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The 'Ins and Outs' of complement-driven immune responses

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ABSTRACT

The complement system represents an evolutionary old and critical component of innate immunity where it forms the first line of defence against invading pathogens. Originally described as a heat-labile fraction of the serum responsible for the opsonisation and subsequent lytic killing of bacteria, work over the last century firmly established complement as a key mediator of the general inflammatory response but also as an acknowledged vital bridge between innate and adaptive immunity.

However, recent studies particularly spanning the last decade have provided new insights into the novel modes and locations of complement activation and highlighted unexpected additional biological functions for this ancient system, for example in regulating basic processes of the cell. In this review, we will cover the current knowledge about complement's established and novel roles in innate and adaptive immunity with a focus on the functional differences between serum-circulating versus intracellularly active complement and will describe and discuss the newly discovered cross-talks of complement with other cell effector systems particularly during T cell induction and contraction.

INTRODUCTION

The immune system of eukaryotes has evolved under the constant selective pressures driven by ever-changing pathogenic organisms trying to exploit the host for their own survival. The broad range of disease-causing pathogens and their various modes of ensuring procreation within the host are staggering, however infectious agents can generally be sub-grouped into being either intracellular or extracellular bacteria and toxins, protozoans, fungi, viruses or complex extracellular

parasites (1). The immune system has evolved complex and multifaceted defence mechanisms involving the interrelated innate and adaptive arms to sense and combat such a variety of pathogens. The innate immune system forms the first line of defence and stands guard at potential sites of entry such as the skin, gut and lung epithelial cells and layers where secreted antimicrobial peptides help to protect the integrity of these physical barriers (2). If a microbe has nonetheless been able to invade the host, a range of 'cellular patrols' such as macrophages and neutrophils within tissues are poised to detect highly conserved pathogen-associated molecular patterns (PAMPs) using a variety of germ-line encoded receptors such as the toll-like receptors (TLRs), the Nod-like receptors (NLRs) and complement system-derived receptors that are called pattern recognition receptors (PRRs) (3, 4). Activation of these PRRs leads to immune cell activation, with the induction of appropriate protective effector responses, specific for the cells that receive these signals, and clearance of the invading pathogen. The host's ability to differentiate between self and non-self is a central governing paradigm of immunology that allows the immune system to clear infection whilst controlling unwanted injury to host tissues. Particularly, the innate responses are important in controlling the early stages of infection and in subsequently guiding the formation of specific cellular and humoral responses. However, although all PRR systems have initially been discovered and defined as sensor and effector systems fighting pathogens, it is now understood that they also play central roles in the detection and removal of harmful self-derived molecules, so-called danger-associated molecular patterns (DAMPs), commonly generated during cell (hyper)activity, stress responses and cell death (5, 6). Furthermore, emerging data is also demonstrating that PRRs are involved in the post-inflammatory tissue repair phases and in general immune homeostasis – and

that PPRs effector functions in all phases of the immune response are unexpectedly closely linked to cellular pathways that direct basic cell physiological processes such as survival, cell death and metabolic reprogramming (7, 8).

The complement system is one of the most ancient of the preformed mediators of host defence and comprises over 50 serum-circulating proteins and cell-surface receptors and regulators which act in concert to form a large and complex effector system (9). Complement circulating in serum and the lymphatic fluids is considered a sentinel system that 'idles' mostly in an inactive pro-enzyme form. It is, however, triggered and activated almost immediately upon pathogen entry and plays a crucial role in controlling the early stages of infection by direct lysis of pathogens and by facilitating the recruitment and activation of various types of leukocytes (9). This critical role of complement as a PPR is underpinned by the fact that complement-deficiencies lead to recurrent severe infections – and were, in fact, a sure death sentence before the discovery of antibiotics (10). Similar to the TLR and NOD PRR systems, complement also recognizes DAMPs and is instrumental in the removal of apoptotic cells (11). However, complement's activity goes well beyond these innate functions during the early phases of the immune response and also heavily impact both B and T cell immunity (12, 13). Furthermore, the recent discovery of intracellular complement activation in immune cells and its importance for normal cellular functioning has led to the emerging concept of an intracellular "complosome" which exists and functions independently of liver derived complement (7, 14). These intracellularly-generated complement products can induce signalling events through their respective receptors located either on intracellular compartments and/or, after secretion of the intracellularly generated fragments, via their receptors expressed on the cell surface (7, 15) and govern, for example, human

