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# Rules of engagement - Regulation of complement response in tissue

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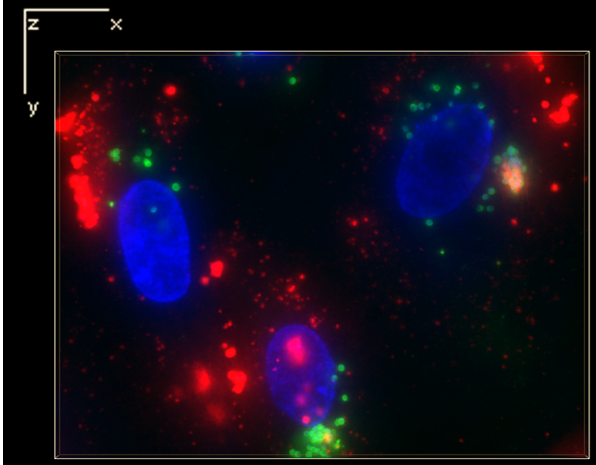
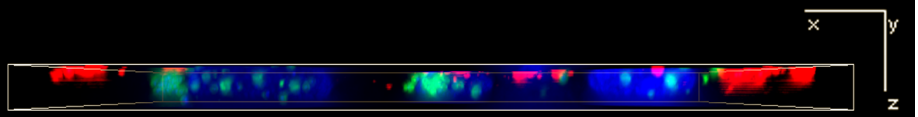
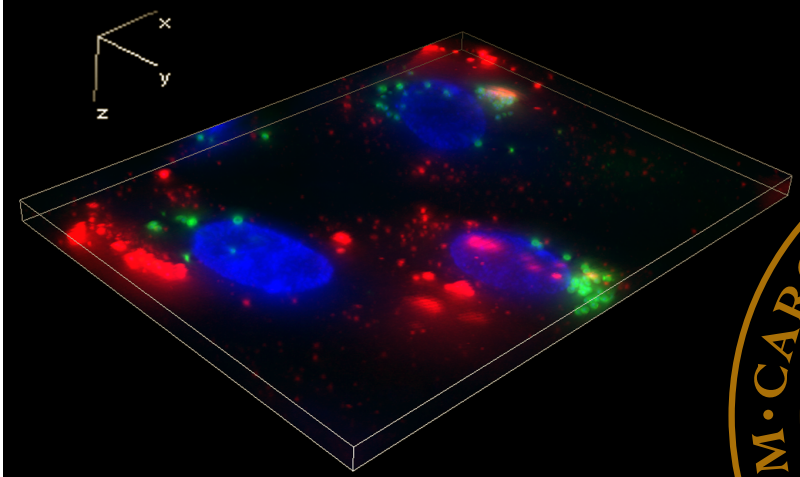


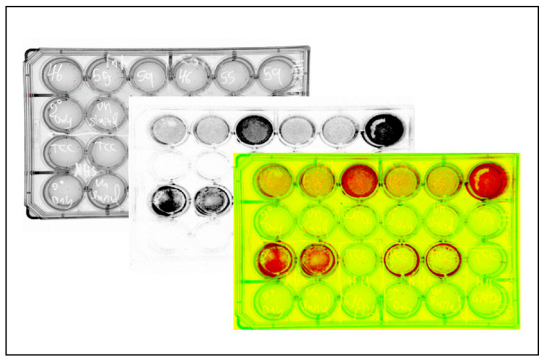

# Rules of engagement

## Regulation of complement response in tissue

ANAS ABU-HUMAIDAN
DEPARTMENT OF CLINICAL SCIENCES | FACULTY OF MEDICINE | LUND UNIVERSITY



Always in motion and probing for danger, complement proteins are found in every space and notch of the body. Their omnipresence combined with an ability to wreak havoc when activated, mandates a strong leash! The how, when, and where to unleash or constrain the complement response remain partly answered questions, despite the significant progress made in the field in the past 100 years.

The work in this thesis aims to answer some of these questions with models that compare health and disease states, using methods that investigate complement response in each. The investigation often followed the lines of queries like: Is complement response relevant to this disease state? Is complement activated or its expression induced? If so, through what mechanisms? And what local effect could the activation or induced expression have?

I hope this book provides some insight into novel mechanisms of local complement regulation. And maybe spark the interest of scientists from outside the field in this exciting and constantly evolving research area.

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# Rules of engagement

## Regulation of complement response in tissue

Anas Abu-Humaidan


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*Faculty opponent*
Professor Tom Eirik Molness

Complement research group, Institute of Clinical Medicine,

Faculty of Medicine, Oslo University

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Abstract

Always in motion and probing for danger, complement proteins are found in every space and notch of the body. Their omnipresence combined with an ability to wreak havoc when activated, mandates a strong leash! The how, when, and where to unleash or constrain the complement response remain partly answered questions, despite the significant progress made in the field in the past 100 years.

The work in this thesis aims to answer some of these questions with models that compare healthy and disease states, using methods that investigate complement response in each. The investigation often followed the lines of queries like: Is complement relevant to this disease state? Is complement activated or its expression induced? If so, through what mechanisms? And what local effect could the activation or induced expression have?

Chapter 1 provides an introduction to the complement system that tackles specific topics like complement's discovery, evolution, function and role in disease. As well as challenges and progress made in complement targeted therapies. Chapter 2 discusses methods and models used in this thesis and in complement research in general. While chapter 3 focuses on the present investigation and where it falls within current knowledge about the local regulation of complement.



Key words Complement system, complement activation, complement regulation, Terminal complement complex, TCC, innate immunity, EGFR, intracellular infection, Staphylococcus aureus, skin, epidermis, HNSCC.

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# Rules of engagement

## Regulation of complement response in tissue

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Cover photo: Immunofluorescence microscopy images of keratinocytes (nuclei in *blue*) infected with intracellular *Staphylococcus aureus* (*green*) with the terminal complement complex deposition (*red*) on the surface. Images were taken at different focal planes, rendered and visualized as a 3-D picture from different angles. Taken by Anas Abu-Humaidan.

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*Dedicated to my mother and father*

# Table of Contents

# Preface

I read the first comprehensive review about the complement system around 5 years ago, I previously had little knowledge of the details, complexity and multi-functionality of complement, which I grew fond of with time. I believe that complement has been overlooked both in research and teaching in the past, but with its increasing clinical relevance, complement is bound to take center stage in immunology textbooks in the future.

Although my time in science has been brief, I believe I have found in complement a life long research interest. Indeed, some researchers spent their whole careers investigating where one small piece fits in the greater complement puzzle.

Whether I continue in complement research or not is for time to tell, regardless, I had great fun writing this book, and I hope my fascination in complement translates in these pages to an easy-to-read and useful knowledge.

*Anas Abu-Humaidan*

April 2018, Lund

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I appreciate the effort done behind the scenes to make our lives easier, starting with B14 very own Anita Berglund. Also, Annette Saltin and Karin Frydenlund.

Some big thanks to our "fika table" active and non-active members for providing the best work environment and interesting conversations. Especially Mohammad (the "other" Arab guy), Jana (good neighbor and party arranger), Marta (and the spooner gang, Ida and Madlen), Malin (for the best Swedish experiences), Sandra P, Jesper, Amanda, Nicklas, Eleni, Frida, Finja, Torgny, Emanuel. And all the awesome people I had the pleasure of sharing a cup of coffee with (I know I forgot some).

Not to forget the people who moved from the BMC and left a pile of theses on my desk. Anele (let's get lunch habib), Johannes (best of luck in Toronto), Sandra J, Sinead, Azadeh, Suado, Jonathan. And to the people I only knew as postdocs, when will you move on?! Lech, Clement and Wael (it's gonna be great!)

Finally, I would like to acknowledge people from outside of work who made me feel less home sick, either through text or in person. Zaytona (you made this work, and life, better), Nader (good falafel all over Lund), Ahmad, Malik. And of course, Osama, Saad, Ali, Baha, Sary (kobbarat who made the effort to visit me in Lund).

# List of papers by the author

Included in this thesis:

**Abu-Humaidan AH***, Ananthoju N*, Mohanty T, Sonesson A, Alberius P, Schmidtchen A, Garred P, Sørensen OE.

*The epidermal growth factor receptor is a regulator of epidermal complement component expression and complement activation*. **The Journal of Immunology** 2014; 192:3355-3364. * Contributed equally.

**Abu-Humaidan AH,** Lars Ekblad, Johan Wennerberg, Ole E. Sørensen.

*EGFR modulates complement activation in head and neck squamous cell carcinoma cell lines.* (**Manuscript**)

**Abu-Humaidan AH,** Malin Elvén, Andreas Sonesson, Peter Garred and Ole E. Sørensen.

*Persistent Intracellular Staphylococcus aureus in Keratinocytes Lead to Activation of the Complement System with Subsequent Reduction in the Intracellular Bacterial Load.* **Frontiers in Immunology** 2018; 9:396

Jane Fisher, Ole E. Sørensen, **Abu-Humaidan AH**.

*A simple and sensitive immunoassay for comparative protein quantification in cells.* (**Manuscript**)

Not included in this thesis:

Mohanty T, Sjögren J, Kahn F, **Abu-Humaidan AH**, Fisker N, Assing K, Mörgelin M, Bengtsson AA, Borregaard N, Sørensen OE.

*A novel mechanism for NETosis provides antimicrobial defense at the oral mucosa*. **Blood**. 2015

# Chapter 1: An introduction to the complement system

Always in motion and probing for danger, complement proteins are found in every space and notch of the body. Their omnipresence combined with an ability to wreak havoc when activated, mandates a strong leash! The how, when, and where to unleash or constrain the complement response remain partly answered questions, despite the significant progress made in the field in the past 100 years.

The work in this thesis aims to answer some of these questions with models that compare healthy and disease states, using methods that investigate complement response in each. The investigation often followed the lines of queries like: Is complement relevant to this disease state? Is complement activated or its expression induced? If so, through what mechanisms? And what local effect could the activation or induced expression have?

To put the complement system in a bigger picture is to miss out on details that make this complex system incredibly intriguing. So this introduction will tackle specificities that will hopefully be of interest to researchers in the complement field as well as the general reader. Topics will include complement's discovery, evolution, function and role in disease. As well as challenges and progress made in complement targeted therapies.

Before delving into those topics, an illustration of complement activation pathways and the most important components in each, can provide a reference point for the reader to return to when those pathways and components are mentioned later in the work (figure 1).

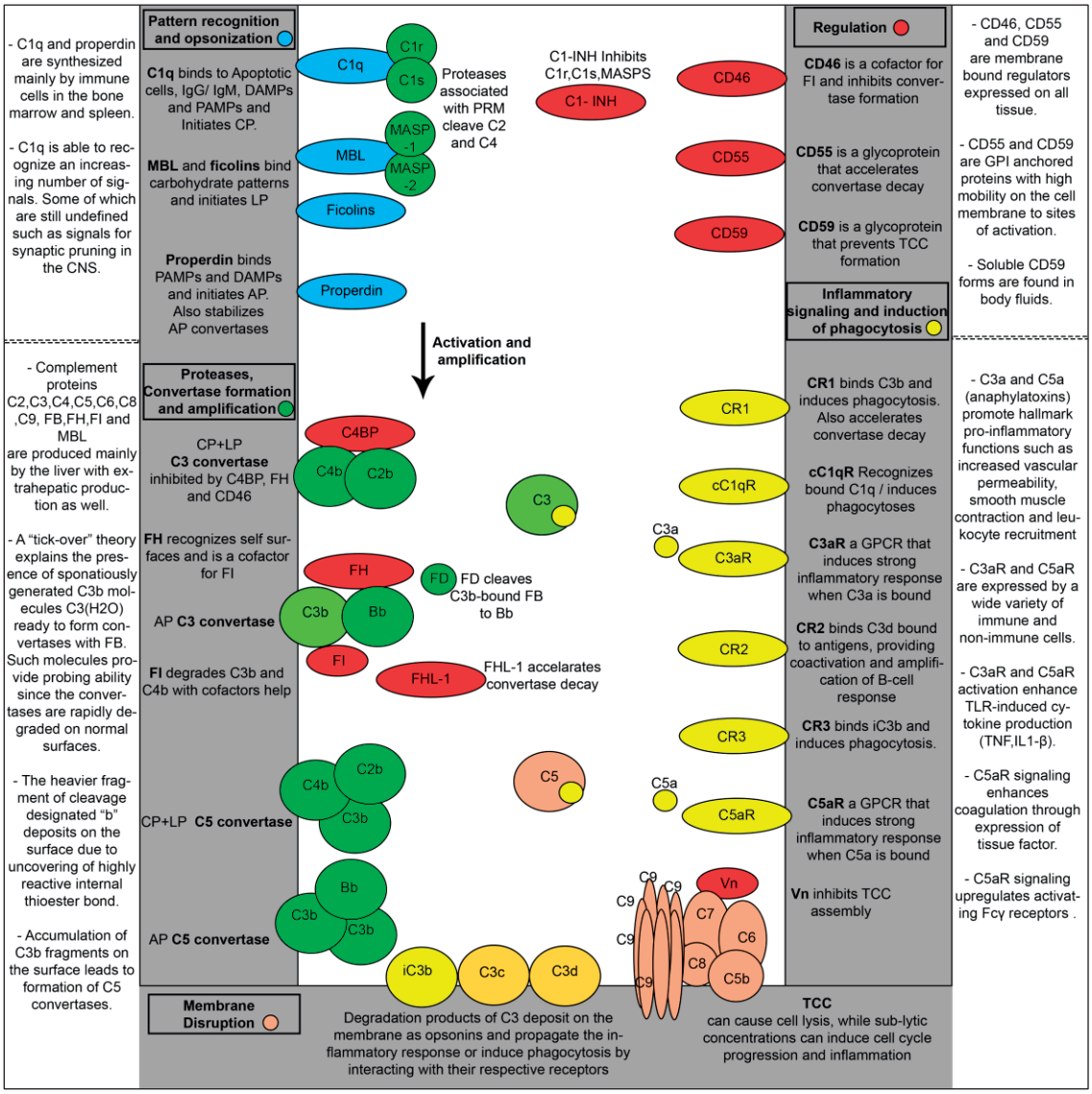



**Figure 1. The complement system, no easy way to put it.** The figure illustrates through color coding the major interactions of the complement cascade, with relevant notes in the margins. (*Grey*) represents any activation surface. The three main pathways Classical (CP), lectin (LP) and alternative (AP) start by pattern recognition (*blue*). This sets in motion a series of proteolytic reactions by proteases and convertases (*green*). If the amplification loop continues, a complex of C5b-8 forms a scaffold for insertion of multiple C9 and formation of the terminal complement complex (TCC), which disrupts lipid bilayers along with other deposited fragments (*cantaloupe*). Released active fragments, and other complement receptors (*yellow*) form an important bridge with adaptive immunity and the coagulation pathway, as well as propagating innate immune responses. Regulation of activation takes place at several steps of the cascade through cell bound and soluble inhibitors (*red*). Note that many of the molecules serve more than one function. D/PAMP (Damage/Pathogen associated molecular patterns), FI (Factor I), FH (Factor H), Vn (vitronectin), GPCR (G-protein-coupled receptor), GPI (glycophosphatidylinositol) *Figure created by author*.

# Unraveling complexity brings controversy - a historical perspective

In Skarnes and Watson's review from 1957 titled "Antimicrobial factors of normal tissue and fluids" [1], one can observe the ambiguity that shrouded humoral immunity at the time, for example, some characteristics used in the review to group antimicrobial factors in tissue fluids were: activity against gram-positive and gram-negative bacteria, tissue of origin and heat stability.

Such simple characteristics were helpful in classifying an increasingly complex profile of antimicrobial serum components. Indeed, one of the earlier observations by Buchner in 1891 was describing a bactericidal activity of serum that was inactivated by heat, he termed this activity *Alexin* (from Greek alexein meaning to ward off) [2]. In 1906, Paul Ehrlich and Jules Bordet further extended the description of this heat-labile activity by showing that it required an additional heat-stable activity (antibodies) [2]. The heat labile activity was termed *Complement* by Ehrlich, since it complemented the killing and phagocytosis of pathogens.

Other important observations by the noble prize winner (often considered the discoverer of complement) Jules Bordet in the early 1900s, is showing that red blood cells could be lysed in a similar way to bacteria. "Hemolysis" as he termed it, required specific antibodies, and this specificity is essential for complement activity to take place. Thus proving that complement is an actual substance and not just an activity of serum as was thought at the time. Bordet's findings were instrumental in the foundation of serology, which aims to describe the immune properties of serum components.

Discovery of the classical pathway came years later, mainly due to advancements in molecular techniques that allowed for purification of individual complement components [3, 4]. By adding purified complement proteins to sensitized sheep erythrocytes, it was possible to study the sequential activation of C4, C2 and later C3 following attachment of C1q to antibodies [4-6]. It is worth mentioning here that names assigned to complement components have changed several times through history [7], although it was first done in the order of their discovery [2]. This is reflected by the name of component C4, which counter intuitively precedes components C2 and C3 in the classical pathway activation cascade.

**The Nobel Prize in Physiology or Medicine 1919 was awarded to Jules Bordet "for his discoveries relating to immunity".**

On the other hand, discovery of the alternative pathway (then called the properdin system), which was described in a study led by Pillemer and published in *Science* some years later (figure 2) [8] involved more controversy [9]. Pillemer noticed that zymosan (a component of yeast cell wall) inactivated serum C3 even in the absence of antibodies, furthermore, he noticed that it was not a simple adsorption to zymosan since it needed specific pH and temperature akin to an enzymatic reaction. Those observations among others (figure 2) made him hypothesize the presence of a substance (properdin) that drives the enzymatic cleavage of C3 without the need for antibodies. This shows that mechanisms governing even the most complex systems like complement could be predicted and confirmed, using simple methods and ingenious deduction.

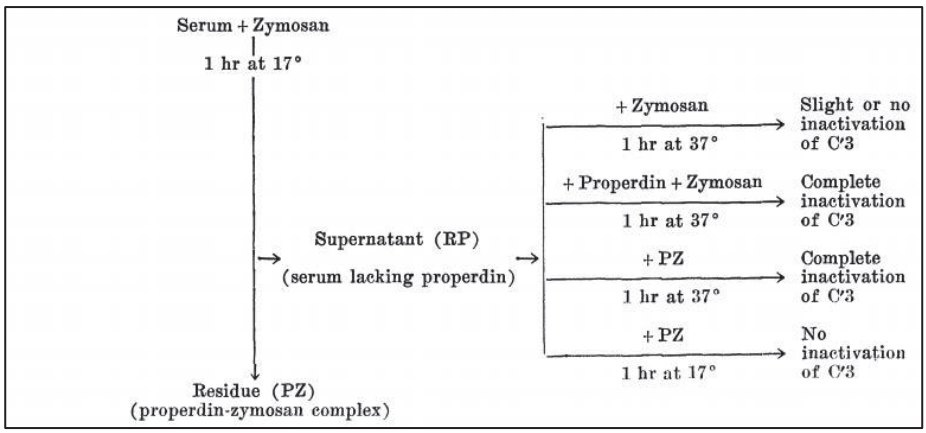



**Figure 2. A reproduced figure from the original study describing Properdin.** Several lines of evidence suggest that a complex between zymosan and a substance in serum is formed, and that the complex can inactivate C3 only in certain temperatures. This substance was named properdin and is now considered an important player in alternative pathway activation.

The evidence Pillemer provided did not go down well with his critics who claimed that the observed C3 depletion was driven by -unaccounted for- natural antibodies that activate complement and deplete C3 [10]. In the late 60s, a few years after Pillemer's death, experiments from researchers outside the controversy supported the presence of properdin. In addition, the theory was accepted by complement authorities of the time like Mayer, whose comment on the issue [9] (presented below) is relevant to complement research to this day. Its note worthy that some controversy regarding properdin's function in pattern recognition still exists [11-13].

*". . . there was controversy ... long before the discovery of properdin . . . Heidelbergern group challenged the work of Ecker and Pillemer during the 1940s for reasons which derive from differences in experimental approach. In Heidelbergern view, evidence presented by the Cleveland group was "soft." ... The difficulties in their work were inherent in the nature of the problem and the methods then available. ... This is why I turned away from this methodology around 1946 and began to develop new methods . . . for measurement of . . . complement components* " … Manfred Mayer, 1974

The discoveries of the classical and alternative pathways and later on the lectin pathway [14], laid the ground work for future research in the complement field. The field has since flourished and is now integrated with other fields like cancer research, neuroscience, and metabolism as discussed later in this chapter.

New pathways and regulatory mechanisms of complement are still being unravelled. Despite the recent paradigm shift viewing the complement system as important for tissue homeostasis and not just innate immunity has gained acceptance [15-18], the methods and models used to recapitulate the *in-vivo* effector and regulatory mechanisms of complement are challenging. This might lead to contradicting results from different research groups, in large due to the complex interactions inherent to the system itself.

# From sea urchins to humans, half a billion years strong - an evolutionary perspective

Research into the evolutionary history of complement provides insight into the function of this ancient and conserved system. Studies investigating the evolution of complement suggest the presence of a primitive predecessor to the complement system in vertebrates [19], one that is made of a few proteins and can carry out simple yet vital functions (figure 3). In a recent review on complement evolution [15], the authors even hypothesize the primitive complement system to have first appeared around a billion years ago in the form of an intracellular C3-like protein in unicellular organisms. They base their hypothesis on: the presence of a C3 homologue in ancient creatures like sea urchins [20] and the horseshoe crab [21], the recent finding of functional intracellular C3 stores as well as C3 receptors [18], and the cleavage of C3 by ancient and conserved enzymes outside of the complement pathway like cathepsins [22].

C3 sits at the centre of the complement system, and is a junction point of the 3 pathways. C3 is thought to have evolved from gene duplication of the structurally close relative alpha 2-macroglobulin [23], both of which are part of a family of thioester containing proteins that includes C4 and C5 as well [24]. Similarities in genes encoding for several complement proteins like the thioester containing family suggest that gene duplication and subsequent divergence of function is a common mechanism for the emergence of new proteins in the cascade [19, 25].

The most primitive complement “cascade” made of C3, Factor B and lectins resembling the alternative and lectin pathways in mammals, is found in early multicellular organisms (figure 3) [26], and could have functioned in tagging intruders before entry into the cellular compartment, as well as tagging damaged neighbouring cells. The evolution to a self-propagating pathway would necessitate the presence of regulators, indeed, some complement regulatory proteins have been found in the earliest vertebrates [27].

Complement evolution and discovery have opposing timelines, with the alternative and lectin pathways coming to the scene millions of years ahead of the classical pathway. The finding of an orthologue of mammalian C1q functioning in a similar fashion to the more ancient lectins in Lamprey (the most primitive vertebrate that lacks immunoglobulins) [28], in addition to structural similarities and sequence of activation of the MBL-MASP complex to the C1 complex , could suggest that the lectin pathway is the evolutionary predecessor of the classical pathway [28, 29].

Further ahead in the evolutionary timeline, higher vertebrates share a very similar complement system, with mammals, aves, and amphibia having an almost complete set of complement genes [30].

Taken together, evolutionary evidence suggests that complement's essential functions like tagging of non-self to facilitate phagocytosis or propagate inflammation, for which the complement system was preserved through millions of years, could be performed by a few molecules like C3 and lectins. Yet new functions like clearance of apoptotic cells, and pore formation emerged with later molecules like C1q and the terminal pathway components respectively.

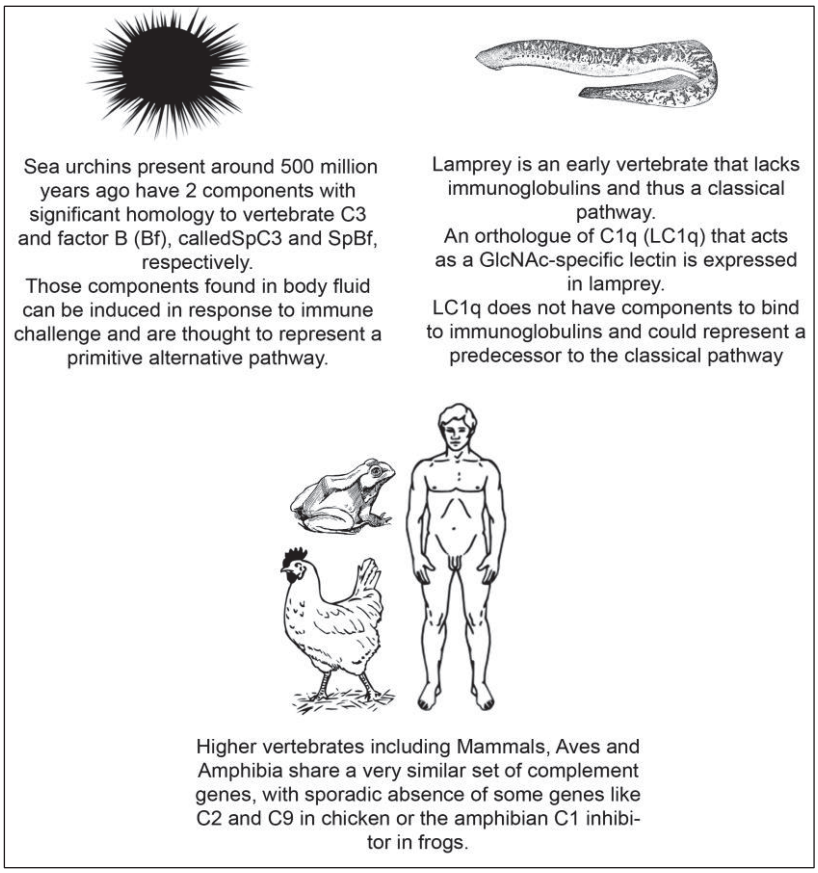



**Figure 3. Ancient origins and conservation of the complement system.** DNA analysis reveals the ancient origins of complement and proposes a primitive complement system from which the mammals complement system has evolved. *Figure created by author using illustrations in the public domain.*

# A jack of all trades, a master of many - a functional perspective

A multitude of functions are ascribed to the complement system [2, 16, 31-34]. Most notable and extensively described is complement's role in innate immunity. Through detection of pathogens by pattern recognition molecules [29, 34-37], and subsequent opsonization or possible lysis of the pathogen [6, 38-41], as well as chemotaxis of immune cells [42-44], complement can help clear microbial intruders. This is further supported by the predictable increased susceptibility to specific infections caused by complement deficiencies [45-47] (figure 4).

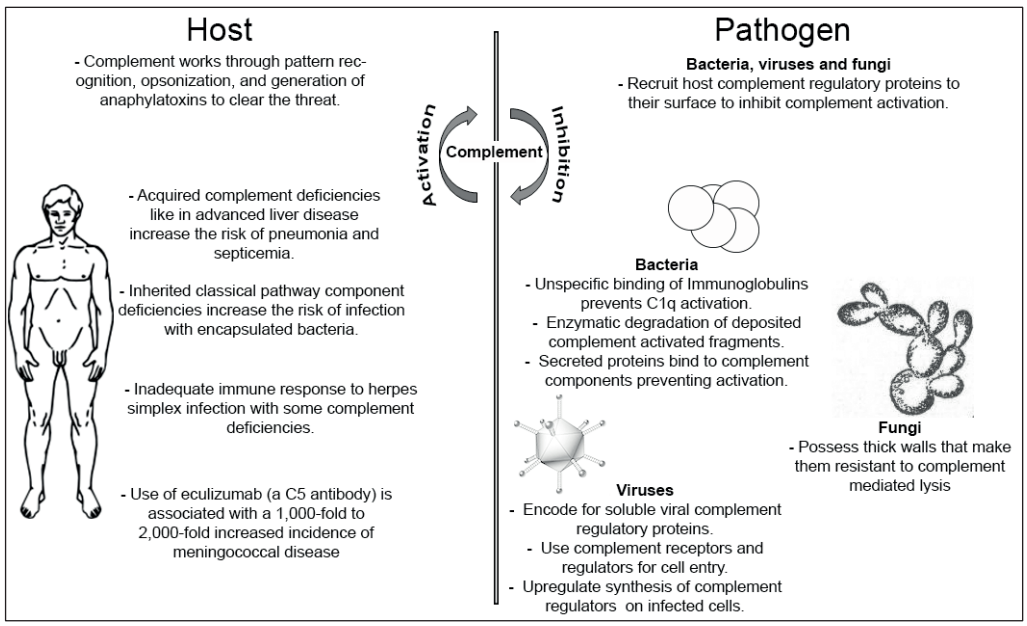



**Figure 4. The complement system in innate immunity, a tug of war.** Several examples highlight the importance of complement in host-pathogen interactions. Since complement activation is vital to clearing pathogens, many pathogens were able to subvert this ancient defence mechanism through co-evolution. *Figure created by author using illustrations in the public domain.*

The role of complement in innate immunity can also be highlighted when seen from the microbe point of view. Through millions of years of co-evolution, many microbial intruders including bacteria [48, 49], viruses [50] and fungi [51] have developed mechanisms to evade complement attack (figure 4).

Newly discovered functions of the complement system have put it in a different light, the functions are so wide spread and varied that complement is described now as important for general homeostasis [16]. A similar scheme in which complement detects the target then alarms and orchestrates immunity with a regulated activation of its cascade, can be extended into other scenarios. For example, complement helps in tagging and removing apoptotic cells [31, 52], pruning of neurons during development [53] and regeneration of tissue after injury [54].

Accordingly, an aberrant complement response is associated with a wide variety of diseases with an inflammatory component (Figure 5) like age-related macular degeneration (AMD) [55], inflammatory bowel diseases [56, 57], rheumatoid arthritis [58, 59], ischemia-reperfusion injury [60, 61], several epithelial cancers [33, 62-64] among others [65-68]. Moreover, complement deficiency of C1q is not only associated with increased risk of infections as mentioned above, but is also known for its relation to the prototypical autoimmune disease systemic lupus erythematosus (SLE) [46, 69, 70], probably due to abnormal handling of apoptotic cells [69, 71-73] further emphasizing the important role complement plays outside of innate immunity.

