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J Immunol 2014; 192:3355-3364; Prepublished online 3 March 2014; doi: 10.4049/jimmunol.1302305
http://www.jimmunol.org/content/192/7/3355

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/03/01/jimmunol.1302305.DCSupplemental

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The Epidermal Growth Factor Receptor Is a Regulator of Epidermal Complement Component Expression and Complement Activation

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The complement system is activated in response to tissue injury. During wound healing, complement activation seems beneficial in acute wounds but may be detrimental in chronic wounds. We found that the epidermal expression of many complement components was only increased to a minor extent in skin wounds in vivo and in cultured keratinocytes after exposure to supernatant from stimulated mononuclear cells. In contrast, the epidermal expression of complement components was downregulated in ex vivo injured skin lacking the stimulation from infiltrating inflammatory cells but with intact injury-induced epidermal growth factor receptor (EGFR)–mediated growth factor response. In cultured primary keratinocytes, stimulation with the potent EGFR ligand, TGF-α, yielded a significant downregulation of complement component expression. Indeed, EGFR inhibition significantly enhanced the induction of complement components in keratinocytes and epidermis following stimulation with proinflammatory cytokines. Importantly, EGFR inhibition of cultured keratinocytes either alone or in combination with proinflammatory stimulus promoted activation of the complement system after incubation with serum. In keratinocytes treated solely with the EGFR inhibitor, complement activation was dependent on serum-derived C1q, whereas in keratinocytes stimulated with a combination of proinflammatory cytokines and EGFR inhibition, complement activation was found even with C1q-depleted serum. In contrast to human keratinocytes, EGFR inhibition did not enhance complement component expression or cause complement activation in murine keratinocytes. These data demonstrate an important role for EGFR in regulating the expression of complement components and complement activation in human epidermis and keratinocytes and, to our knowledge, identify for the first time a pathway important for the epidermal regulation of complement activation. The Journal of Immunology, 2014, 192: 3355–3364.

The online version of this article contains supplemental material.

Abstract

The complement system is an evolutionary ancient part of the innate immune system important in both host defense and tissue homeostasis (1). Complement component deficiencies cause both increased susceptibility to infections and inflammatory conditions such as systemic lupus erythematosus. Although the liver is the major site for synthesis of complement components, local synthesis of complement components is found in many tissues. The importance of the local production of complement components has been highlighted by its role in organ rejection after kidney transplantation (2, 3). Similarly, activation of the complement system is regulated locally in the tissues because systemic deficiencies of complement regulatory proteins give rise to tissue-specific rather than systemic diseases (4). Although the local regulation of complement activations is considered to be a dynamic process (4), the pathways and stimuli that regulate local complement activation are more or less unknown.

In the skin, local production of complement components is found in epidermal keratinocytes (5–7), and the complement system plays an important role in cutaneous manifestations of inflammatory diseases such as bullous pemphigoid (8) and systemic lupus erythematosus (9). Local production of complement components may be important for attraction of neutrophils to the skin because, for example, C1q is a powerful chemotactic agent (10). During cutaneous wound healing, activation of the complement system is important for attraction of neutrophils to the wound (11) and is considered beneficial in acute wounds (12). However, complement activation may be detrimental in chronic wounds, and reduction of complement activation is considered to improve wound healing (12). Despite the importance of the complement system in both skin inflammation and cutaneous wound healing, the regulation of both epidermal expression of complement components and epidermal complement activation is largely unknown.

Because local production of complement components is an important determinant for complement activation, for example, in...
kidney transplants (2, 3), we examined the epidermal expression of complement components during wound healing and found a previously unrecognized role of the epidermal growth factor (EGF) receptor (EGFR) in mediating regulation of epidermal complement component expression and complement activation. EGFR inhibition increased the expression of complement components in epidermal keratinocytes induced by proinflammatory stimuli. Importantly, the EGFR inhibition in human keratinocytes both alone and in combination with proinflammatory stimuli promoted activation of complement after incubation with serum. These data demonstrate a novel role of EGFR in regulation of both expression of complement components and complement activation in the epidermis and, to our knowledge, identify for the first time a pathway important for the epidermal regulation of complement activation.

