $\mu\text{g/ml}$ OX for 1 or 24 h as indicated, and the expression of *CXCL-1* (A, B), *CXCL-2* (C, D), and *CCL-2* (E, F) was analyzed by quantitative PCR. Results are normalized to β -actin, mean $\pm$ SEM for 4–9 mice/group. Significance of differences is indicated.



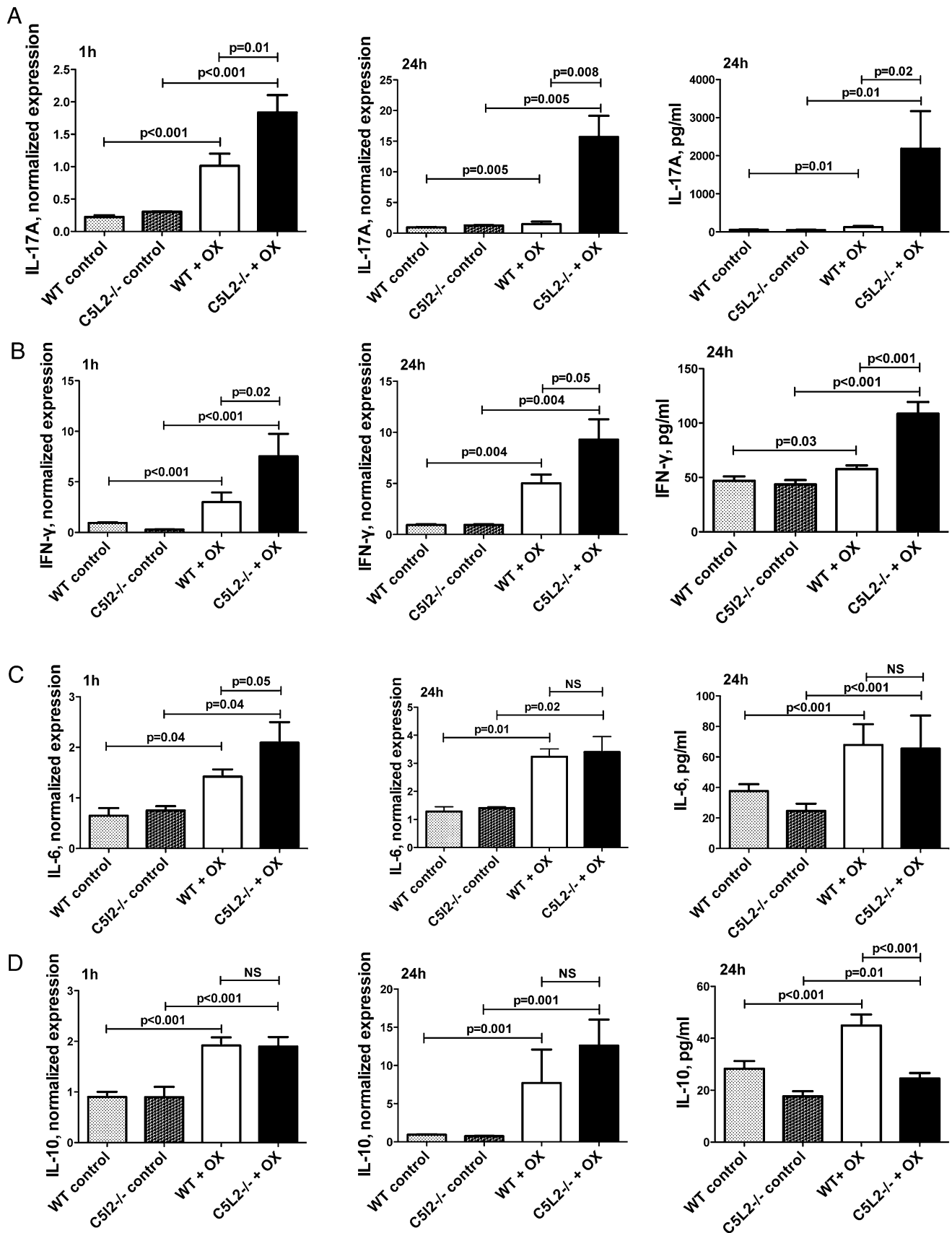


FIGURE 6. Lymph node cells from sensitized *C5L2*^{-/-} mice incubated with OX exhibit increased gene expression for proinflammatory cytokines compared with wild type mice. Cells were incubated with 0 or 1 μ g/ml OX for 1 or 24 h as indicated, and expression of IL-17A (**A**), IFN- γ (**B**), IL-6 (**C**), and IL-10 (**D**) was determined by quantitative PCR or ELISA. Data are the mean $\pm$ SEM for $n = 3$ –8 mice/group. Results are normalized to β -actin for quantitative PCR, mean $\pm$ SEM for 4–9 mice/group. Significance of differences is indicated.