A more straightforward role of excessive complement activation is found in pathologies like paroxysmal nocturnal haematuria (PNH) [74, 75] and atypical haemolytic uremic syndrome (aHUS) [76, 77]. In PNH red blood cells (RBCs) deficient in the complement regulators CD55 and CD59, are unable to modulate complement activation on their surface leading to chronic intravascular haemolysis [74, 75, 78]. While aHUS is a more heterogenous disease, usually manifesting with haemolytic anemia and a low platelet count [79]. Majority of cases have mutations in complement regulatory proteins like Factor H or complement activators like Factor B [77, 80].

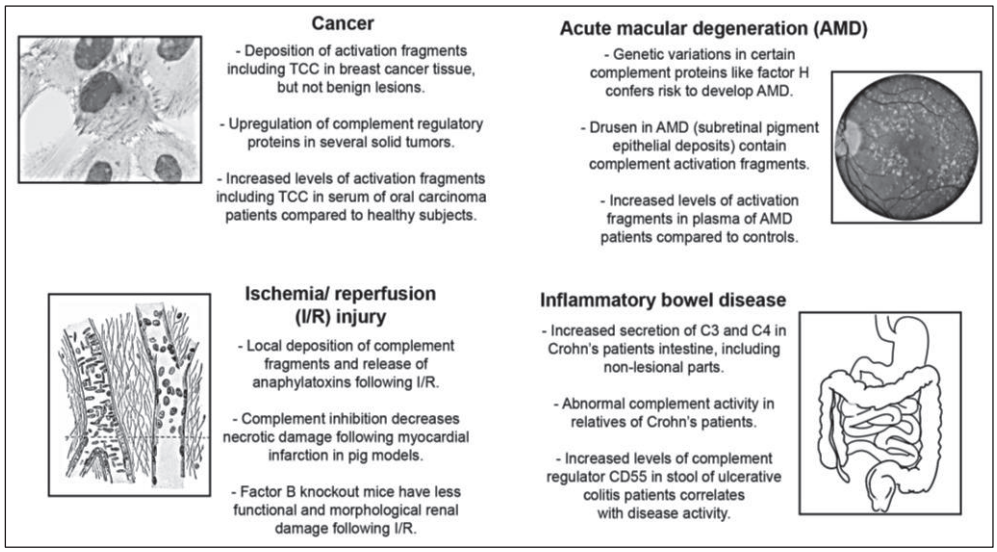



**Figure 5. Complement involvement in inflammation across-the-board.** Evidence for involvement of complement in several diseases often includes local as well as systemic complement activation, raising the questions whether systemic activation drives disease locally or the other way around? A question that needs to be answered distinctly for each condition. *Figure created by author using illustrations in the public domain.*

With its newly assigned functions complement has been "rediscovered", to illustrate this I used the citation report tool from web of science (Clarivate analytics). This tool can be used to generate a graph depicting the total number of times all *records* have been cited over the past 20 years, the *records* are generated by searching for specific *topics.* For example, a citation report generated from a search of *topics* "C1q" + "infection" is probably reflective of the interest in complement's role in innate immunity, "C1q" was used instead of "complement" to increase the specificity of search results. While a citation report generated from search for "C1q" + "cancer" reflects the interest in complement's role in cancer research and so on (figure 6).

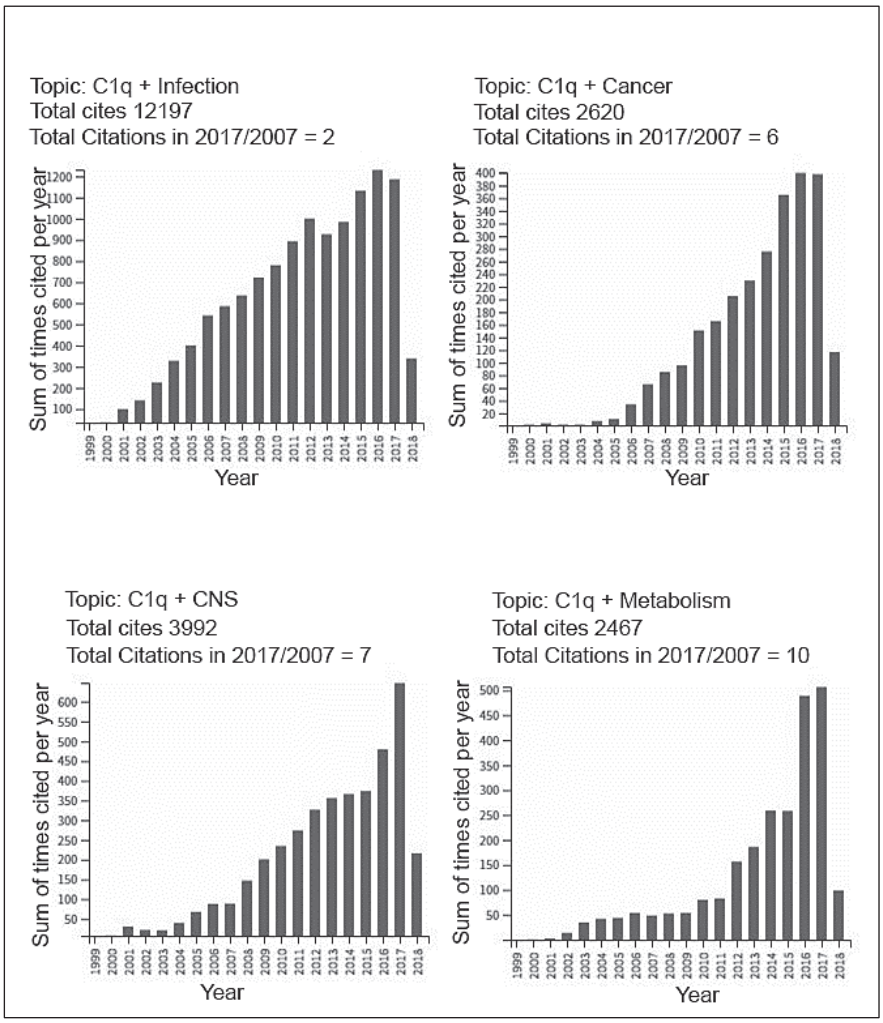


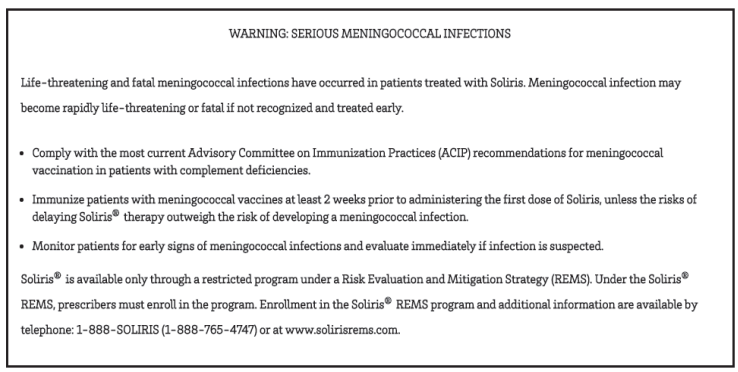

**Figure 6. Rediscovering complement in the past 20 years.** Graphs representing total citations per year of literature containing searched topics, generated from a Web of Science search in April 2018. The graphs show an expected trend of increased citation of literature over the years, yet this increase is more notable when search topics contain newly discovered functions of complement, reflecting a growing interest in such fields. *Figure created by author.*

# Tailoring the therapy, when and what to target? - a translational perspective

A thorough understanding of complement regulation and interactions in health and disease is central in moving complement knowledge to the clinic. Many difficulties arise when targeting a complex biological system such as complement. For example, the extensive crosstalk with other systems like the coagulation and kinin systems would provide an extra level of complexity to consider when targeting complement [29, 81-83]. In addition, the uncertainty of whether complement activation represents an epiphenomenon or a main driving factor in the disease process [55, 84-86], would also discourage the development of drugs that will -most probably- inadvertently suppress complement immunity against infections .

Another aspect to consider when targeting complement is the cascade type of activation, since inhibition of activation at one point (the choice of which is problematic on its own) can lead to undesired downstream interactions. For example, the anti C5 antibody (eculizumab) used for treatment of PNH [78], aims to inhibit the formation of TCC on RBCs that are deficient in the complement regulator CD59. But at the same time eculizumab inhibits the formation of C5a thus probably contributing to the 1000-2000 times increased risk of meningococcal infections in patients undergoing treatment [87, 88]. Upstream events of the cascade should also be considered, since complement activation can happen through more than one pathway concurrently [85, 89].

Finally, the large size of complement proteins [90, 91] and their high abundance in serum [92], could limit the effectiveness of newer drugs that inhibit protein-protein interactions using small molecules [93-95].



WARNING: SERIOUS MENINGOCOCCAL INFECTIONS

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early.

- Comply with the most current Advisory Committee on Immunization Practices (ACIP) recommendations for meningococcal vaccination in patients with complement deficiencies.
- Immunize patients with meningococcal vaccines at least 2 weeks prior to administering the first dose of Soliris, unless the risks of delaying Soliris® therapy outweigh the risk of developing a meningococcal infection.
- Monitor patients for early signs of meningococcal infections and evaluate immediately if infection is suspected.

Soliris® is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS). Under the Soliris® REMS, prescribers must enroll in the program. Enrollment in the Soliris® REMS program and additional information are available by telephone: 1-888-SOLIRIS (1-888-765-4747) or at www.solirisrems.com.

**Black box warning for eculizumab (Soliris®)**

Despite the disheartening obstacles faced in designing complement therapy, there seems to be light at the end of the tunnel [96], and the first complement specific drug eculizumab has made it to the clinic in 2007. Eculizumab was first approved for the treatment of PNH but has since extended its indications to aHUS and generalized myasthenia gravis.

Two important lessons can be drawn from eculizumab treatment in the past decade. First, the risk of serious infections should be addressed in complement therapy, either through targeting pathways that have a less substantial effect on pathogen clearance, or through local administration of the drug (if appropriate for the disease) to decrease systemic effects. Second, the growing list of indications for eculizumab therapy demonstrates versatility of use and promise for the various complement inhibitors currently under development.

Innovation is an important aspect of complement drug development (figure 7), some innovative uses of inhibitors under study include targeted delivery [97, 98], combination therapy [17, 99] and biomaterial coating [100, 101].

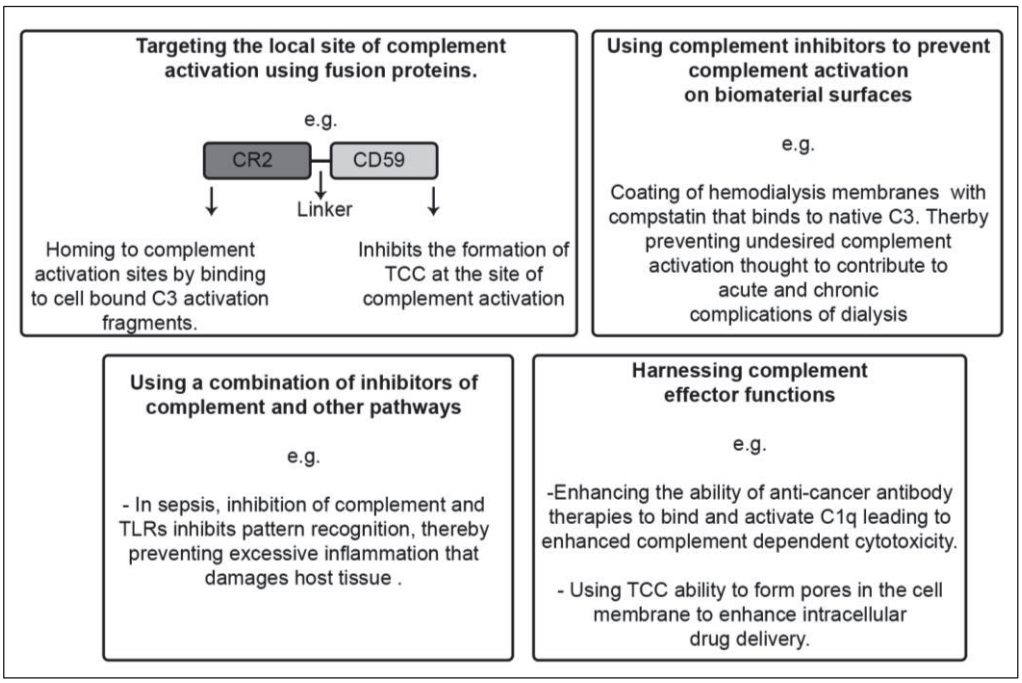



**Figure 7. Innovation in complement therapeutics**. Immunomodulation of the complement system doesn't stop at the use of systemic inhibitors. Several innovative uses are currently under investigation. *Figure created by author*

# Chapter 2: Methodology in complement research

As alluded to in the previous chapter, the complement system has a complex network of interactions in tissue, and through balancing numerous variables complement exerts its function in homeostasis (figure 8). Hence models that investigate complement interactions and complement role in disease cannot account for the entirety of the interactions, nevertheless, these models are vital to analyze this complex network.

This chapter will discuss models and methods used to study the complement system in this thesis as well as in general literature.

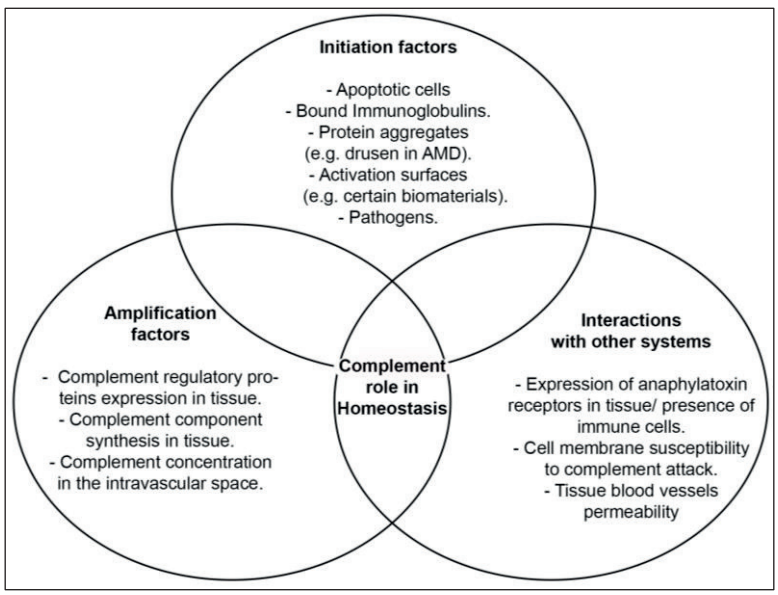
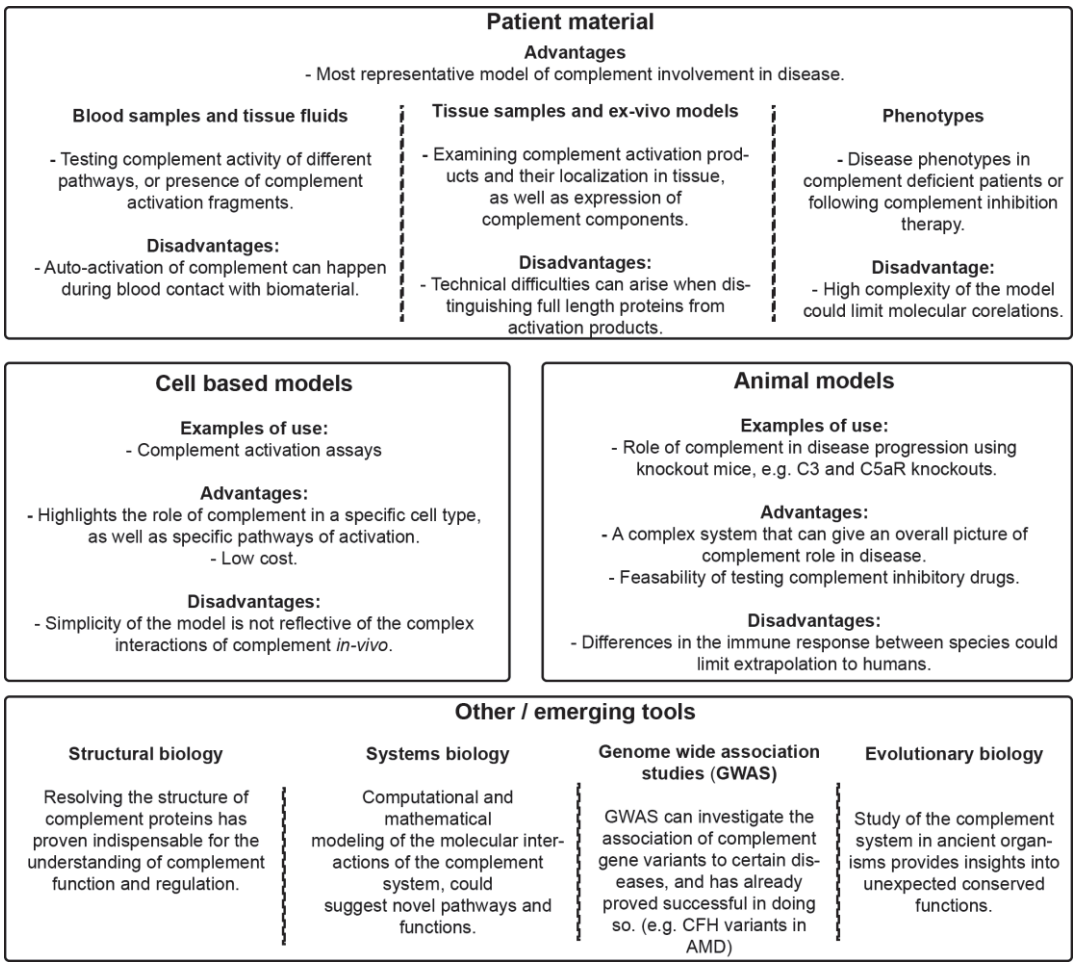



**Figure 8. Complement role in homeostasis, a complicated relationship.** This diagram lists examples of the variables that interact and overlap in a precise and dynamic manner with each other and with the complement system to maintain homeostasis in tissue. Complement role in disease is due to disturbance of this delicate balance, for example, an overactivated complement system can cause direct damage to the tissue, while under activation can predispose to infections and autoimmunity. *Figure created by author.*

# Addressing complexity – models and methods to study local and systemic complement response

The models and methods used to study complement response vary according to the question being investigated [102, 103]. A combination of tools that tackle the question from different angles would be optimal for understanding the role of complement in disease. The boxes below represent some of the more commonly used tools when investigating the role of complement in health and disease.



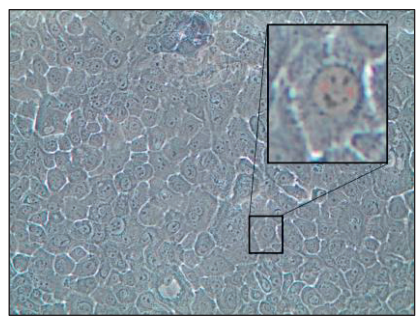
For example, if the involvement of complement in a disease like atopic dermatitis (AD, a chronic inflammatory skin disease) is to be investigated, a simple workflow can be drawn out. Firstly, a good start would be to investigate the deposition of complement activation fragments in lesional vs non-lesional skin, or skin from AD patients compared to healthy subjects. Serum levels of activation fragments can be compared to healthy subjects or correlated to disease flares as well.

Secondly, if increased activation is found the skin lesions, cell-based models can further elucidate the relation. By stimulating primary keratinocytes to mimic the inflammatory and microbial environment in AD then performing complement activation assays, the complement pathway responsible for the activation can be addressed using sera depleted of initiators of each pathway. And a target responsible for the activation can be investigated as well. Thirdly, complement inhibition therapy whether local or systemic can be investigated in animal models of AD, thereby adding another level of evidence to the investigation at hand. Finally, GWAS data bases can be mined to discover a relationship between variations in a complement regulatory protein for example and AD.

A workflow for the investigation of complement's role in a given disease can be prepared in a similar fashion to the example above, with relevant models and methods for the disease investigated.

In most cases, investigating complement effector functions rather than regulation proves more challenging, since effector functions often intersect with other pathways related to immunity or growth [62, 104-106]. For example, we commonly encountered a role for the extracellular regulated kinases (ERK) 1/2 in mediating complement effector functions, as found in paper 2 and 3 in this thesis. Yet further elucidation of downstream effects of ERK activation (paper 2), or upstream effects that led to ERK activation (paper 3) was troublesome.

Other methodological challenges faced when investigating complement activation in cell-based models, is to verify that the cells are not undergoing apoptosis or necrosis, since both processes can induce complement activation [31, 72, 107] and confound the results.



**In our cell-based models, we tried to keep the cells alive and happy, as seen in the above "unedited" microscope picture.**

# Models and methods used in this thesis

**In paper 1**, in efforts to understand the role of EGFR in complement regulation, we used the following models and methods:

- Skin wounds *in-vivo* and *ex-vivo* (figure 9), in comparison to uninjured skin. Those models highlight the role of EGFR activation alone or in combination with proinflammatory stimuli. These models were studied using cDNA microarrays to investigate complement component expression.
- *Ex-vivo* skin, primary keratinocytes, and HaCaT (immortalized keratinocyte cell line) treated with EGFR inhibitors Cetuximab (an EGFR antibody) and AG1478 (a tyrosine kinase inhibitor), in combination with proinflammatory stimuli (prepared from the supernatant of M1 or LPS stimulated PBMC). These models provide data on the role of EGFR activation alone or in combination with proinflammatory stimuli to mimic the wound environment.
  These models were studied using qPCR to compare complement component mRNA, and immunofluorescence microscopy (IFM) and Western blotting of the medium (WB) to investigate complement protein expression.
- Complement activation models. In which primary keratinocytes and HaCat cells following treatment with EGFR inhibitors, are incubated with normal human serum (NHS), heat inactivated serum (HIS), or sera depleted and replenished with essential complement components like C1q and Factor B. The activation of complement, and the pathway important for the activation is then assessed using IFM, by detecting activation products (C3d, C4c or TCC) deposited on the cell monolayers.

**In paper 2,** the important role of EGFR in growth of epithelial cancers and its inhibition as cancer treatment [108-110], among other reasons [64, 111, 112] (figure 9), prompted us to investigate its complement modulatory effects in cancer. To this end, we used the following models and methods:

- Patient-derived cell lines of head and neck squamous cell carcinomas (HNSCC) which overexpress EGFR [108, 113, 114]. The cell lines had different sensitivities to EGFR inhibition treatment. EGFR was inhibited with Iressa (a tyrosine kinase inhibitor), or activated with TGF-α. The expression of complement components and complement regulatory proteins was studied using qPCR.
- EGFR knockdowns of the above-mentioned cell lines using small inhibitory RNA (siRNA). These models provide data on the role of EGFR in complement regulation, especially when combined with EGFR activators and inhibitors. The expression of complement components was studied

using qPCR, and the deposition of C3 following incubation with NHS studied using chemiluminescence imaging of plates (CLIP).

- EGFR inhibition-resistant sublines from the original cell lines. These models provide insight on the resistance mechanism to EGFR inhibitors. Expression of complement components and complement regulatory proteins was studied in these models using qPCR, and the deposition of C3 following incubation with NHS studied using IFM.
- Complement activation models. In which HNSCC cell lines following treatment with EGFR inhibitors, are incubated with NHS, HIS, or sera depleted and replenished with essential complement components like C1q and Factor B. The activation of complement, and the pathway important for the activation is then assessed using IFM, by detecting activation products (C3d, C4c or TCC) deposited on the cell monolayers.

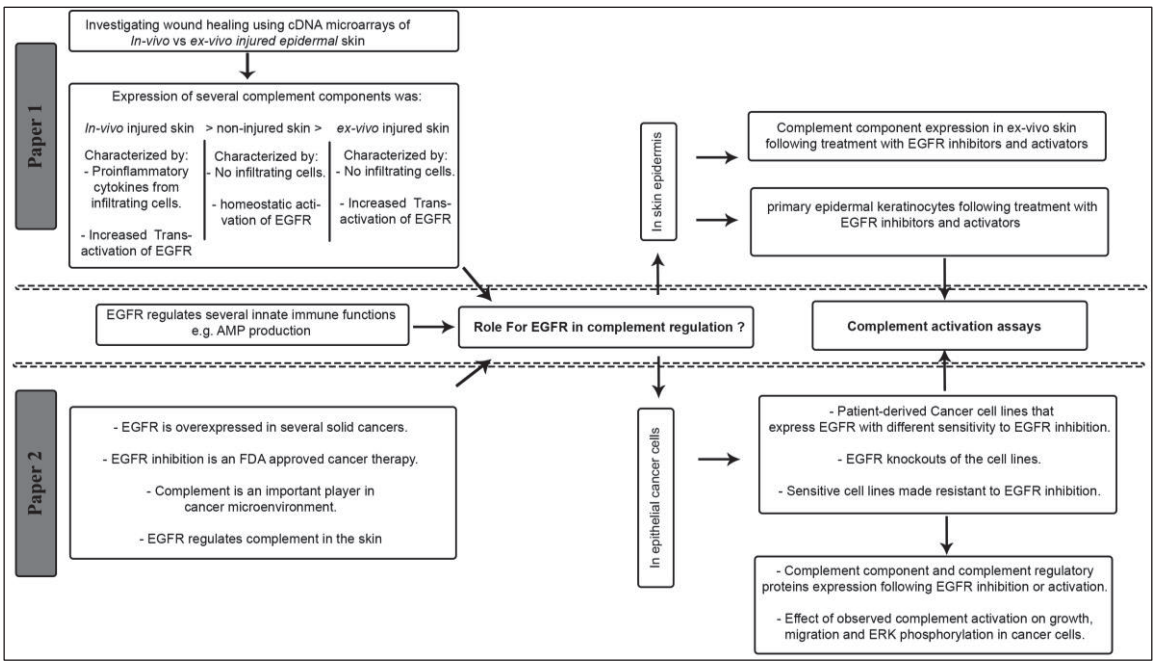



**Figure 9. A flowchart of the methodological rationale in paper 1 and paper 2.**

**In paper 3,** building on previous work indicating that keratinocytes can be activated to kill intracellular bacteria in response to external stimuli (saliva) [115]. And supported by the view regarding keratinocytes as active participants in the immune response, rather than inert building blocks of the skin's physical barrier [116-120]. We examined if complement activation on the surface of keratinocytes affects intracellular bacterial clearance.

We used *Staphylococcus aureus* (SA) in our skin infection model since SA is a common skin pathogen with an impressive immune evasion arsenal [121-123]. Moreover, intracellular persistence in non-immune cells is thought to contribute to SA chronicity and antibiotic resistance [124-126]. Host responses to this persistence are not well understood, and *in-vitro* models are the stepping stone in understanding such complex host-pathogen interactions. We used the following models and methods:

- A model of persistent intracellular SA. In this model primary keratinocyte monolayers are infected with SA for 3 hours, followed by killing of extracellular SA with gentamicin for 24 hours. This model was compared to a model in which intracellular SA is present immediately after infection, and gentamicin treatment lasts for 90 mins only.
  The models above were incubated with NHS, HIS or depleted and replenished sera.
  The activation of complement, and the pathways important for the activation is then assessed using IFM, by detecting activation products (C3d, C4c or TCC) deposited on the cell monolayers.
  The effect of complement activation on intracellular survival was assessed by counting viable colony forming units (CFUs) of intracellular SA, either directly after serum treatment or at 24 hours.
- To investigate if complement activation takes place *in-vivo* with SA infections. We used samples from atopic dermatitis (AD) patients, since those patients are commonly colonized with SA [127-129]. The samples were examined using IFM for SA and TCC staining.

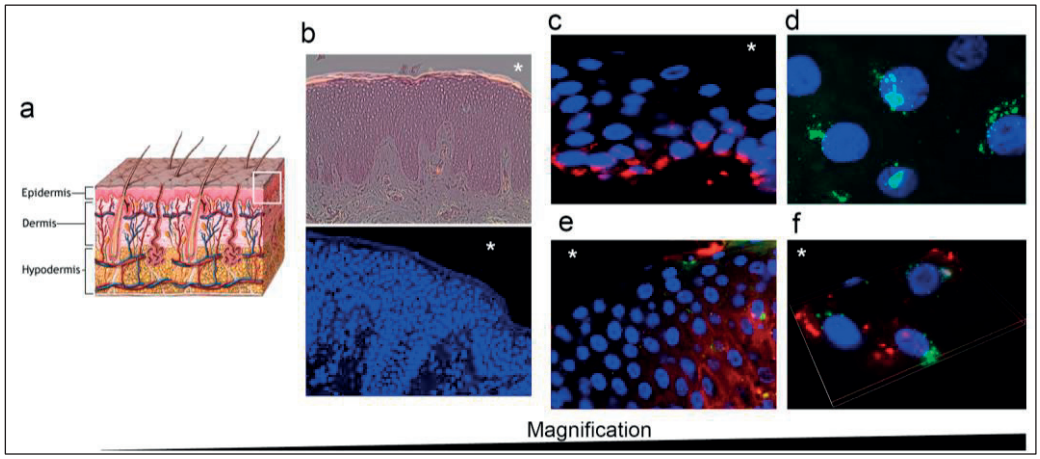



**Figure 10. Examples of models used to investigate complement regulation in the epidermis. (a)** An illustration of skin histology, and an inset around the layer of interest, the epidermis. **(b)** Upper panel: a light microscopy picture of skin epidermis from an AD patient and lower panel: an unstained immunofluorescence microscopy (IFM) picture of the same sample, the skin epidermis part can be easily distinguished under the microscope with epidermal ridges at the bottom and a keratinized layer at the top. AD lesional skin often shows epidermal thickening. **(c)** IFM of an *ex-vivo* skin model where healthy epidermis was incubated with an EGFR inhibitor for 48 hours. Expression of Factor B (red) was found to be increased in comparison to healthy skin, expression was highest in the basal layers of epidermis (refer to paper 1). **(d)** IFM of primary keratinocytes incubated with serum following EGFR inhibition, increased deposition of C3 (green) in comparison to controls reflect complement activation (refer to paper 1). **(e)** IFM of AD epidermis, showing TCC (red) deposition in the vicinity of SA (green). **(f)** IFM showing a 3-D rendering of Z-stacks (several images taken at different depths in the same X-Y field), in order to show the intracellular localization of SA (green) and the deposition of TCC (red) on the infected keratinocytes (refer to paper 3). The (*) depicts the apical side of the epidermis. *Figure created by author, illustration is in the public domain.*

# Chapter 3: Regulation of complement response in tissue

After introducing the complement system in chapter 1, the models used to study it in general and in this thesis in chapter 2. This chapter will discuss the major findings of the present investigation and where they place in the current knowledge of local complement regulation. But before that, the term "complement response" which is used here to describe complement expression and complement activation, warrants further explanation.