Materials and Methods

Reagents

AG-1478, anti-factor B Abs, anti-C1s Abs, and mouse complement serum were purchased from Sigma-Aldrich; cetuximab (Erbitux) was purchased from Merck. Abs against the C3d domain of human C3 and against the C4c domain of human C4 were from Dako (Glostrup, Denmark). Abs against murine C4 were from Thermo Scientific. Anti-C1q Abs, anti-terminal complement complex (TCC) Abs, C1q, C1q-depleted serum, and factor B–depleted serum were from Quidel. Abs raised against the C3d domain of murine C3 were from R&D Systems.

Human skin wounds and microarray

Samples from human skin wounds were obtained under protocols approved by the Ethics Committee at Lund University (Lund, Sweden), as previously described (13). In brief, nonwounded human skin was obtained by taking punch biopsies from three healthy donors, and skin wound samples were retrieved by making new punch biopsies from the edges of the initial biopsies. For analysis by cDNA microarray, as much dermal tissue as possible was removed by dissection from the biopsies, and H&E staining confirmed that >90% of the samples consisted of epidermis (13). RNA was isolated from these samples and used for cDNA microarray analysis. The microarray data are available in the Minimum Information About a Microarray Experiment database (http://www.ebi.ac.uk/arrayexpress; accession number E-MEXP-3305).

Model of ex vivo injured human skin

The skin specimens were obtained as excess healthy tissue from skin surgery, under protocols approved by the Ethics Committee at Lund University. The surgical specimens were cut into slices of 1 × 10 mm, and nonepidermal tissue was removed by dissection. The samples were cultured in KGM Gold Bullet kit from Lonza without insulin or EGF but with 10% human serum. For inhibition experiments, the samples were cultured with 10 μM AG-1478 or 50 μg/ml cetuximab.

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were performed according to the instructions from the manufacturer (Bio-Rad, Hercules, CA). After transfer of proteins from the polyacrylamide gels, the polyvinylidene difluoride (PVDF) membrane was fixed for 30 min in TBS with 0.05% glutaraldehyde (Sigma-Aldrich) and blocked with 5% skimmed milk. For visualization of the proteins, the PVDF membranes were incubated overnight with primary Abs. The following day, the membranes were incubated for 2 h with HRP-conjugated secondary Abs (Pierce) and visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce). The PVDF membrane was stripped for 20 min in 0.2 M glycine (pH 2.5) and 1% SDS, washed twice with TBS with 0.05% Tween 20 (TBBS), and finally blocked before incubating overnight with a different Ab.

RNA isolation

Total RNA was isolated with TRIzol (Invitrogen), according to the recommendations of the manufacturer. The RNA was double purified with TRIzol, then precipitated with ethanol, and resuspended in 0.1 mM EDTA. The concentration was determined by spectrophotometric measurement.

Keratinocyte cultures

Primary keratinocytes were obtained from Lonza (Portland, OR) and grown in serum-free medium (KGM Gold) from Lonza. For 2–4 d after seeding, the cells received 100 ng/ml EGF. Two days before stimulation, the medium was changed to KGM Gold medium without insulin or EGF. The cells were stimulated the day after complete confluence was reached. Murine keratinocytes and medium were purchased from CELLaTec. Murine keratinocytes were cultured in CnT-07 medium. The day of stimulation, the medium was changed to KGM Gold without insulin or EGF.

Complement activation

New medium containing 1.2 mM CaCl2 was added to the cells together with 20% human serum or 20% heat-inactivated serum. After 3-h incubation at 37°C, the cells were washed and fixed with 4% formaldehyde for 1 h (15 min on ice, 45 min at room temperature). After three washes in TBS (10 mM Tris, 500 mM NaCl [pH 7.2]), the cells were blocked with 5% goat serum/5 mg/ml BSA at room temperature for 45 min in TBS. After blocking, inserts were washed once in TTBS. Incubation was performed with Abs diluted in TTBS with 2.5% goat serum/5 mg/ml BSA overnight in cold room under rotation. Slides were washed three times in TTBS and incubated with secondary Abs for 2–4 h at room temperature. The inserts were mounted on slides using Prolong Gold antifade reagent mounting medium with DAPI (Invitrogen).