Th1 responses by driving the signalling pathways and metabolic reprogramming necessary for effector responses (8). In this review we will briefly cover the known roles of liver-derived complement in controlling the early stages of an immune response against a pathogenic invader ('out-side' function of complement) and then discuss in depths the emerging roles of the complosome ('in-side' function of complement) as an independently functioning arm of the complement system in driving the cellular machinery required for the initiation and regulation of T effector cell responses. By the end of this review, we would like to make the case that complement is active in an unexpectedly wide array of cellular processes, of which many are not related directly to immunity and that much remains yet to be discovered about the complement system – for the basic scientist, the clinician and pharmacological industry alike.

'Classic' serum complement activation and regulation

The modes of activating complement are well defined and traditionally divided into three separate activation pathways - the classical, the lectin and the alternative pathway (9) (Figure 1). All three pathways share several common components but differ in the nature of their respective initiation trigger and recognition molecules due to differences in microbial sensing modes (9). This is obviously advantageous to the host as it allows for the detection and subsequent clearance of a broad range of pathogens. The classical pathway, which was the first of the pathways to be discovered and defined, is initiated by the binding of C1q to complement fixing antibodies (primarily IgM and IgG1, 2 and 3 subtypes) bound to their specific antigen on the pathogen's surface. Subsequent to the C1q-antibody interaction, the C1q-associated proteases C1r and C1s undergo auto-activation and transactivation,

respectively, leading to the proteolytic cleavage of C4 into C4a and C4b and then C2 into C2b and C2a. The protein complex of C4bC2a forms the classical pathway C3 convertase, an enzyme complex that activates the complement key component C3. Mannose-binding lectin (MBL), ficolins and collectin-11 of the lectin pathway recognise carbohydrate moieties on the surfaces of pathogens such as those found on members of the *Salmonella spp*, and utilize the mannose-binding protein-associated serine proteases (MASPs) 1 and 2, to activate C4 and C2 to then form the lectin pathway C3 convertase (which is the same as the classical pathway C3 convertase). Activation of the alternative pathway is initiated when C3b-like molecules that are continuously generated in blood via spontaneous hydrolysis ($C3[H_2O]$) bind covalently to target surfaces and recruit factors B and D to then form the alternative pathway convertase (C3bBb). The alternative pathway is also called the 'amplification pathway' since it potentiates the activity of the lectin and classical pathways (9) (Figure 1). C3b is highly reactive by virtue of its activation-exposed thioester group, and binds covalently to nucleophilic groups present on cell surfaces (16). C3b deposition is also the first step towards C5 convertase formation, which cleaves C5 into C5a and C5b fragments. The classical and lectin pathway C5 convertases are C4bC2aC3b and the alternative pathway C5 convertase is C3bBbC3b. C5b is required for the assembly of the membrane attack complex (MAC, a multimeric structure containing complement components C5b-C6-C7-C8-polyC9), which forms transmembrane channels on the surfaces of pathogens or infected cells causing membrane instability and cellular lysis due to osmotic stress (17). C3b and C4b are opsonins and tag pathogens for uptake and destruction by phagocytic cells such as neutrophils and macrophages (18). It is important to note here that it is now becoming increasingly clear that convertase-independent cleavage of C3 and C5 by

specific proteases is of substantial physiological importance. For example, several proteases of the co-agglutination system (19) and of the ancient protease families representing cathepsins and granzymes (7, 14, 20, 21) cleave C3 and C5 into bioactive fragments and these non-classical means of complement activation play key roles not only in the activation of normal Th1 cell responses (see below) but also in several disease settings including trauma and sepsis (14).