Although complement activation and complement component expression are two distinct processes, they often interact in several ways. For example, in hepatocytes, which are the major producers of complement proteins, production of complement proteins like C1q and C5 as well as activation fragments receptors like C5aR is increased following liver injury [130-132], indicating that the inflammatory response involves both processes. Another example is found in the brain, where the blood brain barrier could hinder the passage of systemic complement proteins [133, 134], local complement production by astrocytes is induced by inflammatory stimuli, and complement activation fragments have been shown to deposit in inflammatory and non-inflammatory brain pathologies [133, 135].

The local production of complement and complement activation can form an autocrine cycle in some tissue. In such scenarios, binding of complement activation fragments to their receptors, would induce an inflammatory response that involves the expression of complement proteins. Those complement proteins would in turn be activated through mechanisms controlled in the tissue, and propagate the inflammatory response by binding to their cognate receptors. Such an autocrine loop between C3aR activation and C3 production has been shown in the skin [136].

In this thesis, we propose a role for EGFR in regulating complement expression and complement activation in epithelial tissue in paper 1 and 2. While in paper 3, we describe a functional role for complement activation induced by epidermal keratinocytes infected with intracellular SA. Finally, paper 4 introduces a simple immunoassay for quantification of complement response *in-vitro*.

# EGFR and regulation of complement response

Several inflammatory cytokines have been identified in regulating complement expression. For example, TNF-α and IFN-γ are some of the most common inducers of complement expression [135, 137-139].

Interestingly, tissue specific proteins that are not commonly associated with inflammation have also been shown to exert some form of regulation on complement expression, like the bile acid receptor FXR regulation of C3 expression in the liver [140]. Vitamin D3 regulation of C3 in primary osteoblasts [141]. And our findings on the role of EFGR in regulation of complement expression and activation in the skin [142].

EGFR is an important regulator of cellular growth and proliferation in epithelial tissue [143], and can be activated under physiological and pathological conditions by ligands that are cleaved from the cell surface like TGF-α [144]. EGFR is also known to hold immunomodulatory functions in the skin, where its activation decreases the expression of several chemokines [120, 143, 145], while upregulating expression of antimicrobial peptides [119]. Hence inhibition of EGFR disturbs skin homeostasis [120, 142, 146].

In this sense, our findings in paper 1 on the function of EGFR in epidermal modulation of complement response, fall inline with current knowledge about EGFR and complement functions in immune homeostasis. But those findings can be of importance to other circumstances where EGFR overexpression and and complement aberrant activation contribute to pathology. Namely, in epithelial cancers, which is the the focus of paper 2.

EGFR is overexpressed in many epithelial cancers [143], like head and neck squamous cell carcinoma (HNSCC) [108, 147], colorectal cancer [148], breast cancer [149] and ovarian cancer [150]. Additionally, the role of complement in the tumour microenvironment is often described as a double edged sword [33, 63, 64, 111, 112, 151, 152], where on the one hand complement can help in propagating the immune response against malignant cells. But on the other hand, the aberrant activation of complement can help provide a favourable environment for tumour growth.

The findings in paper 2 which link the EGFR to complenet expression and activation in HNSCC, and further investigates the mechanisim and pathway by which this activation takes place, can be of importance in understanding the complement regulatory mechanisims that are disturbed in the tumor microenviroment. Moreover, our results show that HNSCC cell lines activate complement when incubated with serum to a higher extent than healthy epidermal keratinocytes, this could be related

to the recent finding of increased TCC in serum of patients with oral squamous cell carcinoma [84].

The finding of increased complement activation following iressa treatment in EGFR inhibition-sensitive cell lines, but not resistant cell lines, align with the fact that EGFR inhibition treatment is often associated with inflamatory skin lesions, the severity of which is correlated to treatment success [120, 153].

## Intracellular infections and complement activation

Effector functions of complement against pathogens have been extensively studied in the extracellular compartment and are briefly described in chapter 1. Complement recognizes carbohydrate patterns on the surface of the pathogen, and initiates its cascades in order to clear the threat. This classical view of complement's role in innate immunity has been extended in recent years. Evidence suggest that complement role in fighting infection can extend to the intracellular environment, one study demonstrated activating intracellular defense mechanisms when the pathogen enters the cell decorated with complement activation fragments [154]. While other studies showed the requirement of extracellular complement to induce killing of intracellular pathogens in monocytes, or better outcomes of obligate intracellular infections in C3 knockout mice [155, 156]. Although the effector function by which complement induces the intracellular killing are still elusive [157].

Considering the potential of uncovering new targets for fighting intracellular pathogens, the role of complement in intracellular infections, which seems to be beneficial for the host, should be further explored.

## Future directions

The work in this thesis regarding the role of EGFR in complement regulation, could be taken further to answer some interesting questions like:

- Do complement activation fragments deposit in the tumor microenvironment *in-vivo* following EGFR inhibition treatment? And could this deposition work as a marker for treatment success?
- Does complement activation following EGFR inhibition in HNSCC promote tumor survival or resistance to therapy? And can inhibition of complement be a complementary therapy to current EGFR inhibition therapies?

- How is C1q mediating the observed complement activation following EGFR inhibition?

Such questions can be addressed in part by investigating tissue samples from patients with HNSCC undergoing EGFR inhibition therapy, or cancer xenotransplants in SCID mice, combined with EGFR and complement inhibition therapy. Finally, a deeper investigation in cell-based models can reveal the target of C1q that initiates the activation.

Our investigation into complement role in intracellular infections opens the door for more questions as well:

- Does complement play a similar role in other intracellular infections? Such a question could be of interest in TB infection of macrophages for example, or does obligate intracellular pathogens evade this immune mechanism as they are experts in surviving the intracellular niche?
- How do infected cells induce the activation of complement on their surface? And how does this play out *in-vivo* where a more complex environment of immune and non-immune cells is present?
- Is intracellular survival of SA a common mechanism in colonizing the epidermis of AD patients? And is complement activation associated with intracellular SA important in disease flares?

Again, patient samples from diseases involving intracellular pathogens will provide a wealth of information, and although animal models of intracellular infections of the epidermis are hard to perform, grafting of tissue infected *ex-vivo* could be a starting point. Cell-based models and proteomic approaches in studying cell membrane changes could provide insight into the complement activation initiation steps.

Another future direction could be in investigating functions of intracellular complement. The evidence of a functional activation pathway of intracellular complement [15, 18, 157, 158], in addition to the expression of complement components by several immune and non-immune cells [158, 159], supports a role for complement in the intracellular milieu. Which can also be seen within the bigger picture of complement's role in maintaining tissue homeostasis.

# Concluding remarks

From an esoteric bactericidal activity of serum, to a system that "complements" immune functions, and finally to an orchestrator of vital physiological processes. Much has been achieved in complement research, yet many questions remain open, and scientists from various disciplines are working on the answers.

Currently, some of the main investigation areas in complement include: the role of the newly discovered intracellular complement activation pathway, the targeting of complement for management of a long list of inflammatory diseases, and the mechanisms governing complement activation or expression in tissue in health and disease. Hopefully, the present work helps in providing some answers to the latter.

Improvements in methods and models to study complement are essential for advancing knowledge in the field. But interest from the scientific community is just as important, and while complement research has taken a back seat for many years, it recently hailed back to the spotlight. With any luck, this book might have sparked the reader's interest in this fascinating and ever-evolving field of research.

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# Paper I

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

**Anas H. A. Abu-Humaidan,*[,1] Nageshwar Ananthoju,*[,1] Tirthankar Mohanty,* Andreas Sonesson,[†] Per Alberius,[‡,§] Artur Schmidtchen,[†] Peter Garred,[¶] and Ole E. Sørensen***

**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 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

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Abbreviations used in this article: EGF, epidermal growth factor; EGFR, EGF receptor; PGN, peptidoglycan; PVDF, polyvinylidene difluoride; TCC, terminal complement complex; TTBS, TBS with 0.05% Tween 20.

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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 3% 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 (TTBS), 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 CELLnTEC. Murine keratincoytes were cultured in CnT-O7 medium. The day of stimulation, the medium was changed to KGM Gold without insulin or EGF.

### *Complement activation*

New medium containing 1.2 mM $CaCl_2$ 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 $MgCl_2$, 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 $OD_{280}$.

### *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 3% BSA, subsequently incubated with $^{125}$I-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 poly-lysine–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 (Hamamatsu) 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

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 EGFR activation (13) and not by proinflammatory cytokines. To investigate the role of EGFR 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 EGFR-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 EGFR activation may cause a downregulation of complement component expression. Indeed, EGFR 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). The

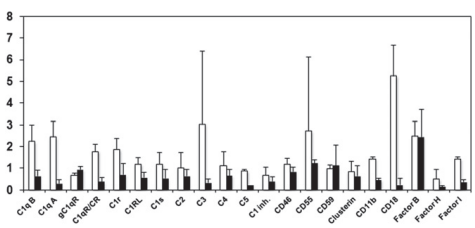


**FIGURE 1.** Expression of complement components in skin wounds in vivo and ex vivo injured skin. cDNA microarray analysis was performed on RNA extracted from skin wound in vivo (white bars, $n = 3$) and ex vivo injured skin (black bars, $n = 3$) day 4 after wounding/injury. The expression of complement components in nonwounded skin was set to 1. Error bars indicate SD.

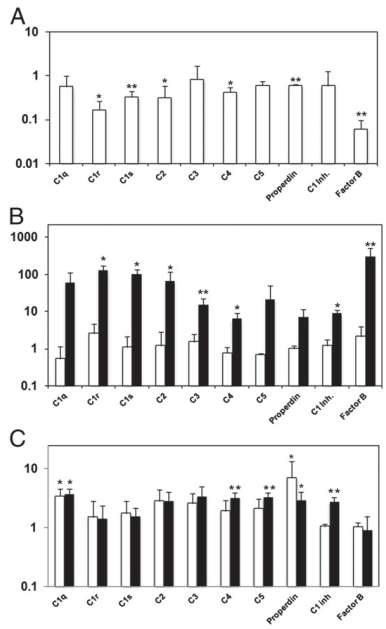


**FIGURE 2.** Expression of complement components in keratinocytes. Human primary keratinocytes were stimulated for 48 h. RNA was purified, and expression of complement components was analyzed by quantitative real-time PCR. Expression of complement components in nonstimulated keratinocytes was set to 1. $*p < 0.05$, $**p < 0.01$. Error bars indicate SD. (**A**) Human keratinocytes stimulated with the potent EGFR ligand TGF-α ($n = 4$). The statistical difference between nonstimulated keratinocytes and TGF-α–stimulated keratinocytes was calculated. (**B**) Human keratinocytes stimulated with supernatant from M1 protein–stimulated PBMC in the absence (white bars) or presence (black bars) of the EGFR inhibitor AG-1478 ($n = 3$). The statistical difference between keratinocytes stimulated with PBMC supernatant in the absence and presence of AG-1478 was calculated. (**C**) Human keratinocytes treated with the EGFR inhibitor AG-1478 (black bars) or EGFR-neutralizing Ab cetuximab (white bars), $n = 3$. The statistical difference between nonstimulated keratinocytes and stimulated keratinocytes treated with AG-1478 or cetuximab was calculated.

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).

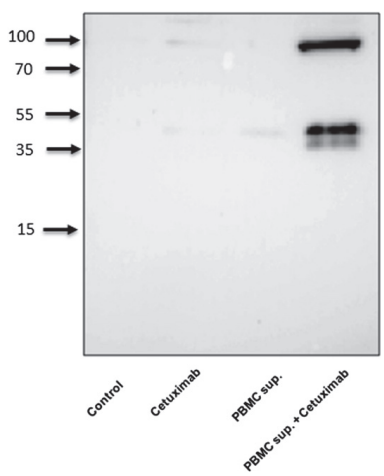


**FIGURE 3.** Western blot of factor B in the medium from stimulated keratinocytes. Human keratinocytes were stimulated for 48 h with supernatant from M1 protein–stimulated PBMC (PBMC sup.) and/or the EGFR-neutralizing Ab cetuximab. Medium was harvested and concentrated by TCA precipitation, and the content of factor B was analyzed by Western blot. Both the unprocessed form (100 kDa) and the processed form (55 kDa) of factor B were observed.

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

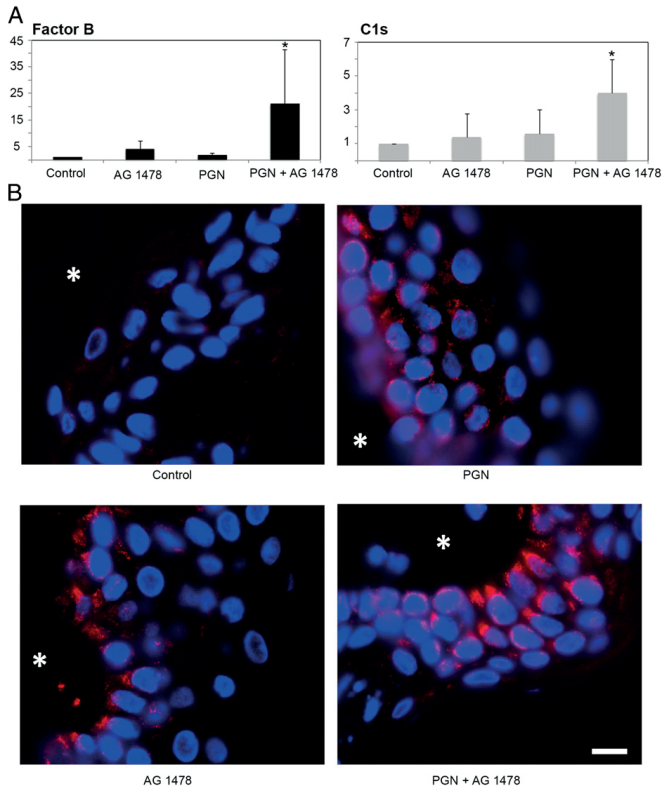


**FIGURE 4.** Expression of factor B and C1s in epidermis. (**A**) Real-time PCR data of factor B and C1s expression in whole skin treated with the EGFR inhibitor AG-1478, PGN, or both for 48 h ($n = 3$). Error bars indicate SD. The statistical difference between nonstimulated epidermis and epidermis treated with AG-1478, PGN, or the combination of AG-1478 and PGN was calculated. $*p < 0.05$. (**B**) Immunofluorescence microscopy of whole epidermis day 2 treated with AG 1478, PGN, or both for 48 h. Factor B (red) expression is increased when PGN-challenged epidermis is treated with AG1478; DAPI stain (blue) was used for nuclear staining. Scale bar, 10 μm. Asterisk indicate the basal side of the epidermis.

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

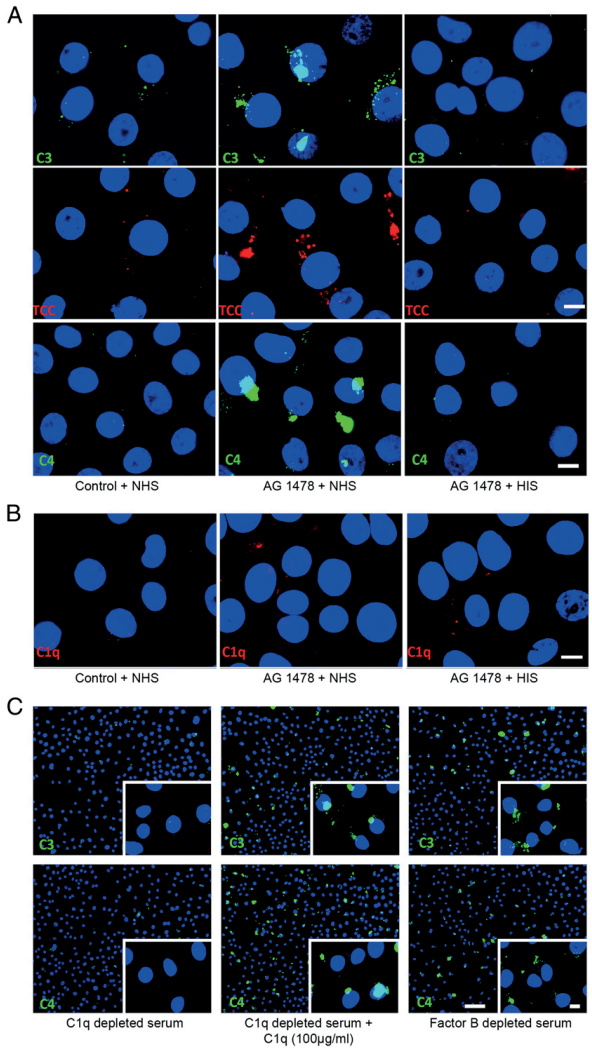


**FIGURE 5.** EGFR inhibition promotes complement activation by the classical pathway through serum-derived C1q. (**A**) Immunofluorescence microscopy showed increased deposition of C3 (green) using an Ab against C3d and the TCC (red) on keratinocytes subjected to EGFR inhibition with AG-1478 compared with nontreated keratinocytes (control) when normal human serum (NHS) was used as a source of complement. Less C3 and TCC staining was found with heat-inactivated serum (HIS). Scale bar, 10 μm. (**B**) Immunofluorescence microscopy showed no additional staining for C1q on AG-1478–treated compared with nontreated keratinocytes after incubation with serum. Scale bar, 10 μm. (**C**) Immunofluorescence microscopy demonstrated no deposition of C3 or C4 on AG-1478–treated keratinocytes incubated with C1q-depleted serum. Addition of C1q to the C1q-depleted serum restored the deposition of C3 and C4 on AG-1478–treated keratinocytes. C3 and C4 deposition was seen on AG-1478–treated keratinocytes after incubation with factor B–depleted serum. Scale bar, 100 μm. *Insets* show higher magnification (scale bar, 10 μm).

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.

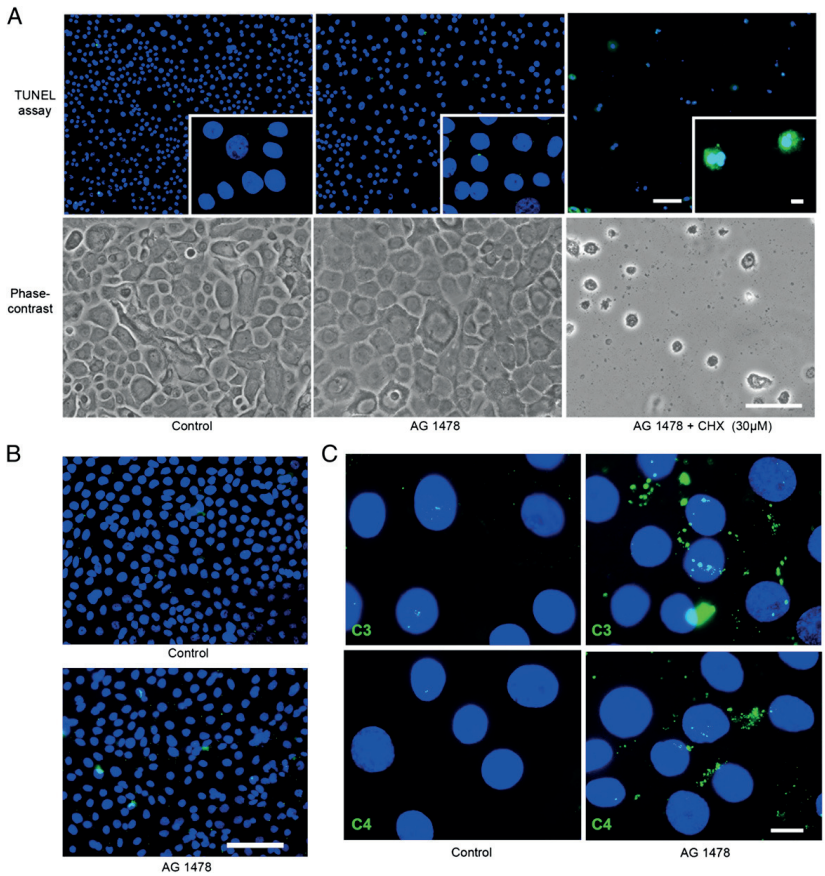


**FIGURE 6.** TUNEL assay and immunofluorescence microscopy of AG-1478–treated keratinocytes. (**A**) Human primary keratinocytes were stimulated for 48 h with AG 1478, and apoptosis was assessed by TUNEL staining. A total of 30 μM cycloheximide (CHX) was used as a positive control for apoptosis. Scale 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.

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

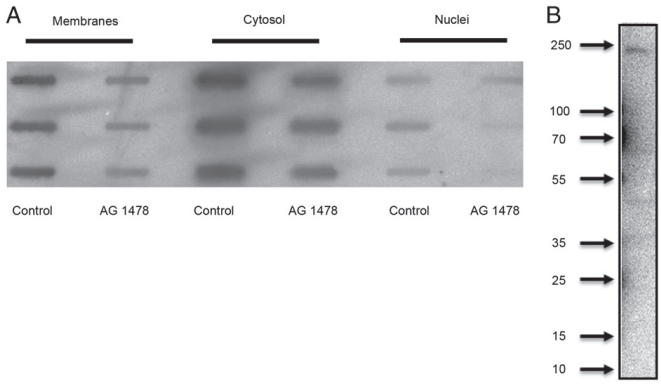


**FIGURE 7.** Binding of C1q to subcellular fractions of HaCaT cells. (**A**) HaCaT cells were treated with the EGFR inhibitor AG-1478 for 48 h or left nontreated. The HaCaT cells were subsequently subjected to subcellular fractionation. Various amounts of subcellular fraction were applied to PVDF membranes, which were probed with $^{125}$I-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 $^{125}$I-labeled C1q. One major band was seen to bind C1q. The same band was found in the membrane fraction from nontreated HaCaT cells.

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 elicited by injury (18), and indeed we found that EGFR activation downregulated the expression of complement components in keratinocytes. To imitate the inflammatory stimuli from infiltrating inflammatory cells during wound healing, we chose to use supernatants from activated PBMC stimulated with the TLR-2 ligand M1 protein from *S. pyogenes* (19) and with the TLR-4 agonist LPS (20) rather than individual cytokines. However, supernatant from stimulated PBMC activates EGFR (15). To unmask the inhibitory effect of EGFR signaling on complement component expression, we stimulated human keratinocytes with supernatant from stimulated PBMC in the presence of an EGFR inhibitor and found that this greatly (>100-fold) enhanced the expression of certain complement components such as C1s and factor B, whereas EGFR inhibition alone had only a minor effect on the complement component expression. These data indicate that the EGFR activation found during wound healing (21) may decrease the local production of complement components induced by proinflammatory cytokines.

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

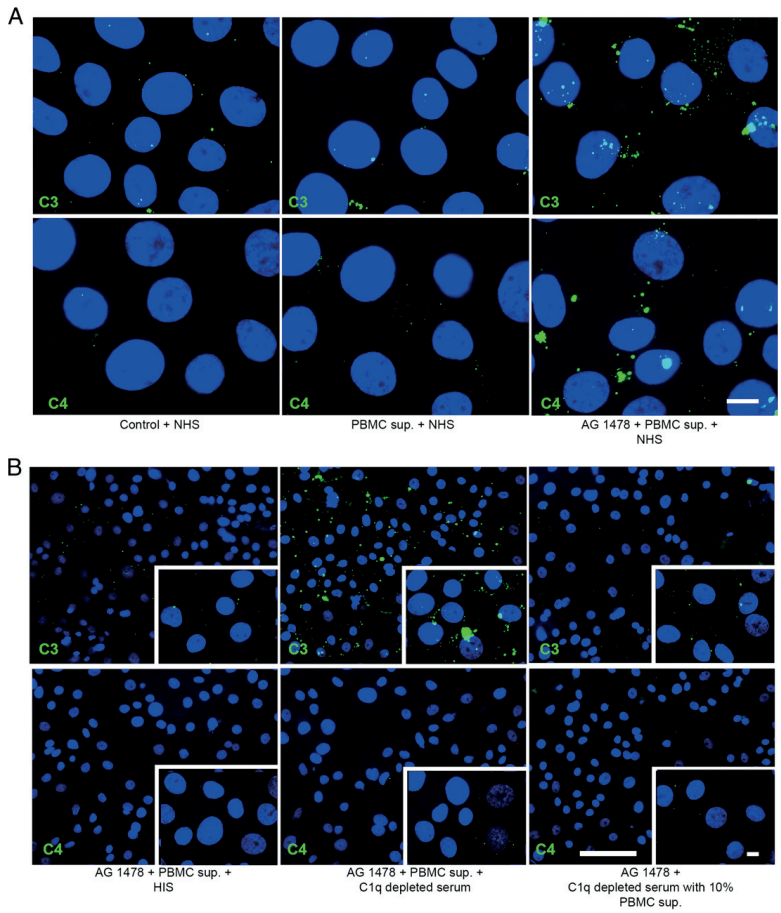


**FIGURE 8.** Complement activation in cells stimulated with PBMC supernatant. (**A**) Immunofluorescence microscopy showed increased deposition of C3 (green) using an Ab against C3d and the C4 (green) using an Ab against C4c on keratinocytes subjected to stimulation with PBMC supernatant in combination with AG-1478 compared with nontreated keratinocytes (control) and keratinocytes stimulated with PBMC supernatant only. Scale bar, 10 μm. (**B**) Immunofluorescence microscopy demonstrated deposition of C3 but not C4 on keratinocytes stimulated with PBMC supernatant in combination with AG-1478 after incubation with C1q-depleted serum. No staining for C3 or C4 was found on AG-1478–treated keratinocytes when C1q-depleted serum was incubated together with PBMC supernatant. Scale bar, 100 μm. *Insets* show higher magnification (scale bar, 10 μm).

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.

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## Disclosures

The authors have no financial conflicts of interest.

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# Paper II

# EGFR modulates complement activation in head and neck squamous cell carcinoma cell lines

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# Abstract

The epidermal growth factor receptor (EGFR) is pivotal for growth of epithelial cells and is overexpressed in several epithelial cancers like head and neck squamous cell carcinoma (HNSCC). Other than its role in growth, EGFR signaling is involved in diverse innate immune functions in the epidermis. Understanding the role of EGFR in modulating the immune response in HNSCC is important not only because of EGFR overexpression, but also because EGFR inhibition is an FDA approved cancer treatment.

Using patient-derived HNSCC cell lines that over express EGFR, we employed clinical EGFR inhibitors, and generated EGFR inhibition resistant cell lines to study the role of EGFR in modulating complement in HNSCC.

We found that HNSCC cell lines deposit complement activation fragments when incubated with human serum. This complement activation was increased following EGFR inhibition using the tyrosine kinase inhibitor Iressa and was dependent on C1q. While EGFR activation did not affect complement deposition, it led to a significant decrease in the expression of several complement components. Sensitive cell line made resistant to EGFR-inhibitors after prolonged treatment displayed complement activation and a decrease in complement regulatory proteins even in the absence of EGFR-inhibitors. Complement activation did not cause lysis of HNSCC cells, and rather led to ERK activation in one cell line.

These data indicate that EGFR has an immune modulatory role in the microenvironment of epithelial cancers, and that a prolonged EGFR-inhibitory treatment in sensitive cancer cells increases complement activation. This has implications in understanding the response to EGFR inhibitors, in which resistance and inflammatory skin lesions are two major causes for treatment cessation.



# Keywords:

EGFR, HNSCC, complement activation, complement regulation, Tyrosine kinase inhibitor, Iressa, cancer microenvironment.

# Introduction

The complement system is a conserved cornerstone of innate immunity. More than 30 proteins comprising the complement system play a role in various immune and homeostatic functions, from killing of pathogens and clearance of apoptotic cells, to recently discovered roles in angiogenesis and tissue regeneration[1]. Activation of the complement system is tightly regulated both at the level of initiation and amplification, since anaphylatoxins C3a and C5a can mount a strong immune response that connects innate and adaptive immunity[2]. An important part of this regulation occurs locally in tissues, hence systemic defects in complement proteins often give rise to tissue specific diseases [3].

The role of the complement system in cancer development is ambiguous[4], but data show deposition of complement fragments in the cancer microenvironment, and elevated anaphylatoxin levels in serum of cancer patients [5],[6]. Complement can recognize a variety of alterations in malignant cells, and complement activation has been traditionally seen as an immune surveillance mechanism against tumors[7], though the initiation and regulation is not fully understood. Consequently, antibody therapies aim to promote complement dependent cytotoxicity (CDC) against tumors[8], but malignant cells tend to upregulate the expression of complement regulatory proteins (CRPs)[9], highlighting a selective pressure exerted on these cells to minimize the harmful effects of complement.

On the other hand, several studies demonstrate a tumor promoting effect of complement activation fragments, either through recruitment of immunomodulatory cells like myeloid-derived suppressor cells (MDSC)[10], or by directly interacting with receptors (e.g. C3aR, C5aR) that activate growth signaling pathways like ERK1/2, or epidermal growth factor receptor (EGFR) transactivation [11, 12]. In many carcinomas, local complement expression and activation promoted tumor growth[13]. The finding that C5aR inhibition retarded tumor growth in mice to a similar extent exhibited by anticancer drugs[10] warrants more detailed studies of complement in the cancer microenvironment.