Subcellular fractionation of HaCaT cells

HaCaT cells were washed once in disruption buffer (100 mM KCl, 3 mM NaCl, 10 mM PIPES, 3.5 mM MgCl2, 1 mM ATP [pH 7.0]). HaCaT in disruption buffer with protease inhibitors (1 mM PMSF, 5 μg/ml E-64, 5 μg/ml garlardin, 1 mM EDTA) was scraped off with rubber policeman, and the cells were disrupted with nitrogen cavitation. Unbroken cells and nuclei (nuclei fraction) were pelleted by centrifugation at 800 × g at 4°C for 15 min. The supernatant containing cytosolic components and plasma membrane vesicle was subsequently pelleted by centrifugation at 100,000 × g for 20 min in Beckman Airfuge. The pellet containing mainly plasma membrane vesicles (plasma membrane fraction) was harvested. The protein content of the cytosol, nuclei, and membrane fractions was quantified by measurements at OD280.

C1q slot-binding assay

Nuclei and membrane fractions (equal amount of proteins in samples from AG-1478–treated cells and control cells) were solubilized in 0.5% Nonidet P-40 and applied to polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore) using a Milliblot-D system (Millipore). Membranes were blocked in TBS (50 mM Tris–HCl [pH 7.5], 150 mM NaCl) containing 5% BSA, subsequently incubated with 125I-labeled protein C1q for 3 h, washed with TBS containing 0.05% Tween 20, and developed by phosphor imaging.

Immunohistochemistry of skin samples

The skin specimens were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Slices (5 μm) were made and placed on polylysine–coated glass slides, followed by incubation for 60°C for 2 h. The slides were then treated with Ag retriever (Biocare Medical, Concord, CA) for 40 min at 97°C in a pressure cooker. After Ag retrieval, the slides were incubated overnight with primary Abs diluted 1:500 for 24 h in TBS with 0.05% Tween 20, 1% BSA, and 5% serum from the same species as the secondary Abs were raised. The slides were washed three times for 20 min in TBS with 0.05% Tween 20 and incubated for 24 h with secondary Abs diluted 1:1000 in the same buffer as the primary Abs. The slides were then washed again three times and mounted with Prolong Gold antifade reagent mounting medium with DAPI (Invitrogen).

Immunofluorescence

For fluorescence analysis, samples were visualized using a Nikon Eclipse TE300 (Nikon, Melville, NY) inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled charge-coupled device camera (Hama-matsu) and a Plan Apochromat objective (Olympus, Orangeburg, NY).

Real-time PCR

cDNA was synthesized from 600 ng purified RNA using iScript cDNA synthesis kit (Bio-Rad), according to the instructions given by the manufacturer. Gene expression of complement components was analyzed with quantitative RT-PCR using iQ SYBR Green Supermix (Bio-Rad). Amplification was measured.
performed at 55°C for 40 cycles in iCycler Thermal Cycler (Bio-Rad), and data were analyzed using iCycler IQ Optical System Software. Gene expression was normalized using GADPH as housekeeping gene. All real-time PCR primers are listed in Supplemental Table 1.

Statistical analysis

Expression of complement components in nonstimulated cells was set to 1, and gene expression in cells treated with PBMC supernatants and/or EGFR inhibitors was measured as fold induction compared with the nonstimulated controls. The expression levels as measured in fold induction were log transformed, and statistical tests were done using paired two-tailed t test using the log-transformed values.