and protein content yielded a similar result. Sensitized and challenged ears from *C5L2*^{-/-} mice exhibited ~4-fold increases in both gene expression and protein compared with wild type animals ($p < 0.01$; $n = 4$ –14 for both), and blocking the C5aR resulted in significant reduction in *C5L2*^{-/-} mouse ears with minor reduction in ears from wild type mice.

OX-stimulated C5L2^{-/-} lymph node cells exhibit increased chemokine and cytokine production compared with wild type cells

To corroborate our *in vivo* findings, we also examined the *ex vivo* response of combined inguinal and axillary lymph node cells harvested 24 h after OX challenge from *C5L2*^{-/-} and wild type mice. As the conditions of these cultures do not include a significant source of complement, the results are consistent with previously described evidence for local synthesis and secretion of alternative pathway complement proteins by T cells and dendritic cells (1, 29–31). These cells have a demonstrated capability for producing anaphylatoxins. Cells were incubated with 0 or 1 $\mu\text{g/ml}$ OX and evaluated for production of the same chemokines and cytokines assessed *in vivo* (Fig. 5). Within 1 h of initiating the cultures, OX-stimulated cells from sensitized and challenged wild type mice exhibited a tendency toward increased *CXCL-1* gene expression relative to unstimulated cells and significant increases in *CXCL-2* and *CCL-2* ($p \leq 0.01$; $n = 4$ –5 mice/group). For OX-stimulated cells from *C5L2*^{-/-} mice gene expression of all three chemokines was significant at 1 h ($p \leq 0.01$; $n = 4$ –5 mice/group), and these cells also exhibited greater chemokine production compared with cells from wild type animals: ~55% for *CXCL-1* ($p = 0.02$), ~35% for *CXCL-2* (NS), and *CCL-2* ($p = 0.002$; $n = 4$ –9 mice/group). After 24 h in culture, these changes were much more dramatic. The increases were significantly different, resulting from OX stimulation of both mouse strains for all three chemokines compared with unstimulated cells. In addition, OX-stimulated cells from *C5L2*^{-/-} animals exhibited elevated gene expression for all three chemokines compared with identically stimulated cells from wild type mice. *CXCL-1* was increased >3.5-fold in *C5L2*^{-/-} cells compared with wild type ($p = 0.03$), *CXCL-2* was increased ~25% ($p = 0.01$), and *CCL-2* was increased >5-fold ($p = 0.002$; $n = 5$ –9 mice/group).

Within 1 h of initiating lymph node cultures, expression of *IFN- γ* , *IL-17A*, *IL-6*, and *IL-10* was elevated in OX-stimulated cells from both *C5L2*^{-/-} and wild type mice (Fig. 6). As for the chemokines, the changes in cytokine gene expression and protein levels were generally more pronounced after 24 h. *IL-6* gene expression and protein were elevated for OX-stimulated cells compared with controls, but at 24 h there was no difference between *C5L2*^{-/-} and wild type cells. Similarly, *IL-10* gene expression was increased with OX stimulation in cells from both mouse strains, equally for *C5L2*^{-/-} and wild type cells. *C5L2*^{-/-} cells exhibited reduced protein relative to cells from wild type mice. In contrast, *IL-10* protein in the ears of OX-sensitized and -challenged *C5L2*^{-/-} mice was elevated relative to wild type animals (see Fig. 3F), possibly reflecting the role of distinct cell types in lymph nodes and intact dermal tissues.