Autonomous growth of malignant cells of epithelial origin is often dependent on EGFR. Consequently, therapies targeting EGFR have been developed[14]. Though EGFR-inhibition therapy is well tolerated by the patients, the effect is often short-lived[14]. We previously found that EGFR regulated complement activation and expression in primary epidermal keratinocytes [15]. To investigate whether EGFR modulates complement in epithelial cancers, we used cell lines from head and neck squamous cell carcinomas (HNSCC), due to EGFR overexpression and activation, and the role of EGFR inhibition as cancer therapy in those cancers[16].

This study investigates a role for EGFR in regulation of complement expression and activation in HNSCC, and proposes that EGFR inhibition treatment can induce complement activation that is not lethal, but rather, at least in some cases, beneficial for the cancer cells.

# Methods

## Reagents

Iressa was purchased from Sigma-Aldrich; cetuximab (Erbitux) was purchased from Merck. Affinity purified polyclonal rabbit antibodies against the C3d domain of human C3, and against the C4c domain of human C4 were purchased from Dako. Monoclonal mouse anti C5b-9 antibody, human purified C1q, C1q-depleted serum, and factor B–depleted serum were from Quidel. Monoclonal mouse antibody against GAPDH, affinity purified rabbit antibodies against ERK and phosphorylated ERK were purchased from R&D systems.

## Cell culture

Head and neck squamous cell carcinoma cell lines LU-HNSCC-(4,5,7,8,19) - *referred to hereafter as HN (4,5,7,8,19) in the figures* - were generated at the Divisions of Ear, nose and throat/ Head and neck Surgery and Oncology at Lund University as previously described [17, 18]. A431 (Human squamous carcinoma, ECACC no. 85090402) and A549 (Human Caucasian lung carcinoma, ECACC no. 86012804) were obtained from Sigma. All cell lines were cultured in DMEM supplemented with 10% heat inactivated FBS and antibiotics (30 µg/mL Gentamicin, 15 ng/mL Amphotericin, Gibco). LU-HNSCC-4 (HN-4) and LU-HNSCC-19 (HN-19) from the floor of the mouth, LU-HNSCC-5 (HN-5) from the gingiva, LU-HNSCC-6 (HN-6) and LU-HNSCC-24 (HN-24) from the tongue, LU-HNSCC-8 (HN-8) from the bucca and LU-HNSCC-7 (HN-7) from a recurrence of a squamous cell carcinoma of the bucca.

## Cetuximab resistant sub lines

Cell lines HN4 and HN5 were treated with increasing cetuximab concentrations doubled every 2 weeks. Dose increase was performed by splitting the cells at the

lower concentration, and after 3 days the medium was changed to medium with double the concentration. The cell lines not treated with cetuximab were grown and split in the same manner as the cetuximab-treated cells. When maximum concentration for each cell line (2560 nmol/L, 0.39 mg/mL) had been reached, the cells were grown for 2 months at that concentration before freezing. Growth was measured using the Sulforhodamine B colorimetric assay.

Before complement experiments, these cells were passaged at least three times with several medium changes in each passage, in medium without cetuximab to avoid possible complement activation due to cetuximab.

## Iressa sensitivity assay

To measure growth inhibition of cell lines HN4, HN5, HN7 and HN8 after Iressa treatment, cells were seeded at densities averaging 2.5*10^5 cells/ well, in 12-well plates in DMEM supplemented with 10% FBS and antibiotics. The next day, medium was changed to KGM bullet kit without EGF or insulin, with or without 5 µmol/L or 10 µmol/L Iressa. Cell counts were done at 24h and 48h after Iressa treatment using 0.4 % Trypan blue staining in LUNA™ Automated Cell Counter (Logo Biosystems).

## EGFR activation and inhibition

On the day cells were confluent, medium was changed to KGM bullet kit without EGF or insulin (Lonza). The day after confluency, cells were treated with either 50 ng/mL transforming growth factor alpha (TGF-α) or 10 µmol/L Iressa for 48 hours in new KGM without EGF or insulin. Non-stimulated control cells were grown in the same medium but without treatment.

## 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. RNA expression of complement components was analyzed with quantitative RT-PCR using iQ SYBR Green Supermix (Bio-Rad). Amplification was performed at 55°C for 40 cycles in iCycler Thermal Cycler (Bio-Rad), and data were analyzed using iCycler iQ Optical System Software. RNA expression was normalized using GADPH as housekeeping gene.

## Complement activation

New KGM medium without EGF or Insulin was added to the cells together with 10% normal human serum (NHS) or 10% heat-inactivated human serum (HIS). After 3-h incubation at 37°C, the cells were washed with PBS and fixed with 4% formaldehyde for 1 h (15 min on ice, 45 min at room temperature). After three washes in TBS (10 mmol/L Tris, 500 mmol/L 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 TBS with 0,05% tween (TTBS). Primary antibodies were incubated in TTBS with 2.5% goat serum/5 mg/mL BSA overnight in at 4 °C under rotation. Slides were washed three times in TTBS and incubated with secondary Abs for 2–4 h at room temperature. After washing the inserts were mounted on slides using Prolong Gold antifade reagent mounting medium with DAPI (Invitrogen).

## Immunofluorescence microscopy

For fluorescence microscopy analysis, samples were visualized using a Nikon Eclipse TE300 (Nikon) inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled charge-coupled device camera (Hamamatsu) and a Plan Apochromat objective (Olympus). C3, C4 and TCC fluorescence around several fields was quantified and normalized to DAPI staining using ImageJ, one representative experiment is shown.

## $^{125}$I-labeled C1q binding assay

HN4 and HN5 cells were treated with Iressa (10 µmol/L) for 48 hours. Subsequently, cells were incubated with 1 µg/mL $^{125}$I-labeled C1q (10,000 cpm) in 3.5 mg/ml bovine serum albumin for 30 min at 37 ºC. Cells were washed three times in PBS and trypsinated. The radioactivity associated with the trypsinated cells was determined in a gamma counter (PerkinElmer).

## Sulforhodamine B (SRB) assay

The colorimetric SRB assay was used to assess cell density, based on the measurement of cellular protein content [19]. Cells were washed and fixed by adding ice-cold 17% (w/v) trichloroacetic acid (TCA) to each well and incubated for 1 hour at 4°C, supernatant was discarded and plates rinsed five times with water and air-dried. Fixed cells were then stained in SRB solution (0.4% w/v SRB in 1%

acetic acid) for 20 minutes at room temperature, rinsed five times with 1% acetic acid to remove unbound SRB and air-dried. The dye was dissolved in 150 µL 10 mmol/L Tris base and the absorbance measured at 565 nm.

## Growth assay

HN5 cells were grown to around 20% confluence. EGFR inhibition and subsequent complement activation were performed as described above. Briefly, cells were treated for 48 hours with 10 µmol/L Iressa, and subsequently incubated for 3 hours in 10% NHS,10% HIS or medium only. Afterwards, growth was measured using an SRB assay either directly (3h time point) or after 21 hours (24h time point) of complement activation.

## Scratch assay

To see if complement activation promotes migration of cells, we used a modified scratch assay. The day prior to confluence of HN5 cells, medium was changed to KGM bullet kit without insulin or EGF and with 10 µmol/L Iressa. This medium was used during the remainder of the experiment. The day after confluence, a scratch was made with a 200 µL pipette tip. Immediately after the scratch, 10% NHS or HIS was added for 3 hours, and pictures were taken. Medium was changed back to medium with Iressa (to minimize the effect of cell growth on the assay) and pictures were taken at 24 hours from serum addition. Open areas not containing cells after the scratch were analyzed using the software TScratch[20]. Area of the scratch at 24 hours was presented as a percent of the original scratch area, using the following formula: $(\boldsymbol{Open\ area\ at\ 24h / Open\ area\ at\ 0h}) * \mathbf{100}\%$, where values below 100% represent closure of the original scratch.

## SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were performed according to the instructions from the manufacturer (Bio-Rad). 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% BSA. For visualization of the proteins, the PVDF membranes were incubated overnight with primary antibody. The following day, the membranes were incubated for 2 h with HRP conjugated secondary antibody and visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce), Quantification of signal was done using Image lab. The PVDF membrane was stripped for 20 min in 0.2 mol/L glycine (pH

2.5) and 1% SDS, washed twice with TBS with 0.05% Tween 20 (TTBS), and finally blocked before incubating overnight with a new antibody.

## ERK activation:

ERK activation was monitored by Western blots using chemiluminescence of phosphorylated ERK and normalized to total ERK.

## Statistical analysis

Values were log-transformed and Student's t-test was performed on log transformed to compare different treatments. * denotes $p< 0.05$, ** denotes $p<0.01$.

# Results

## HNSCC activate the complement system when incubated with human serum

To investigate the role of the complement system in HNSCC, we first investigated the deposition of complement components C3 and TCC, which reflect activation of the complement system, in 4 patient-derived HNSCC cell lines in comparison to human epidermal keratinocytes from healthy adult donors (HEKa). Using immunofluorescence microscopy (IFM), we found that incubation with NHS as a source of complement but not HIS lacking complement activity, led to a significant increase in TCC deposition in HNSCC cells in comparison to HEKa cells (Figure 1a). Interestingly, pooled quantification of C3 and TCC deposition, obtained by subtracting the fluorescence signal of cells treated with NHS from those treated with HIS, showed no significant difference between HNSCC and HEKa in C3 deposition, but only in TCC staining (figure 1b). The significant increase in TCC staining in HNSCC cells prompted us to investigate the expression of CRPs CD46, CD55, CD59 and Factor H which are important in regulating complement activation at the cell surface. Using qPCR, we compared delta CT values ($_{CT}$CRP- $_{CT}$GAPDH) in 4 HNSCC cell lines, HEKa from 3 healthy donors and 2 cancer cell lines of epithelial origin, A431 and A549, and found heterogenous expression of CRPs in the cells tested (Figure 1c). While no significant difference was seen in CD46 and CD55 expression between HNSCC and Heka, HNSCC cell lines had significantly lower expression of CD59 than HEKa, A431 and A549 (Figure 1c). Factor H was expressed more in the HNSCC cell lines in comparison to HEKa which had barely detectable levels of Factor H (Figure 1c). This suggested that the decrease in CD59 expression could be the reason for the observed increased deposition of TCC in HNSCC.

While complement activation was heterogeneously found across the monolayer (figure 1a), we noticed that cells with dysmorphic nuclei did not show a similar pattern of complement activation (sup. Figure 1). So we investigated apoptosis and necrosis in the cell monolayers using Annexin V which binds to phosphatidylserine, a marker of apoptosis when it is on the outer leaflet of the plasma membrane, and EtD-III which is a highly positively charged nucleic acid probe, impermeant to live or apoptotic cells, but stains necrotic cells. We found very few positive cells for apoptosis or necrosis ( less than 5%) (Figure 1d), this is comparable to what we noticed before in HEKa monolayers incubated with NHS [21], such cells had condensed nuclei as shown by DAPI staining (figure 1d, arrow) and are similar in nuclear morphology to cells with a different complement deposition pattern (Sup figure 1). To further validate that the complement activation seen is not related to

apoptosis, we performed a double stain with Annexin V and TCC as well, and while most cells were negative for Annexin V staining, we found faint annexin V staining in cells with normal nuclear morphology (Figure 1e) in HNSCC cell line (HN8), but cells stained with PS showed less TCC staining and the staining did not colocalize with TCC as C3 often does (Figure 1a), further emphasizing that complement activation is not associated with PS or apoptotic cells. These data taken together indicate that viable HNSCC cells activate the complement system with subsequent deposition of TCC to a greater extent than healthy cells.

## EGFR inhibition and complement activation

To examine if EGFR is involved in regulation of complement activation, we first confirmed the expression of EGFR using qPCR and found no significant difference in normalized EGFR expression to GAPDH between the 4 HNSCC cell lines (sup figure 2a). As we wanted to study the role of EGFR in regulation of complement activation, we inhibited EGFR using the tyrosine kinase inhibitor Iressa, an FDA approved EGFR inhibitor, rather than using the monoclonal anti-EGFR antibody Cetuximab, which is reported to activate complement through antigen-antibody complexes [22] .The HNSCC response to Iressa was first tested at different concentrations and time points (sup figure 2b). The average of 24h and 48h growth inhibition following 10uM Iressa treatment for two of the cell lines HN4 and HN7 was 20% and 24% respectively, while cell lines HN5 and HN8 averaged growth inhibition was 39% and 46% respectively. Notably, only cell lines HN5 and HN8 showed a significant difference between 5μM and 10μM Iressa growth inhibition. This indicated that cell lines HN5 and HN8 were more sensitive to Iressa treatment, in accordance with a previous account that tested the sensitivity of those cell lines to Cetuximab [23]. Cell counts and viability following Iressa treatment were measured concurrently using an automated cell counter and trypan blue staining, it is important to note here that growth inhibition did not affect viability, which averaged above 90% in those cell lines at 10μM Iressa concentration (the highest concentration used).

After confirming the response to EGFR inhibition, HNSCC cells were treated with Iressa for 48 hours. In general, we found an increase in C3 and TCC deposition in cell lines more sensitive to Iressa HN5 and HN8, we found increased deposition of C3 and TCC after incubation with NHS but not HIS (figure 2a, c). In cell lines HN4 and HN7 displaying less growth inhibition, Iressa treatment did not increase C3 and TCC deposition after incubation with NHS (figure 2b, c).

To further examine the complement activation mediated by EGFR inhibition on cancer cells, we focused on cell line HN5 with prominent complement activation after EGFR inhibition. Incubation of NHS with Iressa-treated cancer cells led to

increased deposition of C4 (figure 3a). This indicated activation of the complement system by the classical pathway or lectin pathway. Incubation of Iressa-treated cancer cells with C1q depleted serum, or C1q only, did not lead to complement activation. However, complement activation was found after reconstitution of the C1q-depleted serum with C1q (figure 3b). In contrast, incubation with Factor B depleted serum, which lacks an important component in the alternative pathway, led to complement deposition on Iressa-treated HN5 cells (figure 3b). This demonstrated that EGFR-inhibition by Iressa treatment led to C1q-dependent complement activation.

Incubation of HN5 cells with radioactive labeled C1q, showed no increased binding of C1q in cells subjected to EGFR inhibition compared to controls, demonstrating that the observed complement activation was not due to increased C1q binding (Sup. figure 3).

Since the Iressa-mediated complement activation was not regulated by C1q binding, we investigated the effect of the Iressa mediated EGFR inhibition on the expression of complement regulatory proteins (CRPs) CD46, CD55, CD59, and factor H, which are important in regulating complement activation on cell surfaces[24]. EGFR inhibition decreased the expression of CD59 and Factor H in HN5, and CD55 and Factor H in HN8 (Figure 2d). As for cell lines less sensitive to EGFR inhibition, HN4 and HN7, no significant change in CRP expression was observed (Figure 2d).

## EGFR stimulation inhibits complement component expression

EGFR is constitutively activated in cancer cells, often in an autocrine manner through release of its ligands. TGF-α is a potent EGFR activating ligand, abundant in the environment of epithelial cancers. Since local complement production can promote growth[13] and modulate the immune response in the cancer microenvironment [25], we investigated the expression of the following complement components after TGF-α treatment; C1q and C4, essential for the classical pathway initiation and propagation. C3 and C5 which give rise to potent anaphylatoxins C3a and C5a, implicated in tumor growth and progression[26], as well as Factor B, which forms the alternative pathway C3 convertase together with C3b. We found a significant decrease in the expression of several complement components in most cell lines tested (Figure 4). This indicated that EGFR ligands continuously released from cancer cells have an autocrine effect on reducing the expression of complement proteins, and therefore an additional level of control over the immune microenvironment of cancer.

## Complement activation in sensitive cell lines made resistant to EGFR inhibition following prolonged cetuximab treatment

The therapeutic effect of EGFR inhibition in cancers like HNSCC is mostly short-lived. Consequently, the cell lines were made resistant to EGFR inhibition with prolonged treatment with the EGFR neutralizing antibody cetuximab. The cells subjected to prolonged cetuximab treatment were passaged at least three times in medium without cetuximab to avoid that cetuximab contributed to the complement activation. After prolonged cetuximab treatment, the cell line HN5 previously sensitive to EGFR inhibition, did no longer display growth retardation following EGFR inhibition (Figure 5a), the sensitivity to Iressa was measured as well (sup figure 2b). Interestingly, the resistant subline HN5-cet (from HN5), now displayed some degree of complement activation even in the absence of EGFR inhibition (Figure 5b). Moreover, expression of mCRPs CD46, CD55 and CD59 was significantly reduced in the resistant subline HN5-cet, compared to the original cell line HN5 (Figure 5c). This demonstrated that prolonged EGFR-inhibition could promote complement activation independent of growth inhibition.

The cell line HN4, previously resistant to EGFR-inhibition, was subjected to similar prolonged cetuximab treatment. In HN4-cet no complement activation was found and the prolonged treatment with cetuximab did not decrease the expression of CRPs (Figure 5a-c).

## Complement activation was not associated with apoptosis and did not affect growth

To confirm that the observed complement activation was not due to apoptosis, we performed a TUNEL assay after 48 hours of Iressa treatment of cell lines HN4 and HN5, no significant difference was found between control and Iressa-treated cells (sup. figure 4)

Furthermore, we investigated the effect of complement activation on cell growth, cell migration and growth factor expression in HN5 cells. Using the SRB assay cellular growth was measured 3 and 24 hours after addition NHS or HIS to cells at 20% confluency. These cells displayed complement activation after incubation with NHS (data not shown). No significant difference in growth between cells treated with NHS or HIS was observed, indicating complement activation did not affect growth significantly (Figure 6a).

We also investigated if the observed complement activation affected cell migration, using a scratch assay we measured the cell migration s in the presence of the growth inhibitor Iressa, to minimize the effect of cell growth on the assay. We found no

significant difference between migration of cells after NHS compared to HIS treatment, indicating migration was not significantly affected by complement activation (Figure 6b). Finally, we examined the expression of growth-related genes possibly induced by complement activation in HN5, using the cell line HN4 as a control. We found no significant difference in gene induction between the two cell lines and hence no significant induction by complement activation (Figure 6c).

## Complement activation and ERK phosphorylation

Complement activation fragments have been shown to drive several pro survival signals in different cell types, one of which is through ERK activation[11], important in cell proliferation and migration [27]. Using cell lysates after complement activation, we found an increase in phosphorylation of ERK1/2 after incubation of cells with NHS compared to HIS in cell line HN5, which had the most prominent complement activation after EGFR inhibition by Iressa, but not in other cell lines (Figure 6d). To further illustrate the complement role in ERK phosphorylation, and rule out the difference found in the cell line HN5 is solely as a result of heat inactivation of serum components other than complement, we incubated the HN5 cells with C1q depleted serum, or C1q depleted serum reconstituted with C1q. Addition of C1q to C1q depleted serum increased ERK1/2 phosphorylation demonstrating that Iressa-mediated complement activation increased ERK activation in the HN5 cell line (Figure 6e). This indicates that complement activation observed can be beneficial for the cancer since it promoted ERK phosphorylation, which in addition to its role in cell proliferation, can promote resistance to complement lysis [28].

# Discussion

The aberrant growth of epithelial tumors commonly involves EGFR overexpression and activation, and EGFR inhibition is an FDA approved cancer therapy using monoclonal antibodies or tyrosine kinase inhibitors [14]. Other than its role in growth, EGFR regulates several immune processes like production of chemokines and antimicrobial peptides [29], complement component expression and activation in the epidermis [15], and maintains general homeostasis in the skin [30]. Accordingly, many patients undergoing EGFR inhibition therapy develop cutaneous toxicities, which serve as the best prognostic marker for treatment response [14].

EGFR inhibition therapy is frequently used in treatment of HNSCC. Since impairment of EGFR signaling in epithelial tissues induces a local inflammatory environment, and inflammatory environments are integral for the neoplastic process in general [31], and in HNSCC specifically [32-34]. We sought to examine if EGFR in HNSCC has a role in regulation of complement expression and activation.

We first report that viable HNSCC cell lines activated complement when incubated with NHS, the activation led to deposition of TCC to a higher extent than HEKa. HNSCC had lower CD59/GAPDH than HEKa as shown by qPCR, CD59 is an important inhibitor of TCC formation. Other reports confirm the presence of elevated systemic complement activation fragments in patients with HNSCC [35], as well as local complement activation [36]. Yet CD59 was elevated when compared to non-neoplastic epithelium using immunohistochemistry [37], this could be attributed to GAPDH normalization we used in qPCR, or to heterogeneity of HNSCC types [38].

Previous studies on complement activation following EGFR inhibition, focused on the complement fixing potential of monoclonal antibodies like Cetuximab, and on improving consequent CDC [8, 39]. In this study, we performed complement activation assays with the tyrosine kinase inhibitor Iressa, using HNSCC cell lines with varying growth sensitivities to EGFR inhibition. To the best of our knowledge, this has not been previously investigated. We found deposition of complement components C3, C4 and TCC in these cell lines in a manner correlating with the growth inhibitory effect induced by EGFR inhibition. Indeed, cell lines most sensitive to EGFR inhibition showed a significant difference in complement deposition between non-treated and Iressa-treated cells. This activation was C1q dependent, since C1q-depleted serum did not lead to deposition of complement components, but reconstitution of the depleted serum with C1q did. However, Iressa treatment did not lead to increased C1q-binding, thus, complement activation seemed to be regulated subsequent to C1q-binding.

We further investigated EGFR mediated regulation of complement activation in HNSCCs by measuring expression of complement regulatory proteins (CRPs) CD46, CD55, CD59 and Factor H after EGFR inhibition. These CRPs are commonly upregulated in cancers and are important in conferring resistance to complement lysis, hence their expression depends on the surrounding environment and is thought to be a dynamic process [37]. Our data showed that EGFR inhibition using Iressa, decreased expression of one or more CRP, and the decrease correlated to EGFR inhibition sensitivity in cell lines tested.

Following treatment of several epithelial cancer cell lines with the potent EGFR activating ligand TGF-α, we found that expression of complement components essential for activation and propagation of the complement pathway was reduced. This indicates that cancer microenvironments rich in EGFR activating ligands can modulate the synthesis of complement components, thereby affecting cancer growth and progression.

found that a cetuximab resistant subline generated through prolonged treatment with cetuximab, showed significantly decreased CRPs when compared to the original cetuximab sensitive cell line. Moreover, incubation of the resistant subline with serum, demonstrated increased C3 and TCC deposition in comparison to the original sensitive cell line, indicating complement activation in the absence of growth retardation by an EGFR inhibitor.

Additionally, we examined the effect of complement activation seen in the cell line HN5 on tumor cell growth, cell migration and growth factor expression. We found no significant effect on these parameters when comparing NHS to HIS, probably due to the presence of multiple growth factors in serum.

Since inflammation in the tumor microenvironment is commonly modulated for the tumor benefit[31], and the activation seen was not due to apoptosis of EGFR inhibited cells, we hypothesized that cancer cells can modulate this activation when faced by growth inhibition. Although consequences of complement activation are complex and include interactions with several cell types and immune mechanisms[1], we found direct effects of this complement activation on the phosphorylation state of ERK1/2, which drives mitogenic events in malignant cells and is reported to play a role in resistance to complement induced lysis[27].

Inflammation is the best prognostic marker that a tumor is responding to EGFR inhibition therapy, however, the effects is often short lived[14]. In this context, it is interesting to note that tumor cells that responded to EGFR-inhibition by growth retardation, promoted complement activation that led to ERK-activation. Furthermore, when sensitive cell lines became resistant to EGFR-inhibition, complement activation was present at the cell surface even after termination of

EGFR inhibition. It is thus tempting to hypothesize that EGFR-mediated complement activation may ,in certain situations, be beneficial for the cancer.

In conclusion, activation and regulation of the complement system need to be further investigated in cancers, especially in light of recent evidence that complement inhibitors can be used alongside cancer therapy to improve outcome[40, 41]. Our data demonstrate a complement modulatory role for EGFR in epithelial cancers, since activation of EGFR led to decreased complement component expression, and its inhibition led to a C1q-dependent complement activation, and a change in the expression of CRPs. This complement activation could be beneficial to the cancer cells where it was found, since it promoted ERK phosphorylation and possible inflammation. Future *in vivo* experiments using tumor xenografts with varying sensitivities to EGFR inhibition, will hopefully further elucidate the local complement response to EGFR inhibitors.

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# Conflict of interest

The authors declare they have no conflicts of interest regarding this work.

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# Figures

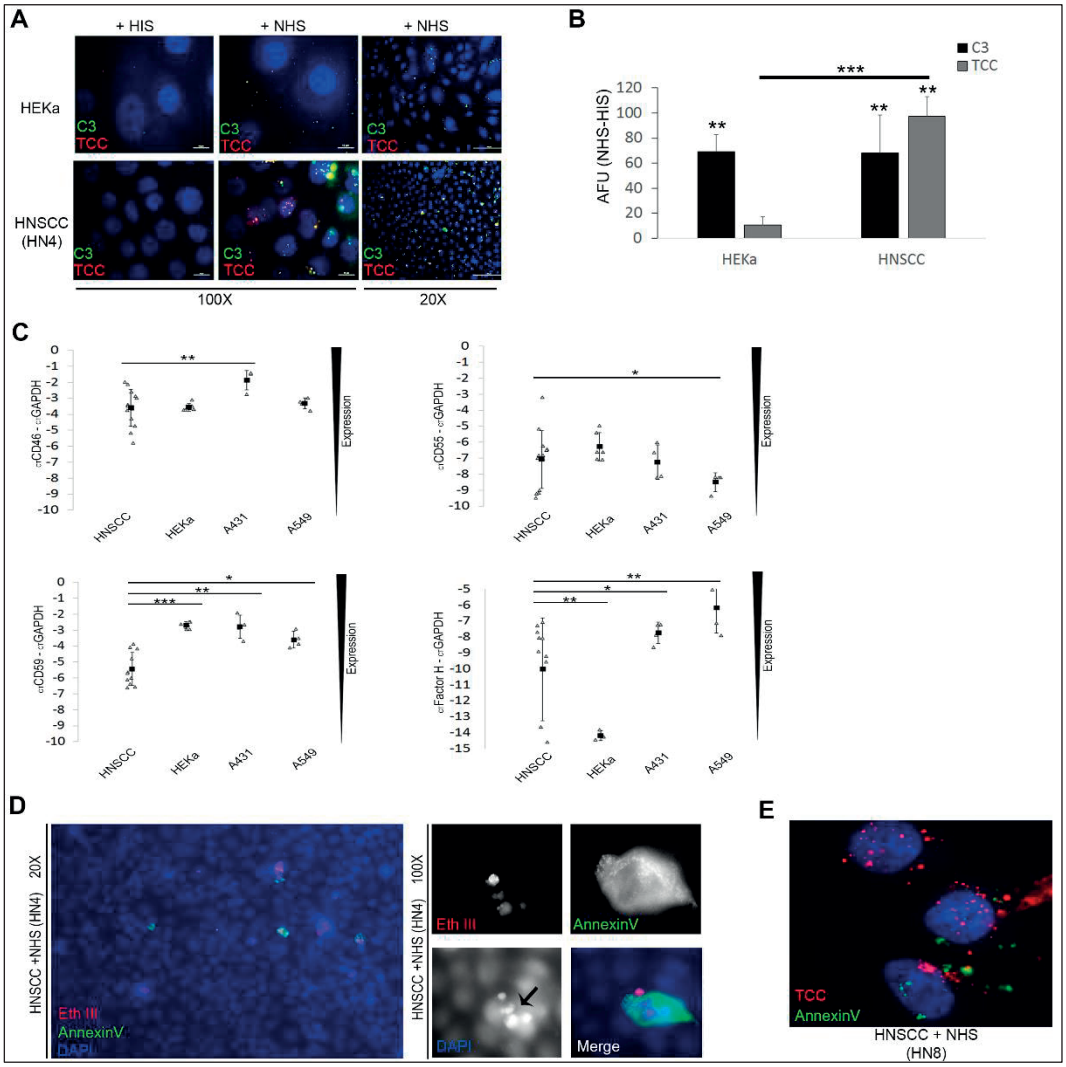



**Figure 1. Complement deposition in viable HNSCC cell lines**. **(a)** Immunofluorescence microscopy (IFM) shows deposition of complement activation fragments C3 and TCC in HNSCC cell line (HN4) when incubated with NHS but not HIS. **(b)** IFM pooled quantification of C3 and TCC deposition in 4 HNSCC cell lines was compared to HEKa cells, HNSCC showed a significant increase in TCC deposition. The quantification was done by subtracting the signal of NHS from HIS treated cells. **(c)** qPCR data of CRPs CD46, CD55, CD59 and Factor H showed significantly less normalized expression of CD59 in HNSCC cell lines in comparison to other cells tested. **(d)** IFM was used to investigate apoptosis using Annexin V, and necrosis using Ethidium bromide homodimer (Eth III) in HNSCC (HN4) monolayers, very few cells stained with either. A close up of a cell that stains for apoptosis and necrosis markers shows abnormal nuclear morphology in the DAPI channel. **(e)** A double stain of TCC and Annexin V was done in HNSCC (HN8), very few cells stained with Annexin V, a close up of the cells that stain for Annexin V showed no colocalization with TCC. This indicated viable HNSCC cells in the monolayer activate the complement system.