Results

We examined the epidermal expression of complement components during the proliferative phase of wound healing day 4 after injury, because we have previously found robust induction of genes involved in innate immunity at this time point (13, 14). However, the expression of many complement components was only induced to a minor extent in epidermis of skin wounds compared with epidermis of noninjured skin (Fig. 1). This very limited induction of complement components was surprising because proinflammatory cytokines have previously been demonstrated to induce the expression of certain complement components in keratinocytes in vitro (5–7) and the expression of antimicrobial peptides induced by proinflammatory cytokines peaks day 4 after injury (13). However, in the epidermis during wound healing, the expression of many innate immunity genes is induced by the growth factor response mediated by EGF activation (13) and not by proinflammatory cytokines. To investigate the role of EGF activation in the epidermal expression of complement components, we examined the expression of complement components in a model of ex vivo injured epidermis with intact EGF-mediated growth factor response but lacking the stimuli from the infiltrating inflammatory cells found in skin wounds in vivo. In the ex vivo injured epidermis, the expression of complement components was in many instances downregulated compared with nonwounded skin (Fig. 1), indicating that the EGF response mediated by EGF activation may cause a downregulation of complement component expression. Indeed, EGF activation by TGF-α significantly downregulated the expression of complement components in cultured human keratinocytes (Fig. 2A).

To further investigate the seemingly opposing effects of EGFR activation and proinflammatory cytokines on complement component expression in keratinocytes, we stimulated primary keratinocytes with medium from PBMCs stimulated with M1 protein from Streptococcus pyogenes. As expected, the PBMC supernatant rich in proinflammatory cytokines induced the expression of some complement components, although to a minor extent (Fig. 2B). Because supernatants from stimulated PBMCs induce gene expression through EGFR activation (15), keratinocytes were also stimulated with the PBMC supernatant in combination with the EGFR inhibitor AG-1478. EGFR inhibition enhanced the expression of complement components in primary keratinocytes stimulated with PBMC supernatants in some instances >100-fold (Fig. 2B). Similar results were found with supernatants from LPS-stimulated PBMC (Supplemental Fig. 1A). Like AG-1478, the EGFR-neutralizing Ab, cetuximab, enhanced the expression of complement components in keratinocytes stimulated with PBMC supernatant (Supplemental Fig. 1B), demonstrating that the observed effect of AG-1478 was due to EGFR inhibition. The EGFR inhibitors, AG-1478 and cetuximab, had only minor effect on the expression of complement components in keratinocytes by themselves in the absence of PBMC supernatant (Fig. 2C).
finding by real-time PCR was corroborated by Western blots of factor B. Factor B was only present in significant amount in the medium from keratinocytes stimulated with both PBMC supernatant and EGFR inhibitor (cetuximab) but not in medium from keratinocytes treated with cetuximab or PBMC supernatant alone (Fig. 3). Similar Western blots were performed with Abs against C1s, C4, and C3. However, the stimulated PBMC produced high levels of these complement components, thereby masking the effect of the EGFR inhibition at the protein level in keratinocytes.

To substantiate the in vitro findings in keratinocyte cell cultures, whole human epidermis was stimulated with peptidoglycan (PGN) and the expression of the complement components C1s and factor B was examined with or without inhibition of EGFR. Inhibition of EGFR boosted the expression of C1s and factor B in PGN-challenged epidermis, whereas the EGFR inhibition by itself had only a minor effect (Fig. 4A). PGN was chosen rather than LPS or M1 protein because we have previously found that PGN stimulation of whole epidermis gave prominent expression of antimicrobial peptides mediated by proinflammatory cytokines (15). Immunohistochemistry revealed that the combination of EGFR inhibitor and PGN led to a more widespread expression of factor B in epidermis than that found by the EGFR inhibitor or PGN alone (Fig. 4B).

Subsequently, we investigated whether EGFR inhibition in keratinocytes promoted complement activation. Human primary keratinocytes were subjected to EGFR inhibition with AG-1478 for 2 d and subsequently incubated with serum for complement activation. Deposition of C3 using an Ab against the C3d domain was found in cells subjected to EGFR inhibition by immunofluorescence microscopy. Only minor staining for C3 was found in cells incubated with heat-inactivated serum, demonstrating that the C3 staining was due to complement activation (Fig. 5A). To further substantiate the complement activation, immunofluorescence microscopy was performed using mAbs against the TCC (C5b-C9). More prominent staining was found for TCC in cells subjected to EGFR inhibition after incubation with serum compared with nontreated (control) cells. Only minor staining was found with heat-inactivated serum (Fig. 5A).