It is critical that complement activation is tightly regulated as activated C3b and C4b not only bind to pathogens but also non-discriminatively to host cells where they could induce unwanted tissue damage and inflammatory disease (18). This vital complement control is achieved through a range of fluid-circulating and membrane-bound complement inhibitors and regulators (18) (Figure 1). A central focus of complement control is on the alternative pathway amplification loop and mediated by proteolytic inactivation of deposited C3b and C4b (cofactor activity by CD46, factor H, C4b binding protein, and complement receptor 1, CR1; CD35), rapid disassembly of C3 and C5 convertases (decay accelerating activity, CD55, and CR1), and inhibition of MAC formation and insertion (CD59, vitronectin) (18) (Figure 1). Interestingly, the complement receptors and regulators not only protect host cells from complement attack but are also able to transmit intracellular signals upon activation (22, 23) – and this is likely the reason why complement is such an active participant in a wide range of effector responses of immune cells during inflammatory processes (13, 22) (see below).

Complement in innate immunity and the inflammatory reaction

The early stages of infection are typically confined to a localized area such as a wound or other portal of entry of infectious agents, and are met with an acute phase

of localized inflammation mediated by a combination of inflammatory cytokines produced primarily by tissue resident macrophages, and, critically, the anaphylatoxins C3a and C5a generated via complement activation. This local inflammation is driven by increased permeability of small blood vessels and smooth muscle contraction (24, 25) allowing plasma proteins and immune cells to enter tissues and this phase is typically associated with the characteristic symptoms of fever, malaise and localised swelling. The anaphylatoxins C3a and C5a are small, soluble mediators of inflammation which have pleiotropic effector functions that orchestrate both local inflammation and the development of adaptive immune responses (26, 27). They exert their effects by binding to their specific receptors, the C3a receptor (C3aR) and the C5a receptor (C5aR1 or CD88), which belong to the superfamily of seven transmembrane spanning G-protein coupled receptors (GPCRs). Additionally, a second C5a receptor, C5aR2 (GPR77) exists which is uncoupled from G-proteins and has a high affinity for the des-Arginated form of C5a (C5a-desArg) (28). Anaphylatoxin receptors are widely expressed on cells of myeloid origin including neutrophils, monocytes/macrophages, basophils, eosinophils, mast cells and dendritic cells as well as on non-myeloid cells such as the epithelia (29), endothelia (30) and smooth muscle cells (31). Anaphylatoxins are key to the influx of innate immune cells into tissue as they possess potent chemotactic properties, with C5a promoting the recruitment of neutrophils, macrophages, DCs and basophils while C3a gradients attract mast cells (32-36). C5a is also required for normal neutrophil degranulation and the oxidative burst in these cells that mediates killing of intracellular bacteria (37, 38), while histamine release from mast cells is driven by C3a (39). Further, C5aR1 signalling activates the lipoxygenase pathway and arachidonic acid metabolism (40) leading to increased eicosanoid production in

neutrophils and monocytes. The anaphylatoxins are also heavily involved in the priming and activation of 'incoming' leukocytes, enhancing their ability to produce pro-inflammatory cytokines. For example, ligation of the C3aR on the surface of macrophages and DCs synergizes with TLR ligation by bacterial endotoxin to induce the assembly of an active NLRP3 inflammasome, which is crucial for the production of the core pro-inflammatory cytokine IL-1β (41). IL-1β exerts numerous effects on the vascular endothelium, and can increase endothelial expression of chemokines and adhesion molecules, thus promoting leukocyte recruitment and extravasation, respectively (42). Furthermore, C5aR stimulation of the endothelium leads to up-regulation of genes encoding various molecules involved in cellular adhesion and transmigration such as E-selectin, intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1), thereby initiating immune cell rolling and adhesion and the first steps of leukocyte extravasation into tissues (43).