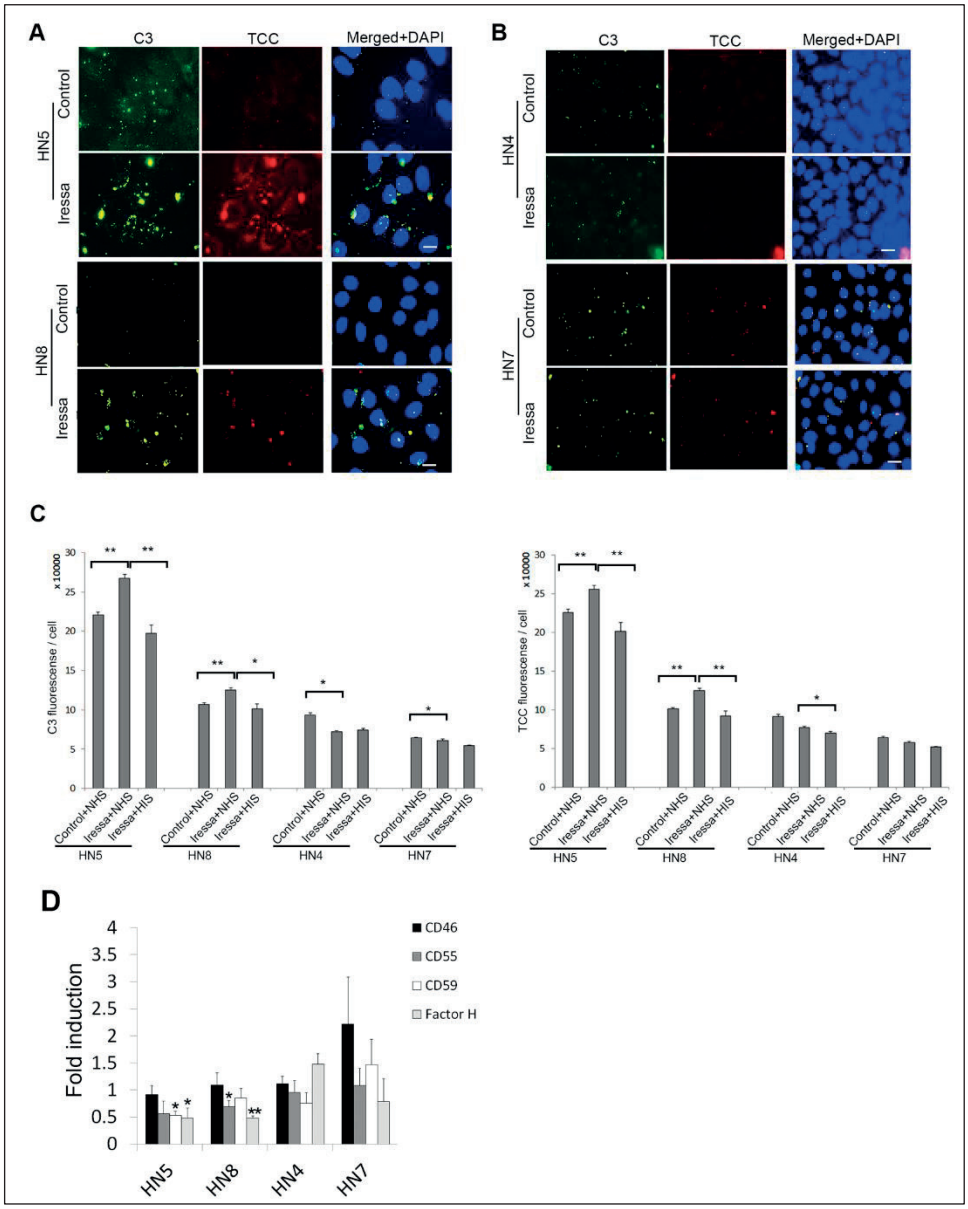



**Figure 2. EGFR inhibition leads to complement activation in sensitive cell lines. (a)** Immunofluorescence microscopy (IFM) shows deposition of complement activation fragments C3 (green) and terminal complement complex TCC (red) on surface of Iressa sensitive cell lines HN5 and HN8 after 48 hours of Iressa treatment and subsequent incubation with NHS. **(b)** whereas no change in deposition of C3 or TCC is seen in Iressa resistant cell lines HN4 and HN7. **(c)** Quantification of IFM following Iressa treatment was done separetly for each cell line . Scale bars, 10μM. Each experiment was repeated at least three times. Error bars represent SEM. * p< 0.05, ** p< 0.01. **(d)** Complement regulatory proteins CD46, CD55, CD59 and Factor H mRNA was then measured in cell lines after 48 hours of Iressa treatment and compared to non-EGFR inhibited controls set to 1, using qPCR. Bars represent the average of 3 experiments. Error bars represent SEM. * p< 0.05, ** p< 0.01

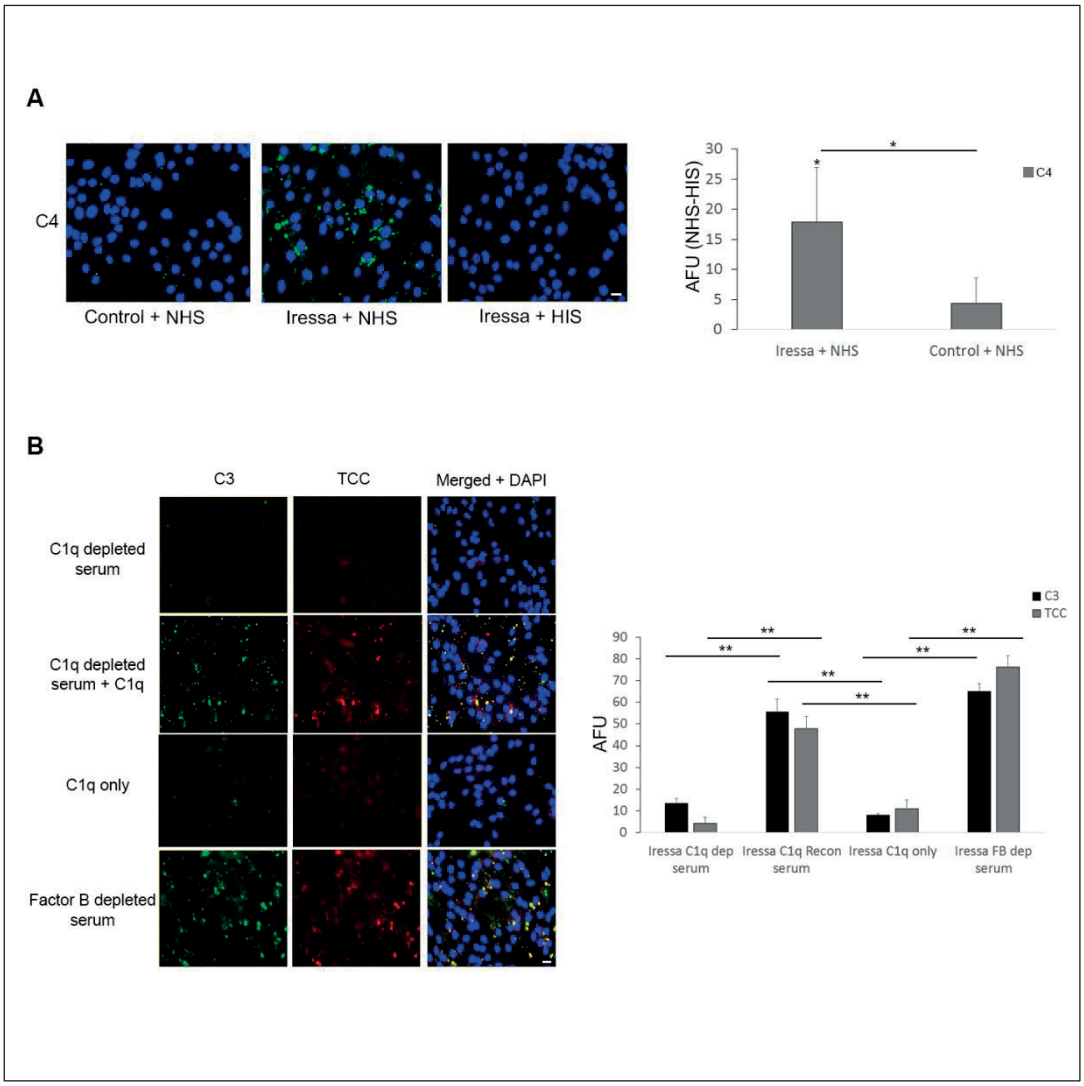



**Figure 3. Complement activation after EGFR inhibition is C1q dependent.** (a) Immunofluorescence microscopy of HN5 cells shows deposition of C4 (green) on surface of Iressa treated cells after incubation with NHS, but not HIS. Controls are not treated with Iressa but incubated with NHS, C4 fluorescence was quantified in the bar chart to the right (b) Immunofluorescence microscopy of HN5 cells show no deposition of complement activation fragments C3 (green) and terminal complement complex TCC (red) on surface of HN5 cells after 48 hours of Iressa treatment and subsequent incubation in C1q depleted serum, or C1q only (100 µg/mL), but when C1 depleted serum was reconstituted with C1q (100 µg/mL), complement activation was restored. While incubation with Factor B depleted serum, lacking ability to form alternative pathway C3 convertase, still shows complement activation, C3 and TCC fluorescence was quantified in the bar chart to the right. Scale bars, 10µM. Experiment was repeated three times.

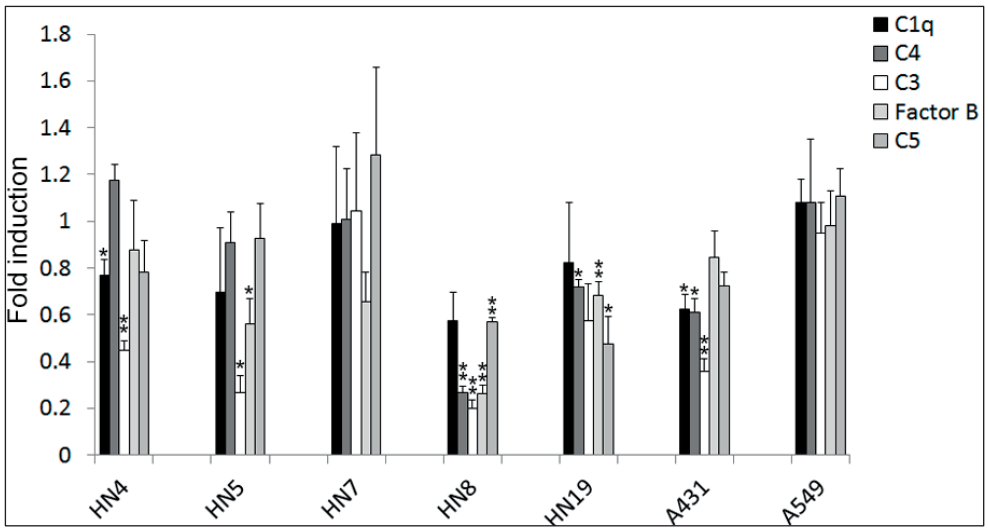



**Figure 4. EGFR activation downregulated complement expression.** Expression of complement proteins C1q, C4, C3, Factor B and C5 was measured using qPCR, in several epithelial cell lines after 48 hours of TGFα treatment. Expression in non-treated controls is set to 1. Bars represent average fold induction of 3 experiments. Error bars represent SEM. * p< 0.05, ** p< 0.01.

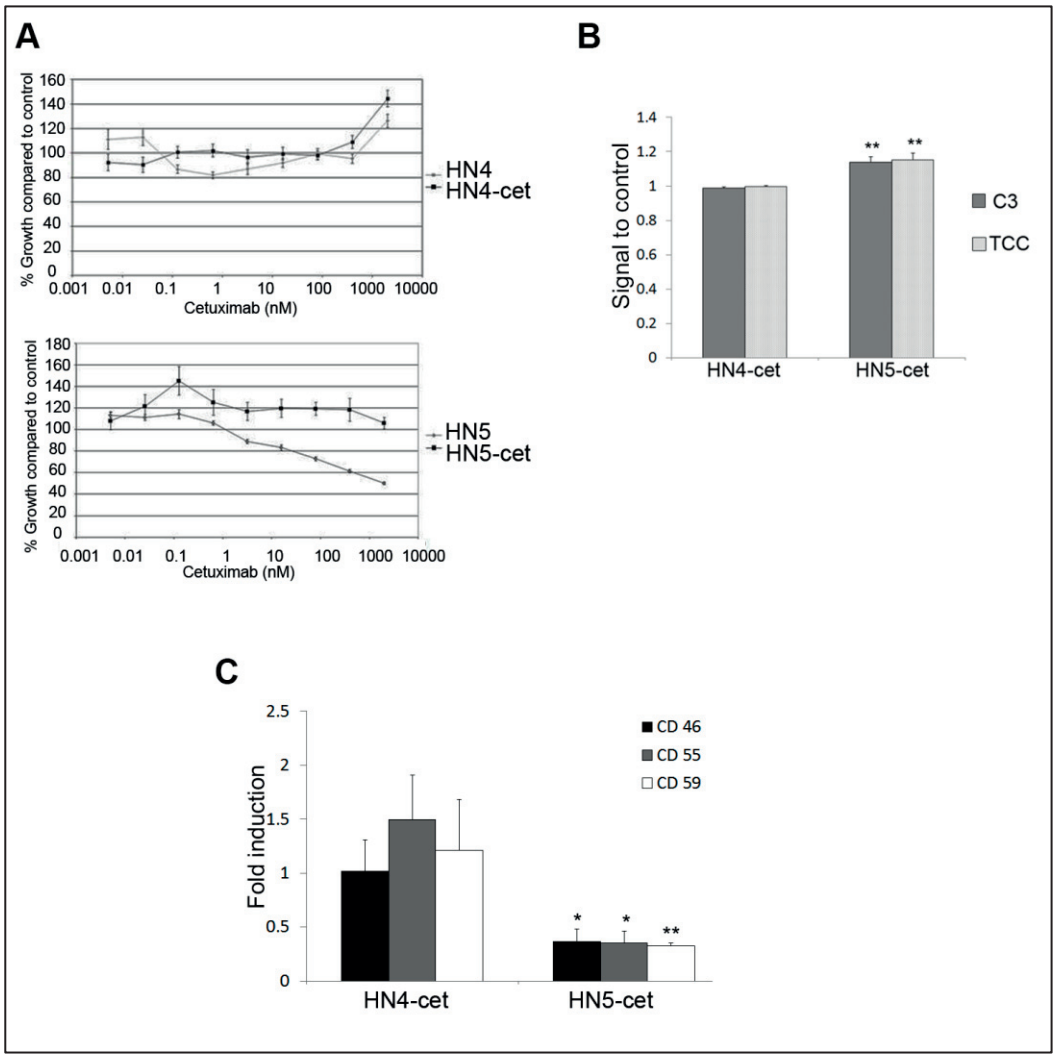



**Figure 5. Complement activation and expression of mCRPs in cetuximab resistant sublines. (a)** HNSCC cell lines HN4 and HN5 were treated with increasing concentrations of cetuximab to yield resistant sublines HN4-cet and HN5-cet. **(b)** Using IFM deposition of C3 and TCC after incubation with NHS was assessed in HN4-cet and HN5-cet, and compared to their respective original cell lines set to 1. Bars represent the average of 10 representative fields. Error bars represent SEM. **(c)** Expression of mCRPs in resistant sublines HN4-cet and HN5-cet, was compared to the expression in the original cell lines HN4 and HN5 set to 1, using qPCR. Bars represent the average of 3 experiments. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$.

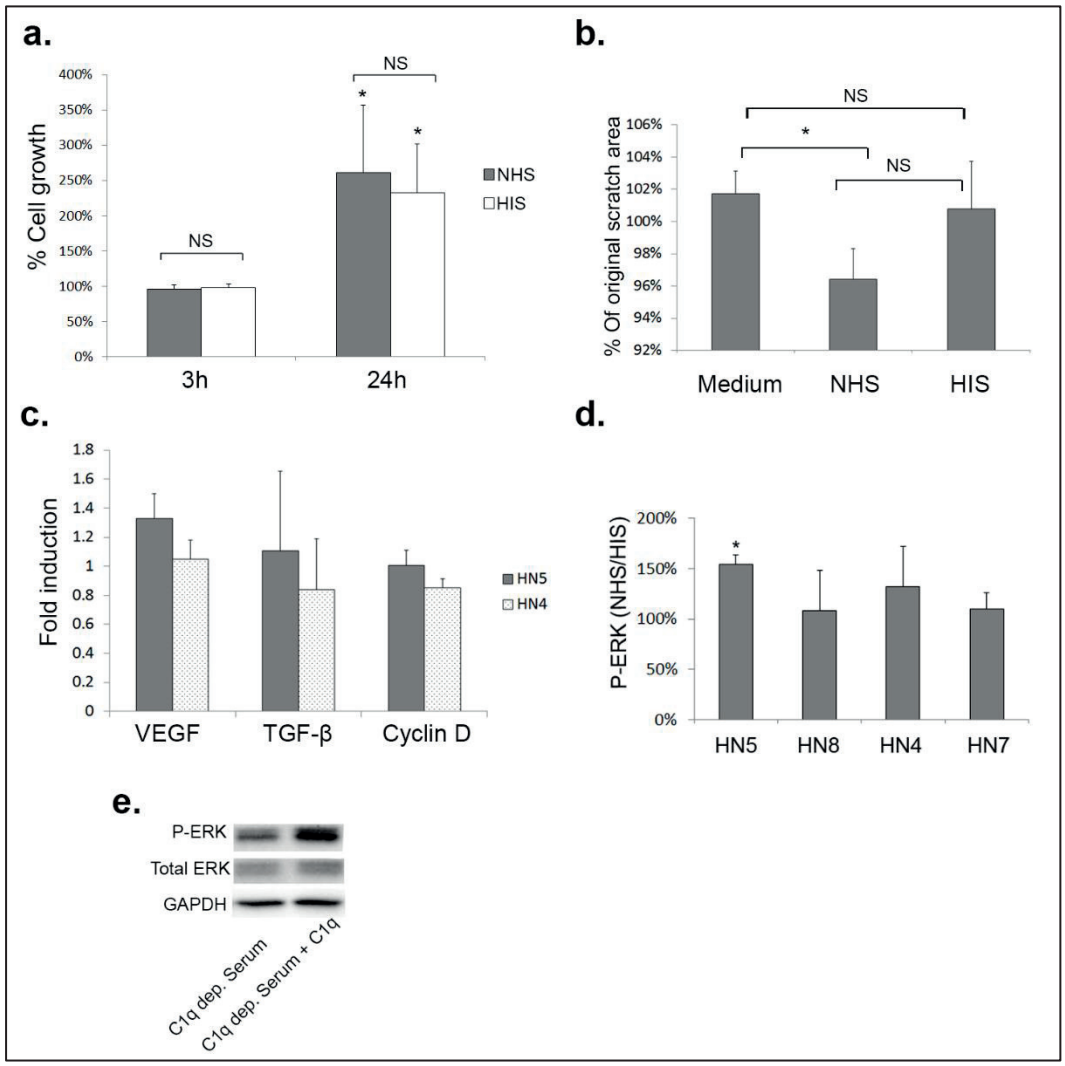
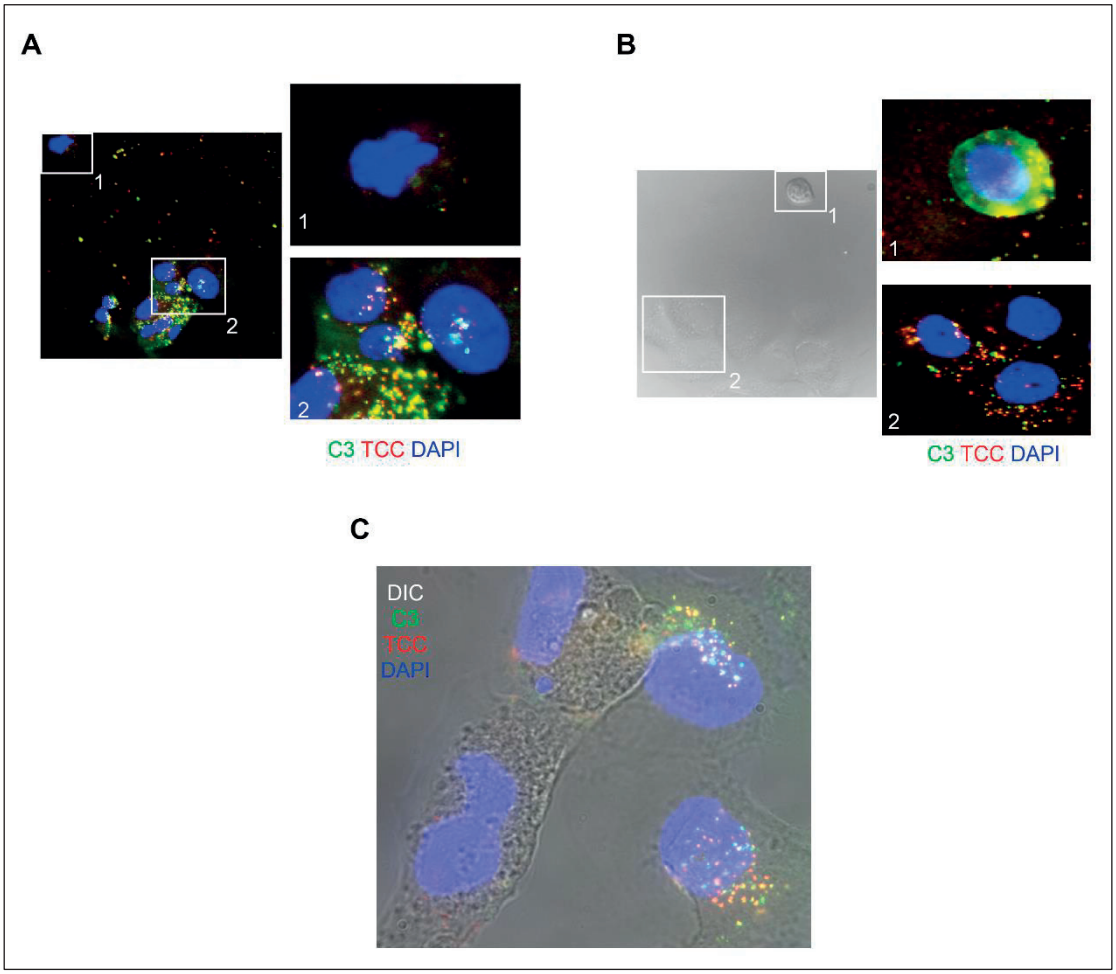
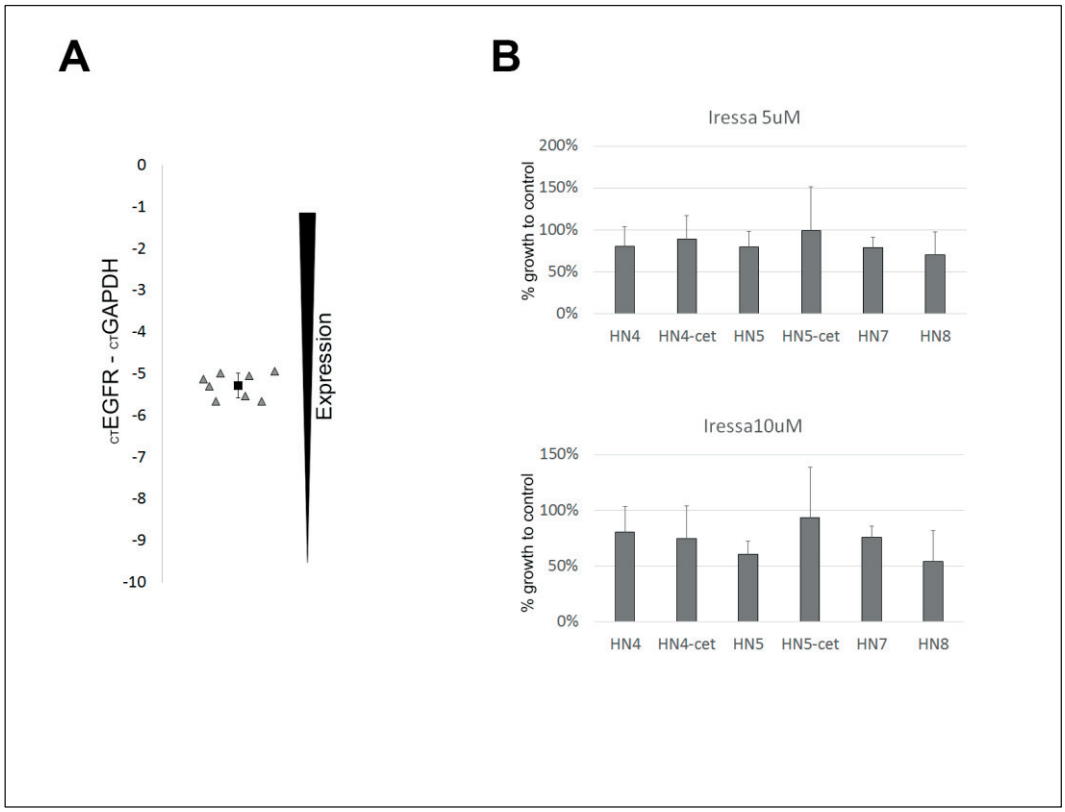



**Figure 6. Growth, migration and ERK-activation after complement activation**. **(a)** Growth of sub-confluent HN5 cells was measured at 3 and 24 hours after adding NHS or HIS, using an SRB assay. Growth of control cells treated with medium only is set to 100%. No significant difference is found between HIS and NHS treatment, indicating complement activation did not affect growth. **(b)** A scratch was made in a monolayer of HN5 cells, and migration of cells into the open area was measured 24 hours after adding NHS, HIS or medium only. The area of the original scratch at 0 h is set to 100%. **(c)** Using qPCR, induction of growth and immune related genes was tested after 48 hours of Iressa treatment and a subsequent 3h complement activation using NHS. In cell lines HN5 and HN4, no significant difference in induction is seen when compared to HIS set to 1. **(d)** HNSCC cell lines were treated with Iressa for 48 hours and subsequently incubated with 10% NHS or HIS, cell lysates were then blotted for phosphorylated ERK (P-ERK) and normalized to total ERK (T-ERK). Bars represent the ratio of ERK phosphorylation after incubation with NHS compared to HIS, average of 3 experiments is used. **(e)** ERK phosphorylation was further tested in HN5 cells after incubation with C1q depleted serum, and C1q depleted serum reconstituted with C1q (100 µg/mL),and was shown with a representative blot. Error bars represent SEM, * p< 0.05

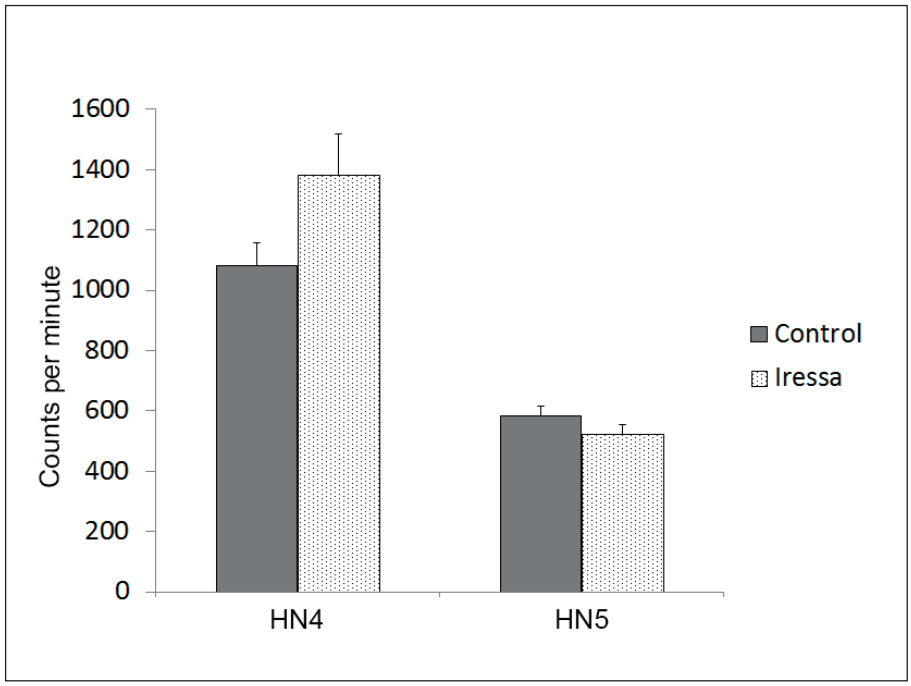
# Supplementary material



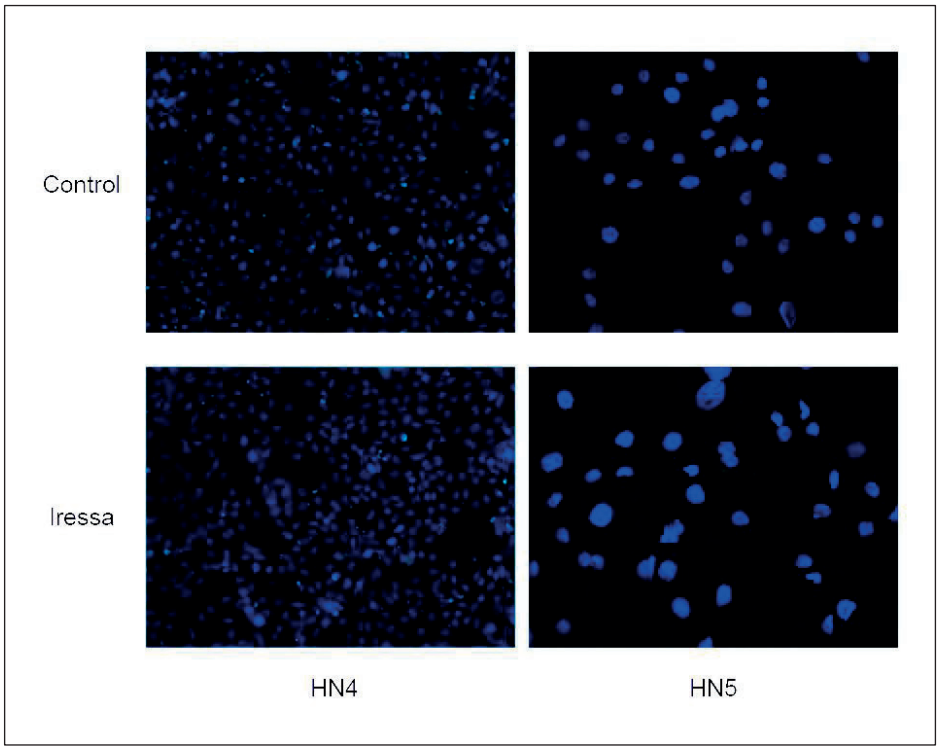

**Supplementary figure 1. Representative images of cells with dysmorphic nuclie in monolayers. (a)** Immunofluorescence microscopy (IFM) showed deposition of complement on cells with normal nuclei, while a cell with a dysmorphic nucleus in the same field showed decreased complement deposition. **(b)** Differential interference contrast (DIC) image shows a shrunken cell with a different pattern of complement staining as shown with IFM than cells with normal morphology. **(c)** Superimposed DIC and IFM images shows that cells with abnormal morphology and nuclei do not deposit complement as cells with normal morphology.