In our experiments, the nuclear morphology of the cells with complement activation did not show the classical signs of apoptosis (shrinkage and nuclear fragmentation). However, to confirm that the observed complement activation was not related to apoptosis induced by EGFR inhibition, cells treated with AG-1478 and nontreated keratinocytes were examined by TUNEL staining to identify apoptotic cells. Treatment with AG-1478 did not increase the number of TUNEL–positive cells, and the TUNEL–positive cells displayed nuclear shrinkage and fragmentation, demonstrating the validity of the assay (Fig. 6A). To rule out the possibility that a naturally occurring Ab bound to the AG-1478–treated keratinocytes, immunofluorescence studies were performed with human serum as primary Ab on nontreated and AG-1478–treated keratinocytes. The AG-1478 treatment did not cause increased immunoreactivity of the human serum (Fig. 6B). Similar results were found with Western blot of cell lysates. The same serum pool that was used for complement activation assays was used for these experiments. To exclude the possibility that the complement activation was due to low amounts of Ab binding to AG-1478–treated keratinocytes with high affinity, complement activation was done with serum preincubated at 4˚C for 1 h with AG-1478–treated keratinocytes before complement activation. The preabsorption with AG-1478–treated cells did not abolish complement activation (Fig. 6C). This indicated that the complement activation observed after EGFR inhibition by AG-1478 was Ab independent.

To identify the complement activation pathway involved in the observed complement activation on keratinocytes, cells were immunostained for C4 by using an Ab raised against C4c after complement activation. Deposition of C4 was prominent in cells cultured with AG-1478 and subsequently incubated with serum, demonstrating that the observed complement activation was due to classical pathway activation (Fig. 5A). Furthermore, C3 and C4 deposition was found after incubation with just 5% serum (lowest concentration tested; data not shown). Incubation of AG-1478–treated keratinocytes with heat-inactivated serum resulted in much less prominent staining for C4, demonstrating that the C4 staining was due to complement activation (Fig. 5A). This indicated a possible role for C1q in the observed complement activation. We found that C1q was locally expressed in keratinocytes and that this expression was increased by EGFR inhibition (Fig. 2C). We did not observe increased C1q staining in AG-1478–treated keratinocytes after incubation with serum compared with nontreated controls incubated with serum (Fig. 5B). To investigate whether the complement activation was dependent on serum-derived C1q, AG-1478–treated keratinocytes were incubated with C1q-depleted serum. No C3 or C4 deposition was found when AG-1478–treated cells were incubated with C1q-depleted serum (Fig. 5C). However, addition of C1q to the C1q-depleted serum restored the C3 and C4 deposition (Fig. 5C). Even when complement activation experiments with C1q-depleted serum were performed with AG-1478–treated keratinocytes in which the complement components produced by the keratinocytes were allowed to accumulate for 48 h, no complement activation was found. When AG-1478–treated keratinocytes were incubated with factor B–depleted serum as a control, C3 and C4 deposition were still found (Fig. 5C). These data demonstrated that in AG-1478–treated keratinocytes the complement activation was dependent on serum-derived C1q.

To further investigate, substantiate, and characterize the role of C1q in the observed complement activation, complement activation
experiments were performed with the keratinocyte cell line HaCaT. As with primary keratinocytes, EGFR inhibition was found to promote complement activation (Supplemental Fig. 2). HaCaT cells were subjected to EGFR inhibition, and subcellular fractionation was performed, separating the HaCaT cells into a nuclei fraction, cytosol, and a membrane fraction consisting mainly of plasma membranes. The fractions were probed for C1q binding in a slot blot-binding assay. C1q bound all subcellular fractions in both nontreated and AG-1478–treated cells (Fig. 7A). The samples for the slot-binding assays were solubilized in detergent to avoid lipid binding to the membranes. The slot-binding assay was validated with Western blots with radioactive C1q. One major C1q-binding band just below 250 kDa was consistently found in the membrane fraction of AG-1478–treated HaCaT cells (Fig. 7B), but the same band was observed in nontreated HaCaT cells (three independent experiments). The C1q-binding band from both AG-1478–treated HaCaT cells and nontreated HaCaT cells was excised from the gels and proteins identified by mass spectrometry of tryptic fragments. There was no difference in the identified proteins from AG-1478–treated and nontreated HaCaT cells, which consisted of mainly different keratins and clathrin. The lack of difference in the identity and amount of C1q binding may be caused by exposure of intracellular C1q-binding components that could mask the relevant extracellular C1-binding components. However, in combination with the immunohistochemistry data, this may indicate that the EGFR inhibition did not promote complement activation by increasing C1q binding to the keratinocytes.