Discussion

Previous investigations have demonstrated a critical role for early complement activation and generation of the C5a anaphylatoxin in allergic contact sensitivity (6–8, 24). C5a exhibits high-affinity interactions with the C5aR, a classical G protein coupled 7-transmembrane segment receptor, as well as the atypical receptor, C5L2, which is deficient in G protein coupling. This study was initiated to determine the effects of C5L2 in a mouse model of

allergic CS induced by OX. Our work in human neutrophils revealed enhanced C5a-C5aR-mediated responses in the presence of a blocking Ab against C5L2 (16); therefore, we expected *C5L2*-deficient mice to reveal exacerbated CS reactions. Examination of the mechanism of action of C5L2 in neutrophils revealed a molecular association between C5L2 and β -arrestin that normally restricts the interaction between β -arrestin and the C5aR following ligand stimulation. When the C5L2- β -arrestin interaction is blocked, C5aR- β -arrestin interactions are potentiated and enhanced signaling through this pathway is observed. In OX-induced CS, we find that C5a-C5aR-mediated responses are similarly potentiated in *C5L2*^{-/-} mice. Indeed, all aspects of CS are exacerbated in *C5L2*^{-/-} mice, including tissue swelling, neutrophil influx, and chemokine and cytokine production, supporting the essential role of early complement activation and generation of C5a. Additional evidence for the specificity of the enhanced CS associated with *C5L2* deficiency is provided by treating animals with an mAb against the mouse C5aR. This not only reverses the increased responses associated with *C5L2* deficiency; in most instances, it ameliorates the responses observed for wild type animals as well. In particular, we observed significant reductions following anti-C5aR pretreatment in the ear swelling, edema, neutrophil influx (as tissue MPO) and increased gene expression of *CXCL-2* and *CCL-2* associated with OX-induced CS. In contrast, this treatment did not alter *CXCL-1* or *IFN- γ* in wild type mice. Although early work showed that C5a production contributes a majority of the inflammatory activity associated with CS, involvement of additional pathways is also evident, particularly at later time points following hapten challenge (7).

An examination of the role of C3a in allergic CS revealed enhanced cytokine production in the sensitization phase in *C3aR*-deficient mice, but no change in ear swelling in the elicitation phase (32). We find that mice deficient in the *C3aR* also exhibit a reduction in the expression of C5L2 protein resulting from the formation of a heterodimer between the two (N.P. Gerard, unpublished observations). Since the impact of *C5L2* deficiency is enhanced responses to C5a, we expect a similar phenotype in *C3aR*-deficient animals as well. Thus, the result in this case is not a reflection of a protective role for C3a, but it is an indirect effect because of an alteration in the expression of C5L2. The absence of observed changes in the elicitation phase may be explained by the transient nature of the reaction, as the contribution of C5a was reduced as a function of time, whereas the apparent role of chemokines became more pronounced (8).

The results of the lymph node cultures closely mirror those of the mouse ears in that the inflammatory parameters associated with allergic contact sensitivity are elevated in OX-stimulated cells from *C5L2*^{-/-} mice compared with wild type animals. The absence of a significant serum source for complement supports the previously identified ability of dendritic cells and T cells to synthesize locally and secrete alternative complement pathway components that might result in the generation of anaphylatoxins (1, 30, 31). Importantly, this observation, coupled with evidence based on Ab blockade of the C5aR, lends further support to the role of C5L2 as a negative regulator of C5a-C5aR-mediated reactions.

Disclosures

The authors have no financial conflicts of interest.

References

1. Lalli, P. N., M. G. Strainic, M. Yang, F. Lin, M. E. Medof, and P. S. Heeger. 2008. Locally produced C5a binds to T cell-expressed C5aR to enhance effector T-cell expansion by limiting antigen-induced apoptosis. *Blood* 112: 1759–1766.