**Supplementary figure 2. EGFR expression and sensitivity to Iressa. (a)** qPCR measured normalized EGFR mRNA in 4 HNSCC cell lines, each triangle represents a monolayer. **(b)** Growth inhibition following 5 μM and 10 μM Iressa treatment was measured at 24h and 48 h, and the average is represented for each cell lines in the bar graph. Uninhibited control growth is set to 100%.




**Supplementary figure 3.** Radioactive C1q binding assay was performed on HN4 and HN5 cell lines, after 48 hours of EGFR inhibition using 10µmol/L Iressa. No significant difference in binding between control and Iressa treated cells was found.




**Supplementary figure 4.** TUNEL staining was performed after 48 hours of Iressa treatment in HN4 and HN5 cells, no significant staining difference is found.

# Paper III

frontiers in Immunology

ORIGINAL RESEARCH
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# Persistent Intracellular *Staphylococcus aureus* in Keratinocytes Lead to Activation of the Complement System with Subsequent Reduction in the Intracellular Bacterial Load

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The complement system is an ancient part of the innate immune system important for both tissue homeostasis and host defense. However, bacteria like *Staphylococcus aureus* (SA) possess elaborative mechanisms for evading both the complement system and other parts of the immune system. One of these evasive mechanisms—important in causing chronic and therapy resistant infections—is the intracellular persistence in non-immune cells. The objective of our study was to investigate whether persistent intracellular SA infection of epidermal keratinocytes resulted in complement activation. Using fluorescence microscopy, we found that persistent SA, surviving intracellularly in keratinocytes, caused activation of the complement system with formation of the terminal complement complex (TCC) at the cell surface. Skin samples from atopic dermatitis patients analyzed by bacterial culture and microscopy, demonstrated that SA colonization was associated with the presence of intracellular bacteria and deposition of the TCC in epidermis *in vivo*. Complement activation on keratinocytes with persistent intracellular bacteria was found with sera deficient/depleted of the complement components C1q, Mannan-binding lectin, or complement factor B, demonstrating involvement of more than one complement activation pathway. Viable bacterial counts showed that complement activation at the cell surface initiated cellular responses that significantly reduced the intracellular bacterial burden. The use of an inhibitor of the extracellular signal-regulated kinase (ERK) abrogated the complement-induced reduction in intracellular bacterial load. These data bridge the roles of the complement system in tissue homeostasis and innate immunity and illustrate a novel mechanism by which the complement system combats persistent intracellular bacteria in epithelial cells.

**Keywords: complement activation, membrane attack complex, classical pathway activation, intracellular infection, *Staphylococcus aureus*, atopic dermatitis, Erk activation**

## INTRODUCTION

Encompassing more than 30 proteins, the complement system is an ancient part of innate immunity (1), and it was originally described for its ability to complement phagocytes and antibodies in microbial killing (2). The complement system combats infections by detection of microbial intruders followed by lysis or opsonization to facilitate microbial clearance. In tissue homeostasis, the complement system plays a role as an intricate surveillance system by detecting altered host cells and facilitates clearance of dead or altered cells (1, 3). The different roles of the complement system are illustrated by complement deficiencies, which increase susceptibility to bacterial infections and/or autoimmune diseases, for example, C3 deficiency is generally associated with increased risk of bacterial infections, while deficiency of late complement component C5–C9 is associated with Neisseria infections in particular (4). Deficiency of C1q, which is important for clearance of apoptotic cells, is associated with systemic lupus erythematosus (5).

*Staphylococcus aureus* (SA) is an important human pathogen and a major cause of soft tissue, respiratory, bone, joint, and endovascular disorders (6, 7). SA is commonly associated with skin infections (8), for example in diseases like atopic dermatitis (AD) (9), a chronic inflammatory pruritic skin disease characterized by dry skin, pruritus, erythema, edema, scaling, excoriations, oozing, and lichenification (9). Disease flares of AD are associated with a microbial dysbiosis with abundance of SA (10, 11) suggested to drive inflammation (12).

*Staphylococcus aureus* possess elaborative mechanisms for shielding against complement mediated killing (13) among its impressive arsenal of immune evasive strategies (14). One important immune evasive mechanism by which SA is suggested to cause chronic and therapy-refractive infections is by intracellular survival in viable immune and non-immune host cells (15–21). SA can survive intracellularly in non-immune cells by switching phenotype to small colony variants (SCVs), a phenotype that does not invoke the same host responses in the cells as the wild type (9, 22, 23). This phenotype has been implicated in failure of antibiotic treatments (24). In AD, the dynamic interplay between SA and keratinocytes is proposed to result in a selection for bacteria that stimulate autophagy, thereby promoting degradation of the inflammasome and consequently promoting intracellular bacterial survival (25).

Immune responses to extracellular SA infections are extensively studied, while the immune responses to persistent intracellular bacteria in non-professional immune cells like keratinocytes, despite its clinical importance, are poorly understood. Since persistent intracellular bacteria presumably result in changes of cellular homeostasis, we investigated whether persistent intracellular SA in epidermal keratinocytes could alert the body's intricate surveillance system, the complement system.

In the current study, we found that persistent intracellular bacteria surviving in epidermal keratinocytes promoted complement activation on the cell surface, this complement activation in turn initiated cellular responses that subsequently reduced the intracellular bacterial burden by an extracellular signal regulated kinase (ERK) dependent mechanism. To the best of our knowledge, this demonstrates for the first time a possible role of the complement system in combating persistent intracellular bacteria in non-phagocytic epithelial cells, thus, bridging the dual roles of the complement system in tissue homeostasis and host defense.

## MATERIALS AND METHODS

### Reagents

Rabbit polyclonal antibody against the C3d domain of human complement component 3 (C3), and rabbit polyclonal antibody against the C4c domain of human complement component 4 (C4) were purchased from Dako. Mouse monoclonal anti human C5b-9 antibody directed against a neoepitope exposed on complement component 9 when incorporated into the terminal complement complex (TCC) is from BioPorto Diagnostics. Mouse monoclonal against human complement component 1q (C1q) is from Quidel. Rabbit polyclonal anti SA antibody is from Thermoscientific. Alexa fluor 488 goat anti rabbit IgG, Alexa fluor 594 goat anti mouse IgG and Alexa fluor 488 goat anti human from Molecular Probes. U0126, CRID-3, and AG1478 all used at concentration of (10 μM) from Tocris. Human purified C1q, Mannan-binding lectin (MBL), complement Factor B, C3, C6 proteins and sera depleted of C1q, C3, Factor B, C6 were from Quidel. The MBL deficient serum was obtained from an individual homozygous for the D (R52C, rs5030737) variant not able to activate the MBL dependent lectin pathway has been previously described (26). IdeS was purified as previously described (27) and was generously provided by Lars Björck and Inga-Maria Frick.

### Cell Culture and Inhibition Assays

Keratinocytes were cultured as previously described (28) to near confluence in KGM medium (Lonza) with additional EGF (100 ng/ml). A day before confluence the medium was changed to KGM without EGF or insulin for 24 h to induce differentiation, and then changed to KGM without EGF, insulin or antibiotics for another 24 h before starting the infection experiments. For some experiments, confluent cells were treated for 48 h with the epidermal growth factor receptor (EGFR) inhibitor AG1478 (10 μM), and the medium was changed to KGM without EGF, insulin, or antibiotics overnight before starting the infection assays. For inhibition of ERK pathway signaling, medium containing 10 μM U0126 was used during complement activation on infected cells and until processing of cells for viable count. For inflammasome inhibition, medium containing 10 μM CP-456,773 (CRID-3) was used during complement activation and until processing of infected cells for viable counts. Both inhibitors were solubilized in DMSO and used at the same volume, and viability of cells following treatment of both inhibitors was checked using microscopy and trypan blue staining (described below).

### Human Skin Samples

Atopic dermatitis patients aged 18 years or over, with AD verified by the UK refinement of the Hanifin and Rajka diagnostic criteria for AD (29, 30), were recruited from the Dermatology Clinic at Lund University Hospital, Lund, Sweden. Tissue biopsies from

AD lesional areas were taken from the skin of the patients. The participants gave informed consent complying with the Helsinki Declaration, and the Regional Ethics Examination Board of Lund, Sweden approved the study (Permit Numbers: 144/2010, 317/2010, 82/2012).

## Immunohistochemistry of Skin Samples

The skin specimens were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Slices (4 µm) were made and placed on superfrost plus slides (Thermofisher), followed by incubation at 60°C for 1 h. The slides were then processed in PT link module (Dako) for deparaffinization, dehydration, and epitope retrieval. EnVision FLEX Target Retrieval Solution, High pH (Dako) was used for this process at 97°C for 20 min. After Ag retrieval, slides were blocked for 1 h at room temperature using a blocking solution of TBS with 0.05% Tween 20 (TTBS), 1% BSA, and 5% serum from the same species as the secondary Abs were raised. The slides were then incubated overnight with primary Abs diluted 1:500 in the same blocking solution for 24 h. The slides were washed three times for 20 min in TTBS and incubated for 24 h with secondary Abs diluted 1:1,000 in the same blocking solution. The slides were then washed again three times and mounted with Prolong Gold antifade reagent mounting medium with DAPI (Invitrogen).

## Bacterial Culture and Intracellular Infection Assay

The bacterial culture and intracellular infection experiments were performed essentially as previously described (31). An invasive clinical isolate of SA from atopic eczema (2957/13) and SA Newman were plated on Todd Hewitt with yeast (THY) agar and subsequently cultured overnight in THY broth, washed and used for experiments. Confluent keratinocytes in antibiotic free medium were infected with a multiplicity of infection (MOI) of 10–20 in 12 and 24-well plates, the plates were centrifuged at 1,000×*g* for 2 min to enhance uniformity of SA attachment to keratinocytes and incubated at 37°C for 3 h. Medium was then aspirated and changed to medium containing 100 µg/ml gentamicin for 90 min to kill extracellular bacteria. Medium containing 10 µg/ml gentamicin was used throughout the remaining of the experiment.

For "*intracellular SA present immediately after infection*" keratinocytes were lysed for bacterial viable count after 90 min with 100 µg/ml gentamicin. For "persistent intracellular SA" keratinocytes were cultured for an additional 24 h in medium with 10 µg/ml gentamicin.

## Complement Activation Assay

Complement activation on keratinocytes was performed as described (28). Briefly, after 0 or 24 h in medium containing 10 µg/ml gentamicin, infected, or control keratinocytes were incubated with 10% normal human serum (NHS), heat inactivated human serum (HIS), depleted serum or medium for 3 h, as a source of complement. Cells were consequently washed in PBS and processing of keratinocytes for intracellular bacterial viable counts or fluorescence microscopy was done either directly or 24 h after addition of the different sera.

## Intracellular Bacterial Viable Counts

Keratinocytes were washed three times in ice cold PBS, scrapped, and lysed in 0.1% Triton X-100 in sterile water and vortexed several times. Lysates were serially diluted and plated on THY agar plates and colony forming units counts were performed the next day as described (31).

## Immunofluorescence (IF) Microscopy

Keratinocytes grown on inserts in 12 well plates were washed three times in ice cold PBS and fixed for 45 min in 4% PFA at room temperature. After two washes in PBS, the cells were blocked with 5% goat serum and 5 mg/ml BSA at room temperature for 45 min in PBS with 0.05% Tween 20 (PBST). After blocking, incubation was performed with primary antibodies diluted in PBST with 2.5% goat serum and 5 mg/ml BSA overnight in cold room under rotation. Next day, inserts were washed three times in PBST and incubated with secondary antibodies for 2–4 h at room temperature. The inserts were washed three times and mounted on slides using Prolong Gold antifade reagent mounting medium with DAPI (Invitrogen). Samples were visualized using a Nikon Ti-E microscope (Nikon) inverted fluorescence microscope equipped with Andor Neo/Zyla camera (Andor) and NIS elements advanced research software (Nikon) and a Plan Apochromat objective (Olympus). Fluorescence quantification was done by acquiring several images of each monolayer, covering around 10,000 cells, then analyzed with IntDen measurement (the product of Area and Mean Gray Value) using Fiji (32). No primary antibody controls are used as a reference for quantification.

## Detection of Apoptosis and Necrosis

Apoptotic and necrotic cells were detected using an annexin V-FITC/Ethidium homodimer III (EtD-III) staining kit (Biotium), according to the manufacturer's protocol. Briefly, control and infected cells were washed twice with PBS then incubated with appropriate dilutions of annexin V-FITC and EtD-III in binding buffer for 30 min. Washed twice in binding buffer and fixed with 4% PFA containing 1.25 mM calcium chloride ($CaCl_2$) at room temperature for 30 min, washed three times in PBS containing 1.25 mM $CaCl_2$ and mounted on slides using Prolong Gold antifade reagent mounting medium with DAPI. Viability of infected cells treated with U0126 and CRID-3 was assessed using Trypan blue staining, briefly, cells were washed twice with PBS and incubated with trypsin for 10 min, cell suspension was then mixed 1:1 (v/v) with 0.4% trypan blue solution (Amresco) for 5 min, and viability was assessed using LUNA Automated Cell Counter (Logo Biosystems).

## SDS-PAGE and Immunoblotting

To examine the medium of control and infected cells for degradation of complement components, medium was collected and centrifuged at 10,000 *g* for 10 min to remove cell debris, and supernatant was precipitated using 10% trichloroacetic acid. SDS-PAGE and immunoblotting were performed on the precipitated medium according to the instructions from the manufacturer (Bio-Rad). 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 3% skimmed milk in TTBS for 30 min. PVDF membranes were then incubated overnight with primary antibody diluted in blocking solution. The following day, the membranes were incubated for 2 h with HRP-conjugated secondary antibody in blocking solution (Jackson Immunoresearch) and visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce). The PVDF membrane was stripped for 20 min in 0.2 mol/l glycine (pH 2.5) and 1% SDS, washed twice with TTBS, and finally blocked before incubating overnight with a new antibody.

## Real-time PCR

RNA was purified from control and infected cells using Direct-zol RNA miniprep (zymo research) according to the manufacturer instructions. cDNA was synthesized from 200 ng purified RNA using iScript cDNA synthesis kit (Bio-Rad), according to the instructions given by the manufacturer. RNA expression of complement components was analyzed with quantitative RT-PCR using iQ SYBR Green Supermix (Bio-Rad). Amplification was performed at 55°C for 40 cycles in iCycler Thermal Cycler (Bio-Rad), and data were analyzed using iCycler iQ Optical System Software. RNA expression was normalized using GADPH as housekeeping gene.

## Statistical Analysis

Student's *t*-test was performed on log transformed values to compare different treatments in the case of viable counts. While for fluorescence quantification, Student's *t*-test was performed on non-log transformed values. * denotes $p < 0.05$, ** denotes $p < 0.01$.

# RESULTS

## Persisting Intracellular SA Cause Deposition of Complement Fragments on Primary Keratinocytes

To investigate if persistent intracellular survival of bacteria in non-immune cells results in complement activation at the cell surface, we set up a model of persisting intracellular infection with SA in epidermal keratinocytes. Primary epidermal keratinocytes were infected with an invasive strain of SA isolated from the skin of an AD patient. Cells were infected one day after confluence was reached, to ensure stable cell number throughout the experiment and to mimic the upper epidermal layer. After 3 h of infection, extracellular bacteria were killed using gentamicin (100 µg/ml) for 90 min, while intracellular SA remained viable (33). At this time point (T0), SA will be described as "*intracellular SA present immediately after infection*" in our model. Keratinocytes were then maintained in a medium containing gentamicin (10 µg/ml) for 24 h (T24). This time point where viable intracellular bacteria have persisted for more than 24 h we termed "*persistent intracellular SA*." The infection model is illustrated in **Figure 1**.

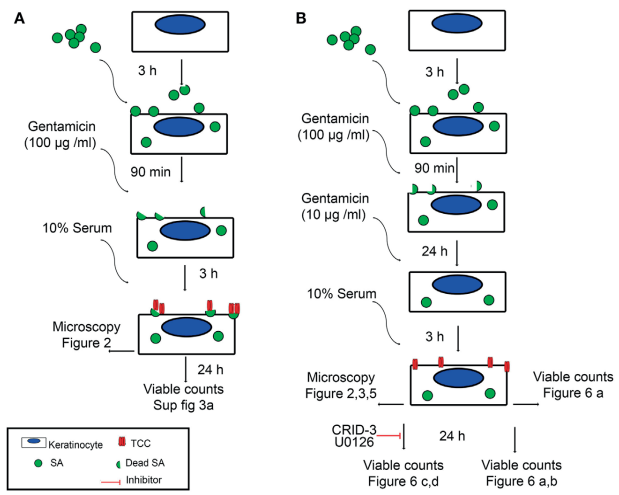


**FIGURE 1** | Experimental setup. An illustration of the two infection models used in this study, as well as the figures corresponding to the time points and analysis methods used. **(A)** Keratinocyte monolayers are infected with *Staphylococcus aureus* (SA) for 3 h, followed by killing of extracellular bacteria using 100 µg/ml Gentamicin for 90 min. At this time point, intracellular SA is referred to in the text as *intracellular SA present immediately after infection*. Incubation with normal human serum (NHS) at this time point leads to deposition of terminal complement complex (TCC) mostly on extracellular bacterial remnants. **(B)** Keratinocyte monolayers are infected with SA for 3 h, followed by killing of extracellular bacteria using 100 µg/ml Gentamicin for 90 min, keratinocytes are further incubated with 10 µg/ml Gentamicin for 24 h, at this time point, intracellular SA is referred to in the text as *persistent intracellular SA*. Incubation with NHS at this time point leads to deposition of TCC on the surface of keratinocytes.

At T24 keratinocytes with *persistent intracellular SA* were incubated with 10% Normal human serum (NHS) as a source of complement. Using fluorescence microscopy, we found deposition of the TCC on keratinocytes with *persistent intracellular SA* (**Figures 2A,B**), this was accompanied by increased deposition of complement C3 and complement C4 on keratinocytes with *persistent intracellular SA* in comparison to non-infected keratinocytes (**Figure 2A**). Keratinocytes with *persistent intracellular SA* incubated with heat-inactivated serum (HIS) lacking complement activity did not show deposition of C3, C4, or TCC (**Figures 2A,B**). Western blots of conditioned medium collected after 24 h of NHS treatment of keratinocytes with *persistent intracellular SA*, showed increased C3 and C4 degradation in medium compared to controls (**Figure 2C**). The combined data of C3, C4, and TCC deposition, along with increased C3 and C4 degradation products in the medium, confirmed that keratinocytes with *persistent intracellular SA* activated the complement system.

To rule out participation of surviving extracellular SA, medium was plated both at T0 and T24, where no viable SA were found. To demonstrate that complement activation was not due to extracellular

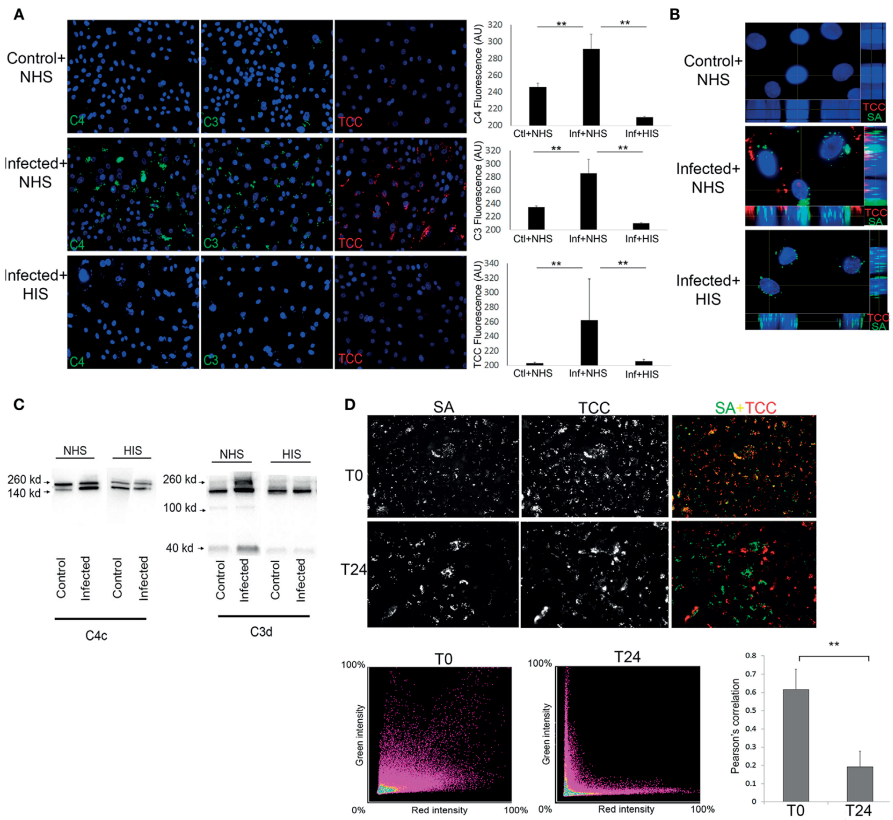


**FIGURE 2** | Keratinocytes with persistent intracellular *Staphylococcus aureus* (SA) activate the complement system on the cell surface. Immunofluorescence (IF) microscopy of primary keratinocytes infected with intracellular SA. **(A)** Keratinocytes were stained for complement activation fragments component 4 (C4), component 3 (C3), and terminal complement complex (TCC) using normal human serum (NHS) or HIS as a source of complement, IF was quantified in the panel to the right. **(B)** Orthogonal view of Z-stacks of keratinocytes infected with intracellular SA (green) show deposition of TCC (red) when incubated with NHS but not HIS. **(C)** New medium was added to the cells after complement activation and collected after 24 h to monitor release of activation fragments deposited on cells, Western blots show higher signal of C3 and C4 degradation products in media of infected cells treated with NHS. **(D)** Keratinocytes with intracellular SA present immediately after infection (T0) or persistent intracellular SA (T24) were incubated with NHS, colocalization (yellow) of SA (green) and TCC (red) indicates complement deposition on extracellular SA, colocalization was quantified over three experiments and represented using Pearson's correlation coefficient in the bar graph in the lower right corner.

bacterial remnants, NHS was added either to keratinocytes with *intracellular SA present immediately after infection* (T0) or to keratinocytes with *persistent intracellular SA* (T24) to compare the pattern of complement deposition. In keratinocytes with *intracellular SA present immediately after infection* at T0, colocalization of TCC and SA was found by IF (**Figure 2D**) demonstrating that complement activation was associated with bacterial remnants present extracellularly. In contrast, only limited colocalization of complement and SA was found when NHS was added to keratinocytes with *persistent intracellular SA* at T24. This indicated that complement activation on keratinocytes with *persistent intracellular SA* was not associated with extracellular bacterial remnants (**Figure 2D**). This was further substantiated using *SA Newman*, a strain defective in host cell invasion (34). We found colocalization of SA and TCC staining even at T24 (Figure S1 in Supplementary Material), indicating that colocalization of staining determines deposition of complement on extracellular bacterial remnants. Taken together, these data demonstrate that complement activation elicited by keratinocytes with *persistent intracellular SA* was not due to the mere presence of extracellular bacteria or bacterial remnants, but possibly involved cellular changes/responses due to the presence of the persistent intracellular bacteria.

## Activation of the Complement System by Keratinocytes with *Persistent Intracellular Bacteria* Involves More than One Activation Pathway

By using sera depleted or deficient of essential complement components, along with C3, C4, and TCC IF staining, we investigated the pathways involved in complement activation initiated by keratinocytes with *persistent intracellular SA*. We found that complement C1q depleted, and MBL deficient sera, both activated the terminal pathway as shown by TCC staining (**Figure 3A**), and reconstitution of both C1q depleted and MBL deficient sera did not cause significant increase in TCC staining (**Figure 3A**). However, reconstitution of C1q depleted serum with C1q significantly increased C4 staining, unlike reconstitution of MBL deficient serum with MBL (**Figure 3A**). This indicated that the classical pathway is a major contributor to the C4 deposition. To investigate the contribution of the alternative pathway, we used Factor B depleted serum. Factor B depleted serum activated the terminal pathway, and reconstitution with complement factor B did not significantly alter C4 or TCC staining (**Figure 3A**). However, unlike reconstitution of C1q depleted serum, reconstitution of Factor B depleted serum increased C3 deposition (**Figure 3B**), indicating a role for the alternative pathway in propagation of C3 activation. As expected, C3 and C6 depleted sera did not give rise to TCC deposition on keratinocytes with *persistent intracellular SA* unless reconstituted (**Figure 3A**), and reconstitution of C3 depleted serum increased C3 deposition (**Figure 3B**).

Since immune complexes are known to activate complement through the classical pathway (35), we assessed binding of natural IgG to keratinocytes with *persistent intracellular SA* by IF. We found increased IgG staining in keratinocytes with *persistent intracellular SA* compared to non-infected keratinocytes when incubated with NHS. The IgG staining was significantly reduced if NHS was treated with IdeS, a specific streptococcal IgG degrading cysteine proteinase (27)—before incubation with keratinocytes (**Figure 3C**). IdeS treated NHS caused a decrease in C4 staining compared to non-treated NHS, but terminal pathway activation was not significantly reduced as shown by TCC staining (**Figure 3D**).

In aggregate, these data suggest a redundancy in activation of the complement system by keratinocytes with *persistent intracellular SA*, since C1q depleted, MBL deficient and Factor B depleted sera all gave rise to deposition of TCC. The classical pathway played a major role in C4 deposition as absence of C1q or degradation of IgG significantly decreased C4 staining, while the alternative pathway seemed to play a role in the propagation of C3 activation.

## Complement Is Activated in Epidermis Colonized with SA *In Vivo*

Atopic dermatitis is a chronic inflammatory skin disease and AD patients are commonly colonized with SA during AD flares (9). To investigate if complement activation takes place on epidermal keratinocytes infected with intracellular SA *in vivo*, we double-stained with IF the epidermis of five AD patients with or without SA colonization and three healthy controls for SA and TCC. We found increased staining of TCC in SA colonized AD epidermis both in comparison to healthy controls and AD epidermis not colonized with SA (**Figure 4A**), demonstrating that SA colonization was associated with complement activation in AD. In some instances, the staining of TCC and SA co-localized, suggesting TCC formation on extracellular SA (arrow heads in inset, **Figure 4A**). In other instances, we found a similar pattern as in our model with *persistent intracellular SA* in keratinocytes where TCC and SA did not colocalize, suggesting that intracellular SA in epidermal keratinocytes may contribute to complement activation (**Figure 4A**).

To substantiate the presence of intracellular SA in AD skin, SA-colonized AD skin (verified by growth of SA colonies on agar) was incubated overnight in medium with or without gentamicin (10 μg/ml) to kill extracellular SA, and then incubated in 10% NHS as a source of complement before processing tissue for IF. Intracellular presence of SA in AD epidermis was demonstrated by the positive staining of SA in AD skin after gentamicin treatment (**Figure 4B**, arrow head in inset). The SA staining in the gentamicin treated skin did not strictly co-localize with TCC staining (**Figure 4B**), thus, displaying a similar staining pattern as found in our model of keratinocytes *with persistent intracellular SA*. Non-gentamicin treated skin, in which extracellular and intracellular SA was present, demonstrated more generalized co-localization of SA and TCC compared to gentamicin treated skin (**Figure 4B**, arrows in inset), as well as more SA and TCC staining in general (**Figure 4B**). These data indicate that intracellular SA is present in keratinocytes of SA colonized AD epidermis and could possibly play a role in activating complement *in vivo*.