To investigate whether proinflammatory stimuli influenced the complement activation observed by EGFR inhibition, primary human keratinocytes were treated for 48 h with supernatant from stimulated PBMC in combination with AG-1478 before incubation with serum. Complement activation was found on keratinocytes stimulated with the combination of PBMC supernatant and AG-1478 judged from C3 and C4 deposition but only after incubation with serum (Fig. 8A). The same result was found when the keratinocyte-produced complement components were allowed to accumulate for 48 h, indicating that the levels of keratinocyte-derived complement alone were too low for detectable complement activation. Stimulation with supernatant from stimulated PBMC alone did not cause complement activation when the keratinocytes were subsequently incubated with serum (Fig. 8A).

When complement activation experiments were performed with C1q-depleted serum, we found C3 deposition but no detectable C4 deposition (Fig. 8B). This was irrespective of whether serum was added in new medium (without secreted complement components) or serum was added to medium in which the keratinocyte-derived complement components had been allowed to accumulate. To rule out that the observed complement activation with C1q-depleted serum was caused by binding of complement components from the PBMC supernatant to the keratinocytes, AG-1478–treated
keratinocytes were incubated with C1q-depleted serum in the presence of the PBMC supernatant. The presence of the PBMC supernatant did not cause complement activation after incubation with C1q-depleted serum (Fig. 8B). These data demonstrated that the complement activation observed in keratinocytes stimulated with a combination PBMC supernatant and AG-1478 was dependent on serum-derived components, but was not strictly dependent on serum-derived C1q.

Because we have previously found that innate immune genes induced during cutaneous wound healing mediated by EGFR activation in humans are independent of EGFR in mice (13), we tested the role of EGFR inhibition for expression of complement components and complement activation in primary murine keratinocytes.

We examined the expression of C1s, factor B, and properdin because these genes were induced by the combination of EGFR inhibitor and PBMC supernatant or EGFR inhibitor alone. The EGFR inhibitor AG-1478 did not enhance the expression of these complement components either alone or in combination with PBMC supernatant (Supplemental Fig. 3A). Because we used a human PBMC supernatant, we tested whether it could induce the expression of antimicrobial protein 24p3 in the murine keratinocytes because 24p3 is induced by proinflammatory cytokines in murine keratinocytes (13). We found that the human PBMC supernatant induced the expression of 24p3 >150-fold in each of three independent experiments, demonstrating that the murine keratinocytes responded to the human cytokines in the PBMC supernatant.
When the murine keratinocytes were incubated with mouse complement serum, no complement deposition was found independently of whether the murine keratinocytes had been treated by the EGFR inhibitor (Supplemental Fig. 3B). As a positive control for complement activation on murine keratinocytes, we did find deposition of murine C4 and C3 on murine keratinocytes treated with cycloheximide following incubation with mouse complement serum (Supplemental Fig. 3B).

**Discussion**

Systemic deficiencies of complement regulatory proteins give rise to tissue-specific diseases, demonstrating that complement activation is regulated locally in the tissues (4). Importantly, the local production of complement components could be an important determinant for complement activation. For example, in the transplanted kidney, local C3 production is important for complement activation, resulting in rejection of kidneys after transplantation (3). This could indicate that local synthesis of complement components and local complement activation may be regulated in parallel. However, studies in the regulation of complement activation have focused on the individual complement regulatory proteins (16) and not on the pathways or stimuli important for regulation of complement activation.