2. Cohen, D. E. 2004. Contact dermatitis: a quarter century perspective. *J. Am. Acad. Dermatol.* 51(1 Suppl): S60–S63.
3. Askenase, P. W., M. Szczepanik, A. Itakura, C. Kiener, and R. A. Campos. 2004. Extravascular T-cell recruitment requires initiation begun by Valpha14+ NKT cells and B-1 B cells. *Trends Immunol.* 25: 441–449.
4. Tsuji, R. F., M. Szczepanik, I. Kawikova, V. Paliwal, R. A. Campos, A. Itakura, M. Akahira-Azuma, N. Baumgarth, L. A. Herzenberg, and P. W. Askenase. 2002. B cell-dependent T cell responses: IgM antibodies are required to elicit contact sensitivity. *J. Exp. Med.* 196: 1277–1290.
5. Krasteva, M., J. Kehren, M. T. Ducluzeau, M. Sayag, M. Cacciapuoti, H. Akiba, J. Descotes, and J. F. Nicolas. 1999. Contact dermatitis I. Pathophysiology of contact sensitivity. *Eur. J. Dermatol.* 9: 65–77.
6. Tsuji, R. F., G. P. Geba, Y. Wang, K. Kawamoto, L. A. Matis, and P. W. Askenase. 1997. Required early complement activation in contact sensitivity with generation of local C5-dependent chemotactic activity, and late T cell interferon gamma: a possible initiating role of B cells. *J. Exp. Med.* 186: 1015–1026.
7. Tsuji, R. F., I. Kawikova, R. Ramabhadran, M. Akahira-Azuma, D. Taub, T. E. Hugli, C. Gerard, and P. W. Askenase. 2000. Early local generation of C5a initiates the elicitation of contact sensitivity by leading to early T cell recruitment. *J. Immunol.* 165: 1588–1598.
8. Tsuji, R. F., M. Kikuchi, and P. W. Askenase. 1996. Possible involvement of C5/C5a in the efferent and elicitation phases of contact sensitivity. *J. Immunol.* 156: 4444–4450.
9. Purwar, R., W. Bäumer, M. Niebuhr, T. Tschernig, M. Kietzmann, and T. Werfel. 2011. A protective role of complement component 3 in T cell-mediated skin inflammation. *Exp. Dermatol.* 20: 709–714.
10. Campos, R. A., M. Szczepanik, M. Lisbonne, A. Itakura, M. Leite-de-Moraes, and P. W. Askenase. 2006. Invariant NKT cells rapidly activated via immunization with diverse contact antigens collaborate in vitro with B-1 cells to initiate contact sensitivity. *J. Immunol.* 177: 3686–3694.
11. Klos, A., E. Wende, K. J. Wareham, and P. N. Monk. 2013. International Union of Pharmacology. LXXXVII. Complement peptide C5a, C4a, and C3a receptors. *Pharmacol. Rev.* 65: 500–543.
12. Gerard, N. P., B. Lu, P. Liu, S. Craig, Y. Fujiwara, S. Okinaga, and C. Gerard. 2005. An anti-inflammatory function for the complement anaphylatoxin C5a-binding protein, C5L2. *J. Biol. Chem.* 280: 39677–39680.
13. Ohno, M., T. Hirata, M. Enomoto, T. Araki, H. Ishimaru, and T. A. Takahashi. 2000. A putative chemoattractant receptor, C5L2, is expressed in granulocyte and immature dendritic cells, but not in mature dendritic cells. *Mol. Immunol.* 37: 407–412.
14. Okinaga, S., D. Slattery, A. Humbles, Z. Zsengeller, O. Morteau, M. B. Kinrade, R. M. Brodbeck, J. E. Krause, H. R. Choe, N. P. Gerard, and C. Gerard. 2003. C5L2, a nonsignaling C5a binding protein. *Biochemistry* 42: 9406–9415.
15. Scola, A. M., K. O. Johsrich, B. P. Morgan, A. Klos, and P. N. Monk. 2009. The human complement fragment receptor, C5L2, is a recycling decoy receptor. *Mol. Immunol.* 46: 1149–1162.
16. Bamberg, C. E., C. R. Mackay, H. Lee, D. Zahra, J. Jackson, Y. S. Lim, P. L. Whitfield, S. Craig, E. Corsini, B. Lu, et al. 2010. The C5a receptor (C5aR) C5L2 is a modulator of C5aR-mediated signal transduction. *J. Biol. Chem.* 285: 7633–7644.