## Complement Activation Takes Place on Viable Keratinocytes

To investigate whether the observed complement activation was due to cell death, we used an apoptosis and necrosis quantification

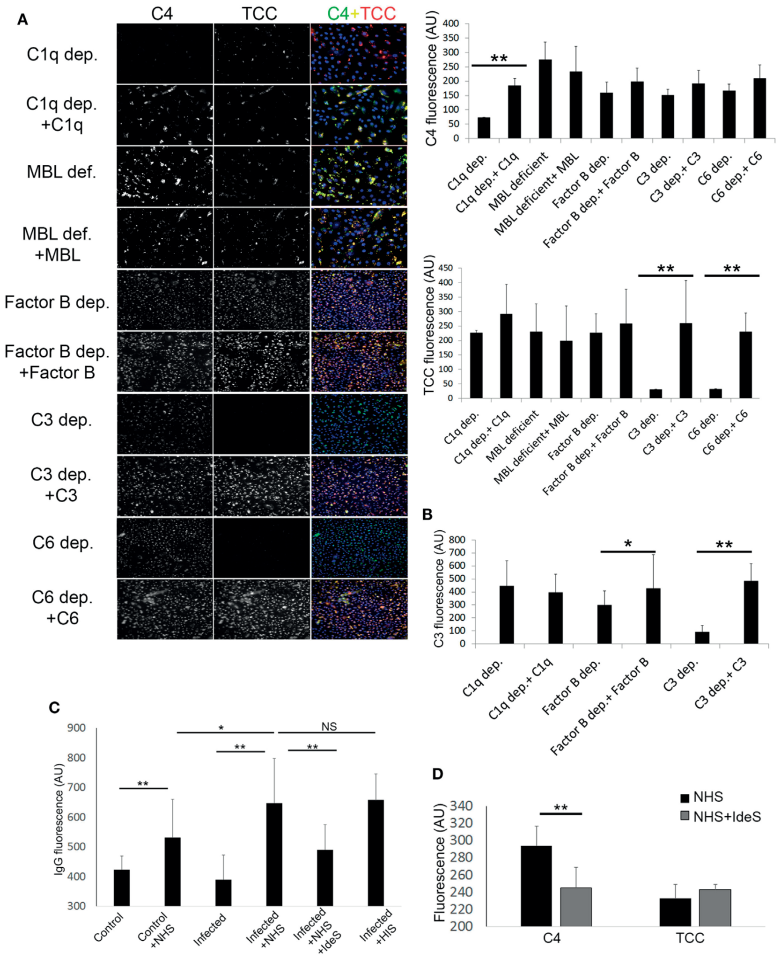


**FIGURE 3** | Pathways involved in complement activation induced by Keratinocytes with persistent intracellular *Staphylococcus aureus*. **(A)** Infected keratinocytes were incubated with depleted or deficient sera along with respective reconstitution proteins as a source of complement, then stained for component 4 (C4) and terminal complement complex (TCC). Immunofluorescence (IF) representative images are shown in the left panel along with quantification on the right. **(B)** IF of C3 was quantified in different depleted and reconstituted sera to confirm participation of the alternative pathway. **(C)** IF of IgG was quantified using anti human IgG antibodies, binding of IgG was found to be increased in infected keratinocytes incubated with normal human serum (NHS) or HIS. Treatment of NHS with IdeS significantly decreased IgG binding. **(D)** IF C4 and TCC were quantified in infected keratinocytes incubated with NHS or IdeS treated NHS.

kit, that employs annexin V binding to the membrane of apoptotic cells (36), and EtD-III to selectively stain necrotic cells (37). We found no significant difference in apoptosis and necrosis between keratinocytes with or without *persistent intracellular SA* in our experimental model (**Figure 5A**). Moreover, morphological hallmarks of apoptosis (cell shrinkage and nuclear fragmentation) were absent in the keratinocytes with *persistent intracellular SA* (**Figure 5A**). This indicated that complement activation occurs in viable keratinocytes. When increasing the MOI three times compared to the MOI used in our study, there was significant

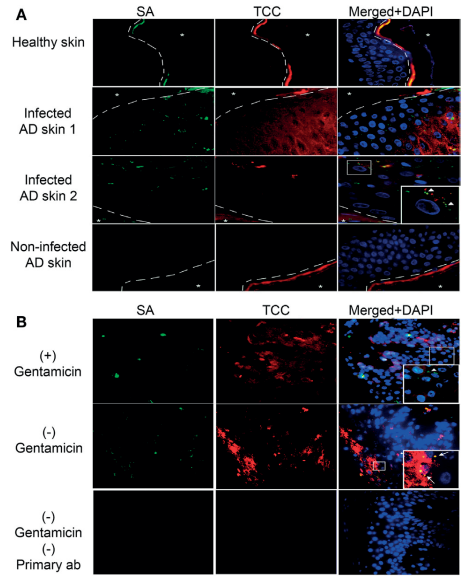


**FIGURE 4** | Complement activation in *Staphylococcus aureus* (SA) colonized atopic dermatitis (AD) epidermis. Representative immunofluorescence images of skin samples from healthy and AD patients stained for SA and terminal complement complex (TCC). **(A)** SA colonized AD epidermis shows an increase in TCC staining near infected keratinocytes, in comparison to healthy epidermis, or AD epidermis not colonized with SA. SA and TCC staining colocalized in some instances (arrow heads in inset), indicating deposition on extracellular bacteria, while in other instances it did not, suggesting intracellular localization of SA. **(B)** To confirm intracellular presence of SA, SA-infected AD epidermis was treated with gentamicin for 24 h to kill extracellular SA or left untreated, then incubated with normal human serum (NHS). Gentamicin treated epidermis showed SA staining, suggesting presence of intracellular SA (arrow heads in inset), while TCC staining did not colocalize with SA to the degree found in non-treated epidermis, which showed increased SA and TCC colocalization (arrows in inset). A no primary control is used for comparison of staining. Asterixis represent the apical side of the epidermis, which is not included in the investigation behind the dashed line due to the high unspecific binding of keratin.

apoptosis and necrosis in the monolayer (**Figure 5A**), in accordance with other studies showing that bacterial load affects the cellular survival upon infection (38).

## Complement Activation Is Found When Persistent Intracellular SA Were Present in SCVs

In our model of *persistent intracellular SA*, we did not observe SCVs commonly implicated in chronic and antibiotic resistant SA infections (20). However, SA survived for up to 7 days in viable keratinocytes (**Figure 5B**), but after 7 days it was mostly in the form of SCVs, as judged by the morphology of the colonies on agar. Increased apoptosis and necrosis (Figure S2A in Supplementary Material) was observed 7 days after the time the cells were infected, both in keratinocytes with and without *persistent intracellular SA*, probably due to the normal life cycle of the confluent culture of primary keratinocytes. At the time point where SCVs were present intracellularly, incubation with NHS still resulted in increased TCC deposition in cells with *persistent intracellular SA* in comparison to controls with no intracellular bacteria (Figure S2B in Supplementary Material). This demonstrated that *persistent intracellular SA* promoted complement activation in viable keratinocytes even when the intracellular bacteria were present as SCVs after prolonged intracellular survival.

## Complement Activation Leads to a Reduction of Persistent Intracellular SA

By viable count, we found that NHS treatment of keratinocytes with *persistent intracellular SA* significantly reduced the number of viable intracellular SA compared to HIS or non-treated cells by more than 60% in absolute colony counts (**Figure 6B**). This reduction of intracellular bacteria was seen 24 h after NHS treatment. No reduction in intracellular bacteria was found directly after NHS treatment (**Figure 6A**), suggesting that bacterial clearance required a cellular response elicited by NHS treatment.

To pinpoint the role of complement pathways and components important in reducing intracellular bacterial load by NHS treatment, we used depleted and deficient sera of complement components, along with reconstituted sera. We found that reconstitution of C1q, C3 and Factor B depleted sera, but not MBL deficient serum led to a significant decrease in intracellular viable counts (**Figure 6B**). This demonstrated that complement activation was responsible for the observed reduction in intracellular bacterial load following NHS treatment, and that this effect was mediated by both the classical and alternative pathways but not the MBL pathway. IdeS treatment of NHS did not influence the reduction in intracellular bacteria, indicating that IgG antibodies did not play a major role in the reduction of *persistent intracellular SA* following complement activation (**Figure 6B**). Reconstitution of C5 and C6 depleted sera also led to reduction of *persistent intracellular SA*; however, this was not statistically significant, demonstrating that TCC was not majorly or solely involved in the reduction of intracellular bacteria, but that activation fragments in particular of C3, played a role in this regard.

## Only Cell Mediated Complement Activation Leads to Reduction in Intracellular Bacterial Load

We then investigated whether only cell mediated complement activation and not complement activation caused by bacterial remnants reduced the intracellular bacterial survival. We have previously demonstrated that EGFR inhibition led to—undefined—cellular responses that cause cell mediated complement activation on the surface of epidermal keratinocytes treated with NHS (28). Thus, EGFR inhibited cells represent a model of keratinocyte mediated complement activation independent of bacteria or extracellular bacterial remnants. Accordingly, we compared intracellular SA viability in cells with *intracellular SA*

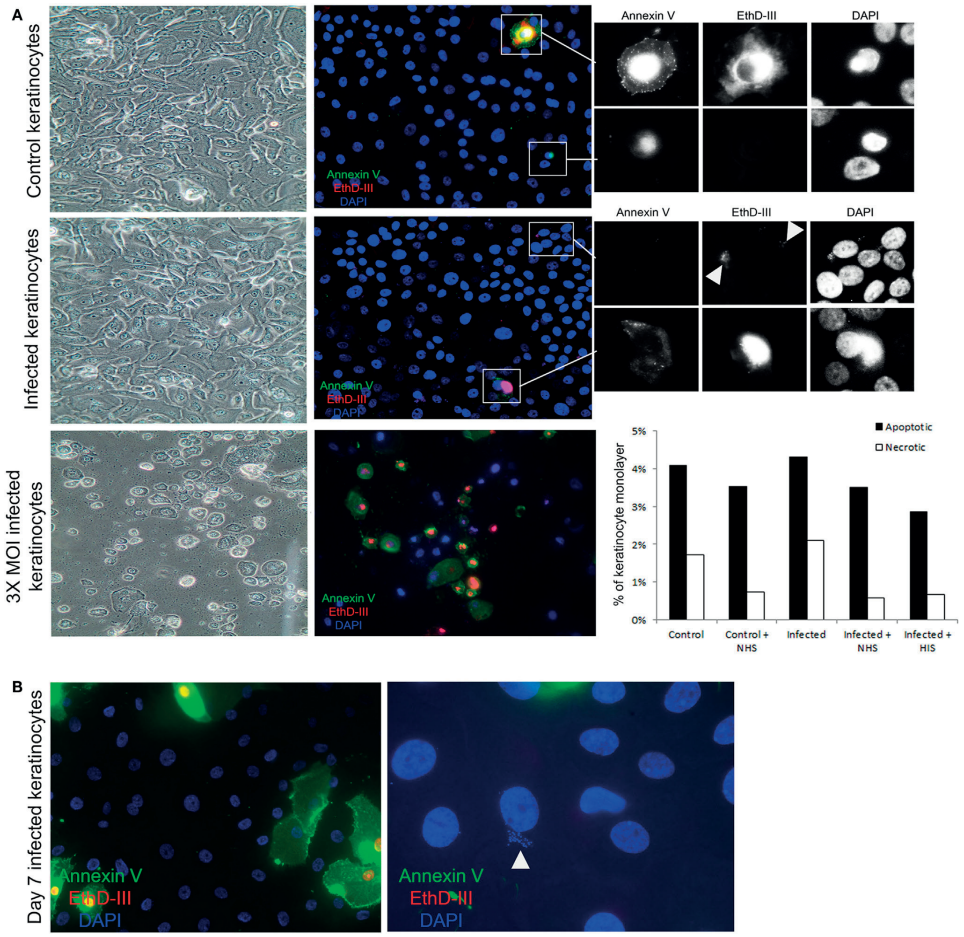


**FIGURE 5** | Complement activation takes place on viable keratinocytes. **(A)** Immunofluorescence microscopy of control and infected keratinocytes shows no significant difference in apoptotic cells stained with Annexin V (green), and necrotic cells with EthD-III (red). A representative experiment was quantified in the bar graph to the right, treatment with normal human serum (NHS)/HIS did not show any significant difference. Insets show a close up of apoptotic and necrotic cells. Arrow heads point to *Staphylococcus aureus* DNA stained with EthD-III. Light microscopy shows intact monolayers of control and infected primary keratinocytes. A higher multiplicity of infection (MOI) induced extensive apoptosis and necrosis in the monolayers and was used as a positive control. **(B)** Keratinocytes infected for 7 days were found to harbor intracellular bacteria, both keratinocytes and bacteria (arrow head) shown in the image are thought to be viable since they did not stain for the apoptosis or necrosis markers.

*present immediately after infection* where complement activation takes place on bacterial remnants at T0 (**Figure 2D**), to EGFR-inhibited cells also with *intracellular SA present immediately after infection* at T0, where the later activate complement both on their surface and on the bacterial remnants. We found that 24 h after NHS treatment, EGFR-inhibited cells had significantly less intracellular SA compared to HIS treated cells, unlike non-EGFR-inhibited cells where no significant difference was seen (Figure S3 in Supplementary Material). This demonstrated that only keratinocyte-mediated complement activation, and not complement activation due to extracellular bacterial remnants, caused a reduction in the intracellular bacterial load.

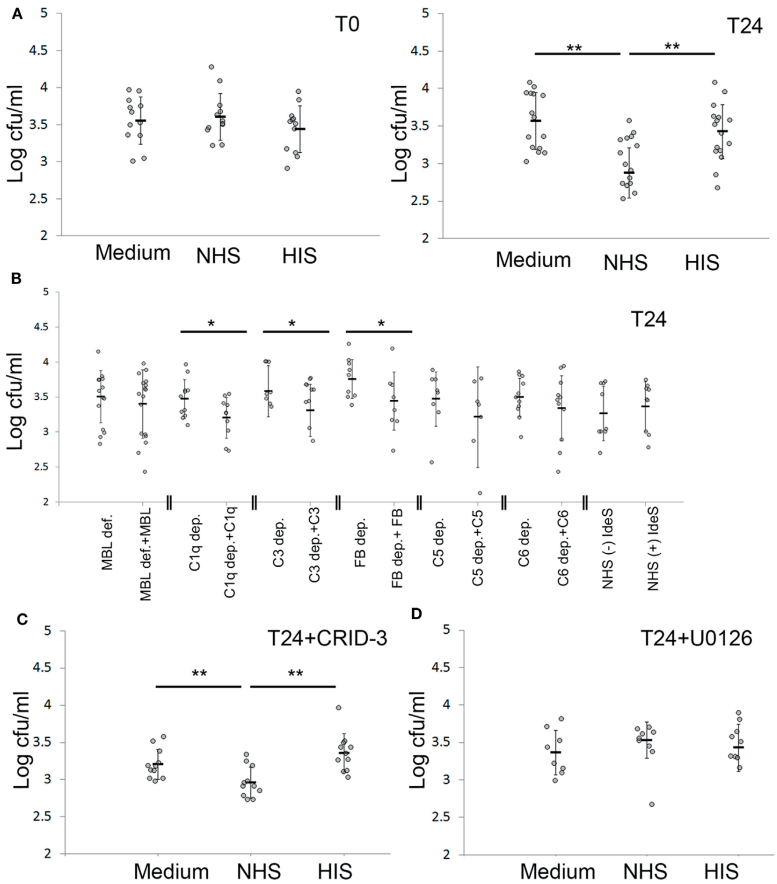


**FIGURE 6** | Keratinocytes actively cleared intracellular *Staphylococcus aureus* (SA) after complement activation. **(A)** Keratinocytes infected with SA for 24 h were treated with normal human serum (NHS)/HIS, then lysed and intracellular bacteria plated, either directly after complement activation (T0) or after 24 h (T24). **(B)** Depleted/deficient sera along with the respective reconstitution proteins were used to underline the complement components essential for bacterial clearance. **(C)** The inflammasome inhibitor CRID-3 was used to investigate if the effect seen is related to inflammasome activation, the effect of NHS on viable counts was not changed. **(D)** Treating infected keratinocytes with the ERK inhibitor U0126 abrogated the effect of NHS on bacterial viable counts. Each gray dot represents a keratinocyte monolayer. Black bars represent the average, and error bars represent standard deviation. * $p < 0.05$, ** $p < 0.01$. Each experiment was repeated at least three times, experiments using depleted sera are independent and are only compared with the respective reconstituted serum.

## Complement Mediated Reduction of Persistent Intracellular Bacteria Is a Cellular Response Dependent on Mitogen-Activated Protein Kinase (MAPK) Pathway

To investigate the role of the inflammasome in the killing of *persistent intracellular bacteria,* we inhibited inflammasome activation in infected keratinocytes using the potent inhibitor of nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome assembly, CRID-3 (39). CRID-3 inhibited interleukin-1 beta release from keratinocytes with *persistent intracellular SA*, demonstrating that CRID-3 inhibited inflammasome assembly in these cells (Figure S4A in Supplementary Material). However, CRID-3 treatment did not influence the number of *persistent intracellular SA* after complement activation (**Figure 6C**), demonstrating that the

complement mediated reduction in *persistent intracellular SA* was not related to inflammasome activation.

To investigate whether the reduction of intracellular bacterial load was related to increased autophagy, we used fluorescence microscopy to detect microtubule-associated protein 1A/1B-light chain 3 (LC3) and lysosomal-associated membrane protein 1 (LAMP-1) and examined the expression of autophagy-related genes Atg5 and beclin-1 using qPCR. We found no significant difference in LC3 or LAMP-1 staining, nor in Atg 5 and beclin-1 expression when comparing cells with intracellular SA treated with NHS or HIS (Figures S4B,C in Supplementary Material). This indicated that the bacterial clearance induced by complement activation was not related to increased autophagy.

However, treatment of keratinocytes harboring *persistent intracellular SA* with U0126, an inhibitor of the MAPKs/extracellular signal-regulated kinases (ERK), abrogated the reduction in the intracellular bacterial load mediated by complement activation (**Figure 6D**), demonstrating that complement activation reduced intracellular SA by an active cellular process mediated through the MAPK pathway signaling. The effect of U0126 was not due to reduced expression of antimicrobial peptides (AMPs), since we tested human β-defensin 2 (hBD-2), RNase 7, and human β-defensin 3 (hBD-3) and none were induced by complement activation on cells with persistent intracellular bacteria (expression of hBD-2 was not detected) (Figure S4D in Supplementary Material). Furthermore, control experiments using trypan blue staining demonstrated that 10 μM of U0126 or CRID-3 treatment did not affect viability of infected cells (data not shown).

## DISCUSSION

The adept skin pathogen SA has been shown to persist in keratinocytes *in vitro* and *in vivo* (19, 20, 40, 41). The intracellular reservoir is suggested to contribute to dissemination, recurrence and antibiotic resistance (16, 42). While host responses to extracellular SA infections are extensively studied, the host response to intracellular bacterial persistence in non-professional immune cells like keratinocytes is poorly understood. Since persistent intracellular bacterial survival of SA likely affects tissue homeostasis, and the complement system is a pivotal surveillance system of tissue homeostasis, we investigated whether persistent intracellular SA in viable epidermal keratinocytes promoted complement activation. To this end, we set up a model of *persistent intracellular SA* where intracellular bacteria persisted more than 24 h after infection.

We found that primary epidermal keratinocytes with *persistent intracellular SA* activated the complement system. In our model of *persistent intracellular SA*, where extracellular bacteria were killed and removed, we found no co-localization between detectable bacteria/bacterial remnants and deposition of complement, demonstrating that the observed complement activation was not mediated by bacteria/bacterial remnants.

Complement activation takes place on apoptotic and necrotic cells (20, 21). Although keratinocytes have been found to be resilient to SA (18), previous studies have also demonstrated that SA infected keratinocytes can undergo apoptosis after internalization of SA (19). However, we found that complement deposition on keratinocytes with *persistent intracellular SA* in our experimental model was not associated with increased apoptosis or necrosis. Thus, we hypothesize that the persistent presence of viable intracellular bacteria may trigger cellular response(s) or change(s) in cellular homeostasis unrelated to apoptosis or necrosis in a way that leads to complement activation.

Complement activation was performed with sera deficient or depleted of essential complement components to identify the complement pathway responsible for complement activation triggered by the presence of *persistent intracellular SA*. Surprisingly, complement activation was found with both C1q and factor B depleted sera as well as with MBL deficient serum, judged by deposition of C3, C4, and TCC. Experiments with sera where IgG was degraded still demonstrated complement activation, ruling out a major role of IgG even for the C1q-mediated complement activation. This demonstrates that other mechanisms are responsible for the C1q-mediated complement activation like direct binding of C1q and/or natural IgM antibodies to the infected cells (43). Furthermore, a contribution of other recognition molecules like the ficolins and collectins in the lectin pathway could also blur the picture (44). Nonetheless, our data clearly indicate that more than one pathway was involved in the observed complement activation on keratinocytes with *persistent intracellular SA*.

Apart from their role in forming a physical barrier, keratinocytes are active participants in the immune response to skin infections, through production of AMPs, complement components, and chemotactic factors (45, 46). Indeed, we previously found that saliva treatment caused reduction of intracellular SA in infected keratinocytes (31), demonstrating that keratinocytes can respond to external stimuli by actively reducing the number of intracellular bacteria. Consequently, we tested whether the complement activation promoted clearance of *persistent intracellular SA*.

Complement activation on keratinocytes with *persistent intracellular SA* did indeed lead to a reduction in the intracellular bacterial load. Sera depleted of C1q, C3, and factor B, all had significantly decreased the number of intracellular bacteria after reconstitution, while this was not the case for MBL deficient serum. Thus, it seems likely that the complement activation required to decrease the load of intracellular persistent bacteria, was initiated by the classical pathway and amplified by the alternative pathway. No effect on the number of intracellular bacteria was seen directly after complement activation. This was not surprising since the persistent bacteria were intracellular while complement activation took place extracellularly, and also since SA is resistant to direct killing by complement activation (13). Instead, complement activation reduced the number of intracellular bacteria through a cellular response mediated by activated complement components. Complement components have previously been shown to activate keratinocytes either through binding to complement receptors (47) or through sublytic TCC insertion (48), and we have previously demonstrated that keratinocytes can reduce the intracellular bacterial load after stimulation (31).

The mechanisms by which keratinocytes reduce the intracellular bacterial load are not well understood, but inflammasome assembly and subsequent caspase-1 dependent cell death or pyroptosis, as well as autophagy, have been suggested as mechanisms to limit survival of intracellular pathogens (49). However, inhibition of inflammasome assembly did not reduce the number of *persistent intracellular SA*, and complement activation did not induce autophagy. We previously found that saliva (as a model of wound licking) reduced the number of intracellular bacteria in keratinocytes, paralleled with expression of AMPs, especially human β-defensin-3 (31). Yet, complement activation did not induce expression of human β-defensin-3 or other AMPs. The MAPK pathway is important for regulating immune functions in keratinocytes (50, 51). Inhibition of MAPK dependent ERK activation abrogated the complement mediated reduction of *persistent intracellular SA*, thus demonstrating that the MAPK pathway is involved in complement mediated cellular responses which reduce the load of persistent intracellular SA.

Although cells with intracellular bacteria have been demonstrated to undergo autophagy, apoptosis and pyroptosis (52), keratinocytes have been found to be resilient to SA (53). Nevertheless, our data demonstrate that keratinocytes seemed to adjust to the persistent intracellular SA with subtle and not yet identified changes that in turn promote complement activation, not unlike what is seen after EGFR inhibition (28). To understand the role of the complement system in the dynamic interplay between bacteria and keratinocytes in chronic and therapy resistant infections, the cellular changes or adaptions to the persistent intracellular bacteria that result in complement activation should be further delineated.

The complement system is known to have important roles in the skin. Locally synthesized complement components in the skin play a role in inflammatory skin diseases (54, 55), and complement deficiencies are associated with pyogenic skin infections (4). Blocking of certain complement proteins alters the skin microbiome (56). Since plasma exudation commonly follows epidermal inflammation, we hypothesize that complement activation induced by keratinocytes with persistent intracellular bacteria could maintain an inflammatory response after clearing the initial insult while at the same time limiting the number of intracellular bacteria. This could be relevant in AD patients commonly infected with SA during AD flares. Although the contribution of intracellular SA in AD has not been examined, and is beyond the scope of this work, our *in vivo* data suggest that intracellular SA in the epidermis could lead to activation of complement, thereby contributing to the inflammation found in SA-colonized AD skin. In this regard, it is worth noting that antistaphylococcal therapies may play a role in severe, superinfected AD (57). Although there is no clear evidence for a clinical benefit of antibiotic therapies in non-infected AD, short courses of systemic antibiotics in more extensive forms of superinfected AD might be needed (9, 57, 58). If complement activation induced by *persistent intracellular SA* plays a role in the inflammation seen in AD flares, it would probably be beneficial if the chosen antibiotic treatment targets not only extracellular bacteria but also the *persistent intracellular SA*.

The complement system is seen as an important surveillance system for maintaining tissue homeostasis. Our study demonstrates that keratinocytes with persistent intracellular SA activate the complement system resulting in reduction of the intracellular bacterial load. This ties together the known functions of the complement system in host defense and tissue homeostasis and demonstrates a novel role of the complement system in combating bacteria like SA, otherwise resistant to complement mediated killing, when present intracellularly in non-phagocytic cells.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Regional Ethics Examination Board of Lund, Sweden. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Regional Ethics Examination Board of Lund, Sweden (Permit Numbers: 144/2010, 317/2010, 82/2012).

## AUTHOR CONTRIBUTIONS

AA-H contributed in experimental design, data acquisition, data analysis and interpretation, writing, and revision of the manuscript. ME, AS, and PG contributed to data acquisition, writing and revision of the manuscript. OS contributed to the study conception, experimental design, data interpretation, writing, and revision of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00396/full#supplementary-material.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Paper IV

# A simple and sensitive immunoassay for comparative protein quantification in cells

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# Abstract

Cell physiology and cellular responses to external stimuli are partly controlled through protein binding, protein localization and protein expression levels. Though quantification of these processes is pivotal in understanding basic cellular biology as well as disease pathophysiology, they can be methodologically challenging. Immunofluorescence is a powerful technique, yet quantification by this method can be hampered by auto-fluorescence. Here we describe a simple, sensitive and robust chemiluminescence-based immunoassay, which we employed first to quantify complement activation in cultured mammalian cells, and to quantify membrane proteins expression, membrane protein shedding, binding and internalization. Moreover, through specific membrane permeabilization we were able to quantify both cytosolic and nuclear proteins, and the translocation thereof. We validated this quantification methods performing parallel experiments with other quantification methods like ELISA, immunofluorescence and Western blots. The workflow of the immunoassay was found to be advantageous when compared to quantification methods used in parallel like Western blotting or immunofluorescence microscopy. Since the reagents used are common to other immunoassays, and no dedicated equipment was needed, in addition to the linearity, dynamic range and signal stability inherent to chemiluminescence, we suggest that this assay can be suitable for both small scale and high throughput protein quantification studies.

# Introduction

Cell culture experiments are useful in understanding basic cell biology and can yield important data of *in-vivo* physiological and disease states [1-3]. These experiments often aim to characterize the cellular response in terms of specific protein expression levels, binding, and localization[4-6]. These cellular responses are often quantitative rather than qualitative in nature, making quantification necessary to draw conclusions. Immunoassays like Western blotting [7, 8], immunofluorescence microscopy [9], immunoflow cytometry [10] and enzyme linked immunosorbent assay (ELISA) [11, 12] are widely employed and relatively cheap methods for comparative protein quantification. The choice of method must be suitable for the experimental model used, for example, quantifying a membrane protein in adherent cells is best done with cells in their natural state, since chemical or enzymatic detachment of cells and cell lysis can often affect protein quantity and the quality of the epitopes, making detection difficult. Another important consideration is the signal detected in the various methods (colorimetry, fluorescence, luminescence). A fluorescence-based detection system is not optimal in a setting with high background fluorescence, since it limits the dynamic range of detection making subtle changes difficult to observe.

Complement activation is a biological process that is tightly regulated and takes place with different intensities *in-vivo* and *in-vitro*. Quantifying the intensity of complement activation is especially relevant in light of studies indicating the presence of continuous and controlled complement activation even in healthy tissue [13, 14]. During complement activation most of the processes take place at the cell membrane, such as deposition of complement activation fragments, and expression of membrane bound complement regulatory proteins [15]. Studying membrane proteins is an arduous task, partly due to their hydrophobic nature. In our previous studies we used immunofluorescence microscopy to detect and quantify deposition of complement activation fragments on cell monolayers [16, 17], since detaching or lysing the cells could affect the proteins deposited on the cell membrane. Although immunofluorescence microscopy provided important results, we found the auto-fluorescence greatly diminished the difference between positive and negative biological and technical controls, and the heterogeneity of activation in the monolayer could introduce operator bias in choosing areas of interest to quantify. This prompted us to investigate a faster, more sensitive and objective way for quantifying complement activation in monolayers with minimum manipulation of cells. We found that chemiluminescence-based imaging provided suitable detections of signal generated from horse radish peroxidase (HRP) tagged proteins in multi-well culture plates. Indeed, we obtained more sensitive and objective quantification data regarding complement activation compared to immunofluorescence microscopy. By extrapolating the method to other proteins in

cultured cells, we could reproduce and quantify established biological phenomena in different cellular compartments, even for more complex responses such as protein phosphorylation and nuclear translocation with minimum optimization steps.

# Methods

## Chemiluminescence imaging of plates (CLIP)

Cells in 6,12,24,96-well plates were fixed with fresh (less than 2-week old in 4°C) 4% PFA for 30-60 mins. Washed twice in TBS and incubated with a blocking solution (5% BSA (w/v) and 5% serum from species of the secondary antibody in TBS) for 30-60 mins at room temperature. Then incubated with primary antibodies in blocking solution overnight at 4°C. The next day cells were washed 3-5 times, 3 mins each in TBS on a shaker. Then incubated with HRP conjugated secondary antibodies in blocking solution for 2-4 hours at room temperature. Afterwards cells were washed 3-5 times, 3 mins each in TBS on a shaker before incubating with ECL (SuperSignal West Pico Chemiluminescent Substrate (Pierce)) for 2-5 mins. For detection of cytosolic proteins, Saponin was added to all solutions at concentration of 0.1% (w/v). For detection of cytosolic and nuclear proteins Triton X-100 was added to all solutions at concentration of 0.1% (v/v). We emphasize that generously covering the bottom of the wells with all solutions involved was important for the heterogeneity of the signal. The dilutions of the primary antibodies ranged from 1:200 to 1:2000, while for the HRP conjugated secondary antibodies a dilution of 1:2000 was used in most experiments.

The plates were then placed in ChemiDoc MP imaging system (Bio-rad), and images are taken using the Chemiluminescence application *Chemi Hi Sensitivity* and the *auto exposure* option. The plates were washed twice in TBS to rid of the ECL and kept in TBS at 4°C for future stripping and re-staining if necessary.

The image files were then analyzed using Fiji [18], by choosing area of interest on each well and then using the *Measure* command under the *Analyze* tab. The results in the form of *mean grey value* or *Integrated density* are then transferred to an excel spread sheet for comparison.

## Stripping of cells from antibodies

Cells are incubated with the stripping solution (reblot strong, Merck) twice 20 mins each, then washed with TBS 3 times 5 mins each. Incubated with ECL for 2-5 mins to confirm absence of signal, if not, extra stripping is needed. If absence of signal is confirmed, CLIP can be performed as above.