Complement activation is an important mechanism for tissue injury after trauma (17) and, accordingly, of interest in wound healing. During wound healing, complement activation seems beneficial in acute wounds but may be detrimental in chronic wounds (12). This indicates that regulation of complement activation is important for successful wound healing. Because expression of local complement components can be an important

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**FIGURE 6.** TUNEL assay and immunofluorescence microscopy of AG-1478–treated keratinocytes. (A) Human primary keratinocytes were stimulated for 48 h with AG 1478, and apoptosis was assessed by TUNEL staining. A total of 30 μM cycloheximide (CHX) was used as a positive control for apoptosis. Scale bar, 100 μm. Insets show higher magnification (scale bar inside insets = 10 μm). (B) Immunofluorescence microscopy of HaCaT cells using human serum as primary Ab. Scale bar, 100 μm. (C) Immunofluorescence microscopy showed deposition of C3 (green) using an Ab against C3d and C4 (green) using an Ab against C4c on AG-1478–treated keratinocytes after incubation with serum that had been preabsorbed with AG-1478–treated keratinocytes at 4°C for 1 h. Scale bar, 10 μm.
determinant for complement activation, we investigated the epidermal complement component expression in cutaneous wound healing. By microarray we found that the expression of many complement components was induced in the epidermis of skin wounds in vivo day 4 after wounding only to a minor extent. This limited induction of complement components was surprising because proinflammatory cytokines induce expression of complement components and at day 4 after wounding the epidermal gene expression is greatly influenced by the cytokines released from infiltrating inflammatory cells. To delineate the mechanism behind the very limited increase in expression of complement components, we therefore also investigated the epidermal expression of complement components in a model of ex vivo injured skin with intact injury-induced growth factor response but lacking the infiltrating inflammatory cells. In this model, the epidermal expression of complement components was downregulated compared with nonwounded skin. This indicated that the injury-induced growth factor response downregulated complement component expression. EGFR activation is an important growth factor response downregulated complement component expression is greatly influenced by the cytokines released from infiltrating inflammatory cells. To delineate the mechanism behind the very limited increase in expression of complement components, we therefore also investigated the epidermal expression of complement components in a model of ex vivo injured skin with intact injury-induced growth factor response but lacking the infiltrating inflammatory cells. In this model, the epidermal expression of complement components was downregulated compared with nonwounded skin. This indicated that the injury-induced growth factor response downregulated complement component expression. EGFR activation is an important growth factor response downregulated complement component expression is greatly influenced by the cytokines released from infiltrating inflammatory cells. To delineate the mechanism behind the very limited increase in expression of complement components, we therefore also investigated the epidermal expression of complement components in a model of ex vivo injured skin with intact injury-induced growth factor response but lacking the infiltrating inflammatory cells. In this model, the epidermal expression of complement components was downregulated compared with nonwounded skin. This indicated that the injury-induced growth factor response downregulated complement component expression. EGFR activation is an important growth factor response downregulated complement component expression is greatly influenced by the cytokines released from infiltrating inflammatory cells. To delineate the mechanism behind the very limited increase in expression of complement components, we therefore also investigated the epidermal expression of complement components in a model of ex vivo injured skin with intact injury-induced growth factor response but lacking the infiltrating inflammatory cells. In this model, the epidermal expression of complement components was downregulated compared with nonwounded skin. This indicated that the injury-induced growth factor response downregulated complement component expression. EGFR activation is an important growth factor response downregulated complement component expression is greatly influenced by the cytokines released from infiltrating inflammatory cells. To delineate the mechanism behind the very limited increase in expression of complement components, we therefore also investigated the epidermal expression of complement components in a model of ex vivo injured skin with intact injury-induced growth factor response but lacking the infiltrating inflammatory cells. In this model, the epidermal expression of complement components was downregulated compared with nonwounded skin. This indicated that the injury-induced growth factor response downregulated complement component expression. EGFR activation is an important growth factor response downregulated complement component expression is greatly influenced by the cytokines released from infiltrating inflammatory cells.