17. Kish, D. D., X. Li, and R. L. Fairchild. 2009. CD8 T cells producing IL-17 and IFN-gamma initiate the innate immune response required for responses to antigen skin challenge. *J. Immunol.* 182: 5949–5959.
18. Li, R., L. G. Coulthard, M. C. Wu, S. M. Taylor, and T. M. Woodruff. 2013. C5L2: a controversial receptor of complement anaphylatoxin, C5a. *FASEB J.* 27: 855–864.
19. Xiao, H., A. Schreiber, P. Heeringa, R. J. Falk, and J. C. Jennette. 2007. Alternative complement pathway in the pathogenesis of disease mediated by anti-neutrophil cytoplasmic autoantibodies. *Am. J. Pathol.* 170: 52–64.
20. Fisette, A., M. N. Munkonda, K. Oikonomopoulou, S. Paglialunga, J. D. Lambris, and K. Cianflone. 2013. C5L2 receptor disruption enhances the development of diet-induced insulin resistance in mice. *Immunobiology* 218: 127–133.
21. Gao, H., T. A. Neff, R. F. Guo, C. L. Speyer, J. V. Sarma, S. Tomlins, Y. Man, N. C. Riedemann, L. M. Hoesel, E. Younkin, et al. 2005. Evidence for a functional role of the second C5a receptor C5L2. *FASEB J.* 19: 1003–1005.
22. Cao, Q., S. M. McIsaac, and A. W. Stadnyk. 2012. Human colonic epithelial cells detect and respond to C5a via apically expressed C5aR through the ERK pathway. *Am. J. Physiol. Cell Physiol.* 302: C1731–C1740.
23. Bozic, C. R., B. Lu, U. E. Höpken, C. Gerard, and N. P. Gerard. 1996. Neurogenic amplification of immune complex inflammation. *Science* 273: 1722–1725.
24. Norman, M. U., S. Hulliger, P. Colarusso, and P. Kubes. 2008. Multichannel fluorescence spinning disk microscopy reveals early endogenous CD4 T cell recruitment in contact sensitivity via complement. *J. Immunol.* 180: 510–521.
25. Engeman, T., A. V. Gorbachev, D. D. Kish, and R. L. Fairchild. 2004. The intensity of neutrophil infiltration controls the number of antigen-primed CD8 T cells recruited into cutaneous antigen challenge sites. *J. Leukoc. Biol.* 76: 941–949.
26. Pennino, D., K. Eyerich, C. Scarponi, T. Carbone, S. Eyerich, F. Nasorri, S. Garovich, C. Traidl-Hoffmann, C. Albanesi, and A. Cavani. 2010. IL-17 amplifies human contact hypersensitivity by licensing hapten nonspecific Th1 cells to kill autologous keratinocytes. *J. Immunol.* 184: 4880–4888.
27. Lajoie, S., I. P. Lewkowich, Y. Suzuki, J. R. Clark, A. A. Sproles, K. Dienger, A. L. Budelsky, and M. Wills-Karp. 2010. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat. Immunol.* 11: 928–935.
28. Rittirsch, D., M. A. Flierl, B. A. Nadeau, D. E. Day, M. Huber-Lang, C. R. Mackay, F. S. Zetoun, N. P. Gerard, K. Cianflone, J. Köhl, et al. 2008. Functional roles for C5a receptors in sepsis. *Nat. Med.* 14: 551–557.
29. Heeger, P. S., P. N. Lalli, F. Lin, A. Valujskikh, J. Liu, N. Muqim, Y. Xu, and M. E. Medof. 2005. Decay-accelerating factor modulates induction of T cell immunity. *J. Exp. Med.* 201: 1523–1530.
30. Strainic, M. G., J. Liu, D. Huang, F. An, P. N. Lalli, N. Muqim, V. S. Shapiro, G. R. Dubyak, P. S. Heeger, and M. E. Medof. 2008. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. *Immunity* 28: 425–435.
31. Kwan, W. H., D. Hashimoto, E. Paz-Artal, K. Ostrow, M. Greter, H. Raedler, M. E. Medof, M. Merad, and P. S. Heeger. 2012. Antigen-presenting cell-derived complement modulates graft-versus-host disease. *J. Clin. Invest.* 122: 2234–2238.
32. Niebuhr, M., W. Bäumer, M. Kietzmann, K. Wichmann, A. Heratizadeh, and T. Werfel. 2012. Participation of complement 3a receptor (C3aR) in the sensitization phase of Th2 mediated allergic contact dermatitis. *Exp. Dermatol.* 21: 52–56.