## Reagents

Mouse monoclonal anti human C5b-9 antibody directed against a neoepitope exposed on C9 when incorporated into the TCC is from BioPorto Diagnostics. Affinity purified Goat anti human CD46, CD55, CD 59 and Mouse monoclonal anti human GAPDH are from R&D Systems. Mouse monoclonal anti human EGFR is from Oncogene. Mouse monoclonal anti human beta-actin is from Santa Cruz biotechnology. Mouse monoclonal against human HLA-ABC antigen, clone W6/32 (referred to in the text as MHC-1) and HRP conjugated polyclonal goat anti mouse are from Dako. Polyclonal rabbit anti Staphylococcus aureus is from Pierce.

## Cell culture

Keratinocytes were grown to near confluence in KGM-gold keratinocyte growth medium (Lonza) with additional EGF (10µg/ml). A day before confluence the medium was changed to KGM without EGF or insulin for 24 hours to induce differentiation, and then changed to KGM without EGF, insulin or antibiotics for another 24 hours before starting the infection experiments. Head and neck squamous cell carcinoma cell lines LU-HNSCC-(4,5,7,8) - *referred to hereafter as HN (4,5,7,8)* were generated at the Divisions of Ear, nose and throat/ Head and neck Surgery and Oncology at Lund University as previously described [17], and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated Fetal bovine serum (HI-FBS) and antibiotics (30 µg/mL Gentamicin, 15 ng/mL Amphotericin, Gibco). EA.hy926 (ATCC® CRL-2922™) were cultured in DMEM supplemented with 10% HI-FBS and antibiotics (30 µg/mL Gentamicin, 15 ng/mL Amphotericin). HaCaT immortalized keratinocyte cell line were cultured in KGM-gold keratinocyte growth medium (Lonza). THP-1 (ATCC® TIB-202™) leukemic monocyte cell line was cultured in RPMI supplemented with 10% heat inactivated FBS and antibiotics (30 µg/mL Gentamicin, 15 ng/mL Amphotericin).

## Immunofluorescence microscopy

Cells were fixed for 45 mins in 4% PFA at room temperature. After 2 washes in TBS, the cells were blocked with 5% goat serum and 5 mg/ml BSA at room temperature for 45 min in TBS . After blocking, Incubation was performed with primary antibodies diluted in TBS with 2.5% goat serum and 5 mg/ml BSA overnight in cold room under rotation. Next day, inserts were washed three times in TBS and incubated with secondary antibodies for 2–4 h at room temperature. The inserts were washed three times and mounted on slides using Prolong Gold antifade reagent mounting medium with DAPI (Invitrogen). Samples were visualized using a Nikon Ti-E microscope (Nikon) inverted fluorescence microscope equipped with Andor Neo/Zyla camera (Andor) and NIS elements advanced research software (Nikon) and a Plan Apochromat objective (Olympus). Fluorescence quantification was done by acquiring several images of each monolayer, then analyzed with *IntDen* measurement using Fiji [18].

## SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were performed according to the instructions from the manufacturer (Bio-Rad). 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% BSA. For visualization of the proteins, the PVDF membranes were incubated overnight with primary antibody. The following day, the membranes were incubated for 2 h with HRP conjugated secondary antibody and visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce).

## Quantitative polymerase chain reaction (qPCR)

RNA was purified from cells using Direct-zol RNA miniprep (Zymo research) according to the manufacturer instructions. cDNA was synthesized from 150 ng purified RNA using iScript cDNA synthesis kit (Bio-Rad), according to the instructions given by the manufacturer. EGFR expression was quantified using iQ-SYBR Green Supermix (Bio-Rad). Amplification was performed at 55°C for 40 cycles in iCycler Thermal Cycler (Bio-Rad), and data were analyzed using iCycler iQ Optical System Software. mRNA levels were normalized to GADPH as housekeeping gene.

## Intracellular infection and complement activation assay

Intracellular infection of keratinocytes was done as previously described . Briefly, Confluent keratinocytes in antibiotic free medium were infected with a multiplicity of infection (MOI) of 10-20 SA 2957/13 (invasive clinical isolate Lund University) in 24-well plates, the plates were centrifuged at 1000*g for 2 mins to enhance uniformity of SA attachment to keratinocytes and incubated at 37°C for 3 hours. Medium was then aspirated and changed to medium containing (100 µg /ml) gentamicin for 90 mins to kill extracellular bacteria. Cells were incubated for another 24 hours in medium containing (10 µg /ml) gentamicin, afterwards new medium containing 10% Normal human serum (NHS) or heat inactivated serum (HIS) was added to cells for 3 hours. Keratinocytes are finally washed twice in TBS and processed for CLIP.

## EGFR knockdown and internalization assay

Knockdowns of EGFR in Cell lines (HN4, HN5, HN7, HN8) were generated using Dharmacon™ Accell™ siRNA according to manufacturer instructions. Briefly, cells are seeded in 96-well plates at a density of 2*10^4 in DMEM with 5% FBS and antibiotics, the next day cells are treated with 1µM of EGFR siRNA or ntRNA for 48-72 hours in Accell delivery medium. Afterwards, cells were washed twice in TBS and then processed for CLIP or qPCR. In the EGFR internalization assay, the knockdowns were treated with (10µM) gefitinib or (50 ng/ml) TGF-α for 48 hours in KGM, then washed twice before processing for CLIP.

## Syndecan-1 shedding assay

EA.hy926 cells were seeded into multi-well culture plates and once they formed a confluent monolayer, they were stimulated with 1µM PMA in DMEM without FBS for varying lengths of time. Cells were washed twice in TBS and processed for CLIP. Cell culture supernatants were collected and Syndecan-1 concentrations in the medium were measured by ELISA (Diaclone) according to the manufacturer's directions.

## STAT-1 Nuclear translocation assay

THP-1 cells were seeded in multi-well plates, the next day new culture medium containing (200 ng/ml) PMA was added for 72 hours to obtain a macrophage-like phenotype, then fresh culture medium with 1% HI-FBS without PMA was added

for 24 hours as a rest period for the cells. New medium containing 1% HI-FBS and (10 ng/ml) Interferon-gamma (IFN-γ) was added for 30 mins. Afterwards cells were washed twice in TBS and processed for CLIP.

# Results

## Workflow of the immunoassay and quantification of complement activation

Briefly, following the desired stimulation of cells cultured in multi-well plates, cells were fixed and the proteins to be quantified were labeled using an HRP-conjugated antibody. Chemiluminescence was generated by addition of enhanced chemiluminescence substrate (ECL). Image of the chemiluminescence signal is taken and later analyzed with an image analysis software (Figure 1a), for chemiluminescence imaging of plates (CLIP) we used Chemidoc MP (Bio-rad) commonly used for imaging of Western blots.

We have previously used fluorescence microscopy to quantify deposition of complement activation fragments on keratinocytes using monoclonal antibodies against the terminal complement complex (TCC) [17]. However during quantification of the obtained images we noticed that the biological negative controls (cells treated with heat inactivated serum (HIS) lacking complement activity), or technical controls (cells stained with only a secondary fluorophore-conjugated antibody) were plagued with a high background fluorescence, reducing their contrast with the true signal. Quantification of fluorescence microscopy images yielded a TCC signal only a 20-30% above the background. However, when we quantified the same model using CLIP, the difference in signal between infected cells treated with normal human serum (NHS, positive biological control) and various negative biological and technical controls was substantially higher, ranging from 500-600% (figure 1b).

We then compared the CLIP method to Western blotting for the detection of two membrane-bound complement regulatory proteins, CD46 and CD59 in primary keratinocytes (Figure 1c). Because CLIP does not require several steps that are needed in Western blotting, including lysis of cells, denaturation of proteins, electrophoresis, and protein transfer to a membrane, the assay time was reduced by approximately three hours compared to Western blotting. Additionally, CLIP gave a strong, robust signal that was more reproducible than that obtained by Western

blotting. We also examined whether CLIP could provide information on the uniformity of the cell monolayer by performing a scratch assay of a monolayer of A431 cancer cell line. We observed a strong contrast between the acellular (scratch) and cellular part of the monolayer. This information could potentially be used to optimize the quantification of uneven monolayers, by selecting an area of interest that excludes acellular parts from the analysis (Figure 1d). Often it is desirable to measure the level of several proteins in the same sample for example to normalize measured protein levels to housekeeping proteins to correct for variations in cell number, or to normalize the level a phosphorylated protein to the level of its unphosphorylated form. We examined whether the CLIP method is compatible with stripping of antibodies and re-probing of the same cells. We stained 2 cancer cell lines HN4 and HN8 for a membrane protein EGFR, and then stripped the antibodies using a commercial stripping buffer. After re-probing with 2 different antibodies we stripped again and re-probed for EGFR, we found little to no loss of signal after 3 rounds of striping (Figure 1e), suggesting that the level of several proteins could be measured in the same set of cells.

To test the linearity and dynamic range of the CLIP assay signal, we performed a dilution series of Staphylococcus aureus bacteria in a concentration range of around 10^7 CFU/ml, attached to a 96-well plate. We detected the fixed bacteria using polyclonal anti-Staphylococcus aureus antibodies followed by detection according to the CLIP method. Quantification of the signal yielded a good coefficient of determination ($R^2$) value of 0.99 in this range, indicating that the assay has good linearity (Figure 1f).

## Quantification of membrane proteins expression, internalization, and shedding

To further validate the usefulness of CLIP, we used CLIP to quantify expression levels and internalization of an extensively studied membrane protein, the epidermal growth factor receptor (EGFR). EGFR is a transmembrane tyrosine kinase receptor, involved in cell growth and proliferation, and is over expressed in head and neck squamous cell carcinoma (HNSCC). We used small inhibitory RNA (siRNA) to knockdown EGFR in 4 patient-derived HNSCC cell lines. After 48 hours of treatment with EGFR siRNA or non-targeting RNA (ntRNA), cells were washed, fixed and stained for EGFR. After quantification of EGFR signal, cells were stripped and stained for GAPDH for normalization, intact monolayers in all conditions were confirmed using light microscopy (figure 2a). CLIP quantification of EGFR to GAPDH was compared to quantification made by quantitative PCR (qPCR), with GAPDH as a reference gene. We found that cells treated with EGFR siRNA, but not with ntRNA, had a significant decrease in EGFR expression, both on the mRNA

level as measured by qPCR, and on the protein level as measured by CLIP (Figure 2 a,b). Furthermore, to quantify EGFR internalization, we treated another EGFR knockdown model with either transforming growth factor alpha (TGF-a) a potent ligand of EGFR that induces EGFR internalization, or with the EGFR tyrosine kinase inhibitor gefitinib as a negative control. As expected, CLIP showed a significant decrease in surface EGFR signal in cells treated with TGF-a, but not with Iressa, demonstrating that CLIP is suitable for detection of internalization of membrane proteins (Figure 2c).

We also used CLIP to quantify shedding of a membrane protein, since shed proteins can be measured in the cell medium using ELISA, thereby validating CLIP quantification. Shedding of the ectodomain of syndecan-1, a transmembrane proteoglycan involved in growth and proliferation signaling, was previously shown to be induced by Phorbol 12-myristate 13-acetate (PMA) within minutes of treatment in endothelial cells. Quantification of the signal of syndecan-1 in cells treated with PMA at different time points, showed a time dependent decrease in the signal of Syndecan-1 on EA.hy926 cells (Figure 2d), that was abrogated in the presence of a TNF-alpha protease inhibitor (TAPI) known to inhibit syndecan-1 shedding. The human major histocompatibility complex (MHC) class I was used as a control and did not show similar response (Figure 2d). A time dependent increase of nanogram concentrations of Syndecan-1 in the medium was confirmed using ELISA (Figure 2e).

This data suggest that CLIP can be used in comparative quantification of a variety of membrane proteins, and as a quick method to verify knockdown models, as well as internalization or shedding of membrane receptors.

## Quantification of cytosolic and nuclear proteins

For quantification of stably expressed proteins in different cellular compartments, we used the keratinocyte cell line HaCaT which expresses beta actin as a cytoplasmic protein, lamins a/b as a nuclear protein, and MHC class 1 as a membrane protein. We then compared the signal from those proteins using 3 different permeabilizing agents, Saponin and Triton X-100 at a concentration of 0.1%, and Tween-20 at a concentration of 0.05%. Saponin specifically permeabilizes cholesterol containing membranes like the plasma membrane but has little effect on nuclear membranes, and Triton X-100 permeabilizes both cytoplasmic and nuclear membranes. While 0.05% Tween-20 did not show any significant difference in any protein signal compared to TBS alone, we found that Saponin significantly increased the signal of beta actin, but not the nuclear lamin signal, while Triton X-100 significantly increased the signal of both beta actin and nuclear lamin (Figure 3a). Interestingly, we found a similar increase in signal when

performing the quantification using fluorescence microscopy at 100X (figure 3b). This indicated that cytosolic and nuclear proteins can be accurately quantified using CLIP after appropriate permeabilization.

To further validate quantification of cytoplasmic and nuclear proteins, we aimed to quantify specific protein phosphorylation, which is important in activating or deactivating several signaling pathways in the cell. Signal transducer and activator of transcription-1 (STAT-1) is a protein involved in cellular proliferation and survival pathways, its phosphorylation leads to enhanced translocation to the nucleus. Using phospho-specific antibodies and a model of Interferon-gamma (IFN-γ) stimulated THP-1 cells, we were able to quantify the increase in phosphorylated STAT-1 (pSTAT-1) following 30 mins of IFN-γ stimulation (figure 3c). We also noticed a significant increase in pSTAT-1 signal when using Triton X-100 as opposed to Saponin as a permeabilizing agent, indicating translocation of the pSTAT-1 to the nucleus (Figure 3c). These data suggest that CLIP is suitable for a quick and sensitive quantification of protein phosphorylation and protein translocation and can be done in parallel.

# Discussion

In this study we describe the simple and robust comparative quantification method of chemiluminescent imaging in plates (CLIP). We have shown here that CLIP can be used to visualize and quantify several known biological phenomena as well or better than other commonly used methodology. We have shown that CLIP can be used to detect the level of deposition of proteins on the membrane, the level of membrane receptor proteins, and the shedding of membrane proteins. We showed that CLIP can be used as a rapid method to verify knockdown of specific proteins. It can also be used to quantify the level of expression of cytosolic proteins and quantify differences in their localization between cytosolic and nuclear compartments.

There are several advantages of CLIP over current immunoassays. Assays that use fluorescence detection methods often suffer from a high background signal due to cell or reagent autofluorescence that makes quantification of subtle changes difficult[19]. We showed the CLIP method was more sensitive in detecting subtle differences in complement activation, most likely because chemiluminescence signal has low to no background in cells. Moreover, the time to obtain quantitative results was less than that needed for fluorescence microscopy, since acquiring representative images with a microscope, and then choosing areas of interest from

those images to quantify the signal proved to be laborious and prone to operator bias.

We also showed that the simple workflow of CLIP is advantageous compared to Western blotting. The minimal manipulation of cells helped to yield faster and more reproducible results than a Western blot, likely because CLIP does not use several steps that can affect reproducibility. Specifically, the cell lysis step in Western blotting releases many proteases and phosphatases that can degrade the protein of interest over time, requiring the use of protease inhibitor cocktails and cold temperatures [20, 21]. CLIP does not require cell lysis and therefore minimizes the possible degradation of proteins and the reagents required. CLIP also does not require denaturation of proteins by SDS, gel electrophoresis, and transfer of proteins to a membrane which reduces both the time and the number of steps at which error can be introduced. CLIP was particularly advantageous when investigating membrane proteins. Due to their poor solubility, membrane proteins are particularly difficult to detect by Western blot. We used CLIP to detect several membrane proteins including CD46, CD59, Syndecan-1 and MHC I without having to use any special steps compared to soluble proteins.

We did not compare CLIP to immunoflow cytometric methods, however we believe that CLIP will also hold several advantages over this methodology. First immunoflow cytometry uses fluorescence detection which can suffer from background fluorescence similar to that we found in immunofluorescence microscopy. Secondly, flow cytometric methods require cells to be in solution. Therefore, adherent cells must be detached by using enzymatic or chemical methods. Enzymatic methods of cell detachment such a trypsin or accutase can remove or alter the protein of interest if it is expressed on the cell surface[22]. Chemical detachment, for example using EDTA, work by chelating magnesium and calcium ions that are required for many adhesion proteins. The loss of these ions can also affect the shape of membrane proteins and can make specific epitopes more difficult to detect. Lastly the cell detachment process could potentially stress the cells and cause changes in expression of some proteins in a stress response.

Methods that are similar in concept to CLIP, such as in-cell ELISA using immunostaining of cells followed by colorimetric detection, are also available. However, we believe that CLIP is advantageous because it uses any generic culture plates and common detection reagents that are available in most labs. Therefore, it does not require specialized and costly ELISA kits. Colorimetric readers typically read 96-well assay plates while chemiluminescence detection systems can ready any size and shape of culture vessel. Many cell types also require a coating of specific matrices such as collagen or matrigel, which can potentially change the optical properties of the plate. Because colorimetric detection requires an optically clear path, it is not compatible with cells that require these specific coatings.

Chemiluminescent detection on the other hand reads the signal from the top of the plate, and therefore is unaffected by matrix coatings of the plate and compatible with any cell type.

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# Figures

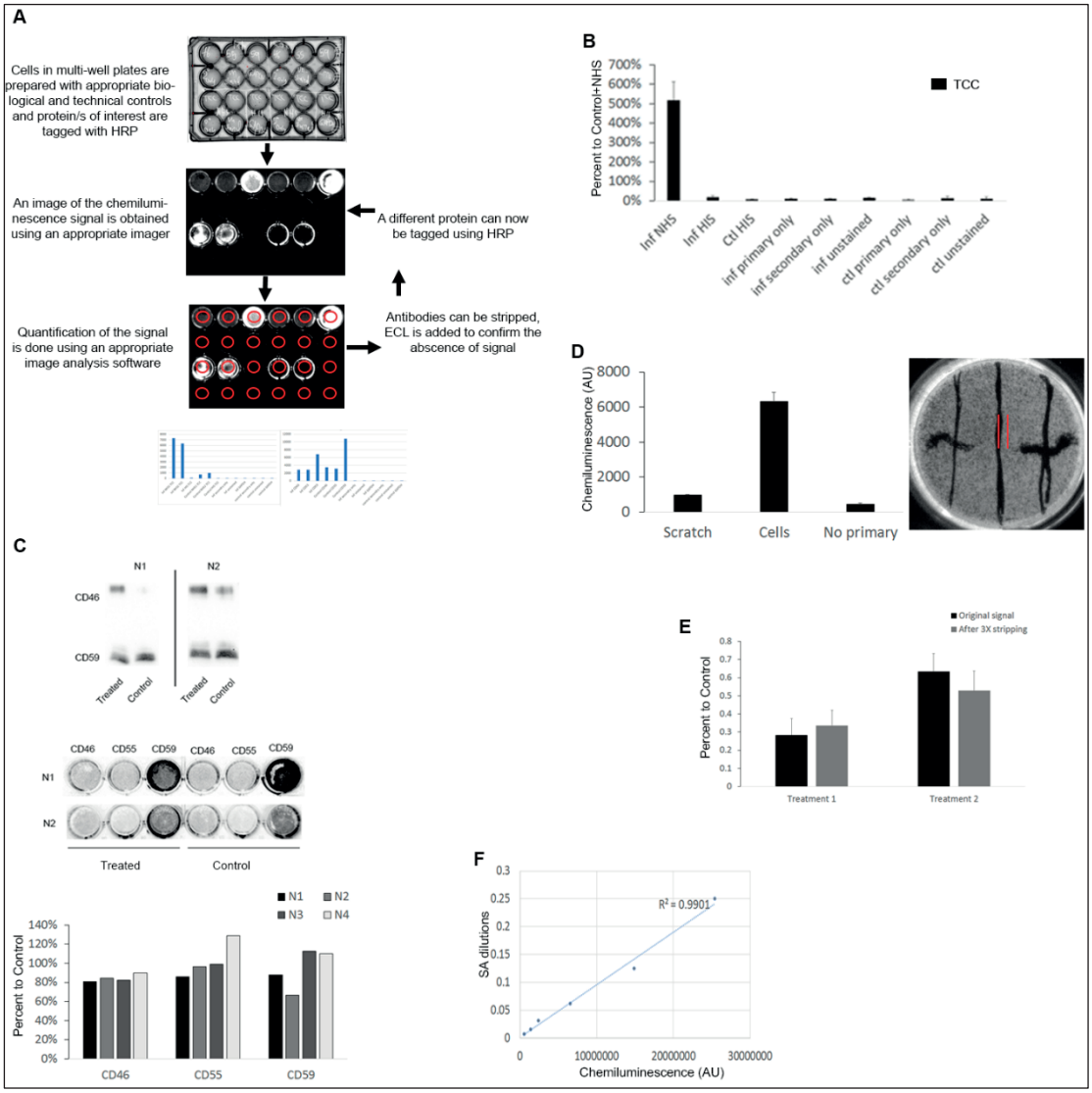


**Figure 1. Workflow of CLIP**. (a) a schematic depiction of the workflow of CLIP. (b) CLIP quantification of the TCC signal in keratinocytes infected (inf) or non-infected (ctl) with intracellular Staphylococcus aureus shows a significant increase in signal over ctl cells treated with NHS, only when infected cells are treated with NHS, but not with HIS, other technical controls are included as well. (c) a comparison of reproducibility between Western blotting (up) and CLIP (middle) when investigating membrane proteins CD46 and CD59in keratinocytes treated with different treatments, and the variation in the quantification made over 4 experiments (bottom). (d) A comparison of the CLIP quantification of the signal using area of interest that involves the scratch, the monolayer or an unstained monolayer (not shown). (e) EGFR was quantified in 2 cancer cell lines using CLIP, the cells were then stripped and proped for 2 other proteins, and later stripped and probed again for EGFR and the signal was quantified to compare the effect of stripping on signal. (f) To assess CLIP linearity, Staphylococcus aureus was serially diluted on a 96-well plate, and the fixed bacteria was quantified using CLIP, and regression curve was made with the obtained values.

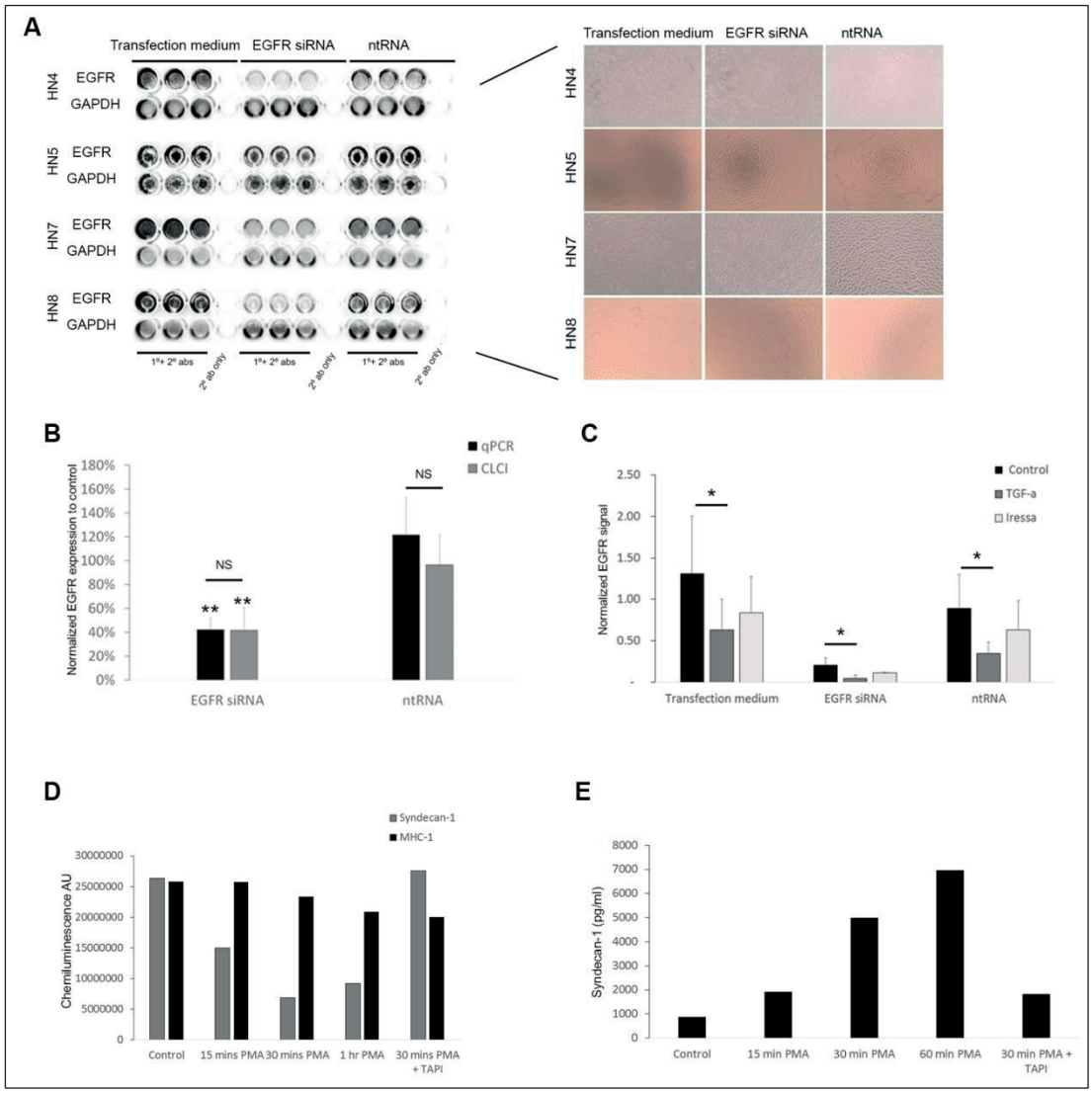


**Figure 2. Quantification of membrane proteins**. (a) EGFR was knocked down in HNSCC cell lines HN4, HN5, HN7, HN8 using siRNA, and the knockdown was investigated by staining for EGFR in cell monolayers using CLIP and appropriate controls, the chemiluminescent signal image is presented on the left and shows quadruplicate wells in different 96-well plates, the same wells were stripped and stained for GAPDH for normalization. Light microscopy images on the right confirm the presence of intact monolayers in all the conditions investigated. (b) The EGFR signal from CLIP was quantified and normalized to GAPDH and compared to the quantification made by qPCR. (c) Knockdown models of EGFR were treated with TGF-a or Iressa and the normalized EGFR signal obtained by CLIP was compared in different treatments. (d) CLIP quantification of time dependent PMA induced shedding of the membrane protein Syndecan-1 from EA.hy926 cells, which is inhibited in the presence of TAPI. MHC-1 was used as a control membrane protein. (e) Media from the PMA treated cells were collected and Syndecan-1 levels were measured using ELISA.

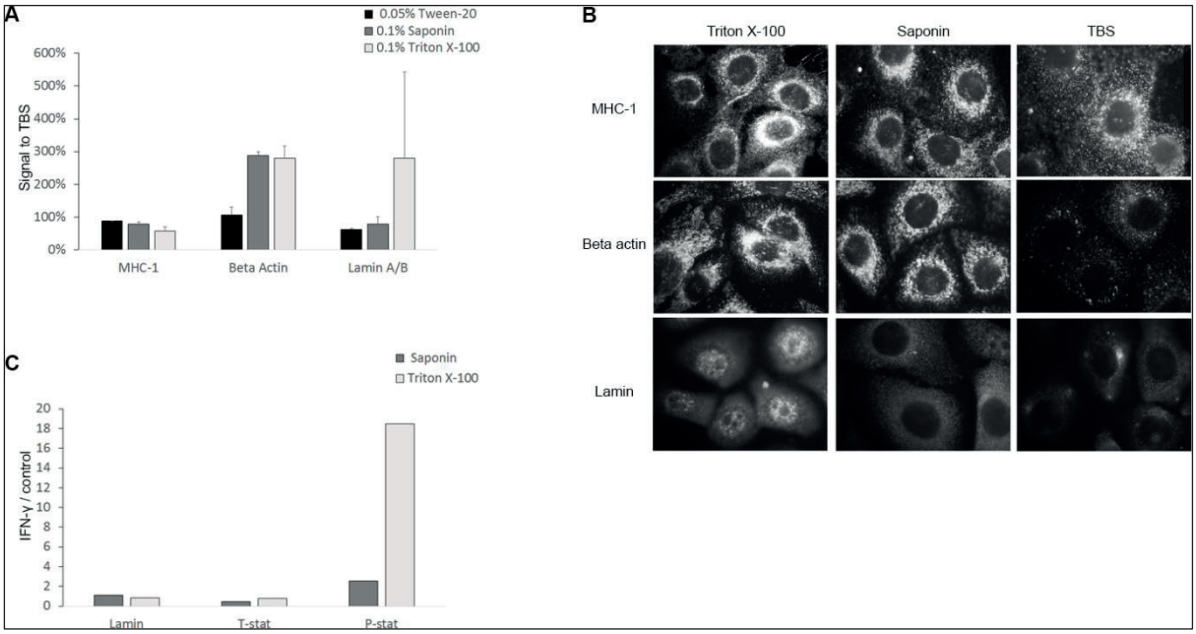


**Figure 3. Quantification of cytosolic and nuclear proteins.** (a) HaCaT cell monolayers are stained for proteins in different cellular compartments using different detergents, and quantification of the signal is done using CLIP. (b) The CLIP signal and protein localization was confirmed using Immunofluorescence microscopy. (c) After inducing differentiation of THP-1 cells to macrophage like phenotype, THP-1 cells were treated with IFN-γ for 30 mins and the level of total STAT-1 or phosphorylated STAT-1 was measured using CLIP, the different detergents are used to specifically permeabilize the cytosolic vs nuclear membrane. Lamin was used as control nuclear protein.