Because complement component expression in some instances is paralleled by complement activation, we investigated whether EGFR signaling also played a role for complement activation. EGFR is activated through an autocrine mechanism named EGFR transactivation, in which membrane-bound growth factors are cleaved by metalloproteases and subsequently bind and activate EGFR (22). Accordingly, a certain amount of EGFR activation will be present in cell cultures even without exogenous EGF (or other EGFR ligands) in the medium. Consequently, we added EGFR inhibitors to the cell culture medium to unmask the effect of EGFR signaling for complement activation.

Indeed, EGFR inhibition promoted complement activation by the classical pathway mediated by serum-derived C1q. However, we did not observe increased binding of serum-derived C1q to the keratinocytes by immunofluorescence microscopy in our experiments with complement activation after EGFR inhibition. To further characterize whether EGFR inhibition caused increased C1q binding to keratinocytes, we performed subcellular fractionation of HaCaT cells and probed the subcellular fractions for C1q binding. We saw less C1q binding to subcellular fractions from HaCaT cells subjected to EGFR inhibition compared with nontreated controls. Western blots demonstrated that C1q bound to the same band (containing the same proteins) in membrane fractions from both nontreated HaCaT cells and HaCaT cells subjected to EGFR inhibition. In the aggregate, these data indicated that EGFR inhibition did not cause complement activation by promoting C1q binding to the keratinocytes but allowed complement activation to proceed after binding of C1q possibly by altering the expression or activity of complement regulatory proteins in the keratinocytes. Apoptosis promotes C1q binding to keratinocytes (23); however, this does not lead to complement activation and deposition of the TCC. We did not find that the cells displaying complement activation, including the deposition of the TCC promoted by EGFR inhibition, were apoptotic. This demonstrates that the complement activation found after EGFR inhibition was unrelated to apoptosis.

However, although the combination of proinflammatory stimuli and EGFR inhibition did not greatly enhance complement activation compared with EGFR inhibition alone, it was no longer strictly dependent on serum-derived C1q because complement activation was found with C1q-depleted serum. However, in this instance, only deposition of C3 was found but no C4 deposition. This indicates that, in the absence of serum-derived C1q, the observed complement activation could be dependent on the alternative pathway either for initiation or amplification of the observed complement activation or by the mannan-binding lectin pathway bypassing C4 (24).

To study the relevance of our findings in cell cultures and in ex vivo injured skin, it would be relevant to look at mice models. However, we have previously found that EGFR activation does

**FIGURE 7.** Binding of C1q to subcellular fractions of HaCaT cells. (A) HaCaT cells were treated with the EGFR inhibitor AG-1478 for 48 h or left nontreated. The HaCaT cells were subsequently subjected to subcellular fractionation. Various amounts of subcellular fraction were applied to PVDF membranes, which were probed with 125I-labeled C1q. (B) The membrane fraction from AG-1478-treated HaCaT cells was electrophoresed on SDS gradient gel, blotted to PVDF membrane, and probed with 125I-labeled C1q. One major band was seen to bind C1q. The same band was found in the membrane fraction from nontreated HaCaT cells.
not play the same role in skin innate immunity in mice as in humans (13). Consequently, we looked at complement component expression and complement activation in primary murine keratinocytes. Contrary to the situation in human keratinocytes, EGFR inhibition did not influence complement component expression alone or complement activation either alone or in combination with the proinflammatory stimuli in murine keratinocytes. Consequently, mice are not suitable to study the role of EGFR for regulation of the complement system in the epidermis.

In conclusion, we have identified a novel role of EGFR for regulation of both expression of complement components and complement activation in the human epidermis. Accordingly, the level for epidermal EGFR activation could be important for the regulation of the complement system in the epidermis during wound healing. To our knowledge, these findings identify for the first time a pathway important for the epidermal regulation of complement activation.

Acknowledgments
The outstanding technical assistance of Malgorzata Berlikowski and Ingbritt Gustafsson is greatly appreciated.

Disclosures
The authors have no financial conflicts of interest.
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