Together with the anaphylatoxins, the opsonins C4b, C3b, iC3b, C3dg and C3d are also generated during serum complement activation. These latter fragments bind to the surface of particles or cells and enhance their uptake by phagocytes (44). For example, CR1 (CD35) is a transmembrane glycoprotein that can bind C3b and C4b that has been deposited onto the pathogen or other target surface upon complement activation. CR1 engagement on neutrophils and macrophages induces the phagocytic uptake of opsonised pathogens (45) – an event that is synergistically supported by C5aR1 activation (46). The Complement Receptors 3 (CR3, CD11c-CD18) and 4 (CR4, CD11b-CD18) belong to the β2 integrin family of adhesion molecules (47). They are also present on neutrophils, monocytes and macrophages and can bind the inactivated form of C3b (iC3b) attached to a pathogen surface or cell (48, 49). Activation of CR3 has recently been shown critical to the induction of

phagocytosis in neutrophils, monocytes and macrophages and to the neutrophil oxidative burst (50). In addition, CR1 activation on macrophages induces secretion of the proinflammatory cytokine interleukin (IL)-12 which is a potent activator of T cells. In sum, serum-derived complement activation fragments are critical mediators of the immediate inflammatory reaction driven by innate immune cells and aiming at containing pathogen breach in its earliest stages.

Complement as key instructor of adaptive immunity

Direct effects of complement on APC function

The increasing awareness over the last decades that complement is not a mere pathogen killing machine but that complement receptors and regulators evoke catered effector responses by a range of immune cells delivered an explanation for the long-known but ill-understood fact that complement dysregulation also contributes to autoimmune diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS) etc. Among the first identified cells (aside from B cells, see below) that demonstrated a clear need for incoming complement-mediated signals for normal maturation and effector functions were macrophages and antigen presenting cells (APCs) – the cells that are key to bridging innate and adaptive immunity. It is in the tissues, that immature professional APCs, namely dendritic cells, capture antigens and process them for presentation as MHC-peptide complexes to T cells in the draining lymph nodes. In the immature state, DCs are highly efficient at capturing antigen, but are poor stimulators of T cells as they lack the co-stimulatory molecules CD40, CD54 and CD86 required for effector cell differentiation (51). Upon receiving maturation signals the DCs lose their ability to

take up antigen but, instead, increase their costimulatory molecule expression (52). Mouse studies have shown a strict requirement for anaphylatoxin-mediated signals for normal and efficient DC maturation and the production of polarizing cytokines that subsequently direct T cell differentiation and therefore adaptive immunity (53, 54). C1q is now also emerging as an important regulator of APC function as C1q enhances surface expression of CD83, CD86, HLA-DR, and CCR7 on DCs (55) and 'C1q-experienced' DCs secrete more IL-12p70 compared to immature DCs, with C1q-primed mature DCs inducing production of IFN-γ by co-cultured T lymphocytes (55). Furthermore, to 'meet' and stimulate cells of the adaptive immune system, the activated DCs must migrate to the draining lymph nodes and complement has been shown to play a role in this activation induced migration of the DCs to the secondary lymphoid organs: Lack of serum complement C3 affects CCR7 expression, a lymph node homing chemokine receptor, on pulmonary DCs, thus altering their traffic to the draining lymph node upon pathogen challenge (56). However, aside from impacting adaptive immunity via the 'DC route', complement has also profound direct effects on adaptive immune cells.

Direct effects of complement on B and T cell effector responses

Both B cells and T cell express a range of complement receptors and regulators (57-59). A prominent role for complement in B cell response had been suggested already in the 1970's by the observation that B cells bound C3 fragments to their surface and that mice depleted of serum-circulating C3 via cobra venom factor treatment have an impaired humoral response against T cell-dependent antigens (60, 61). Subsequent studies then established that CD21 (CR2), and to a lesser extent CD35 (CR1), is

required for normal antibody production (62, 63). On mature B cells, engagement of the CD21-CD19-CD81 coreceptor complex by C3d-opsonized antigen favours their migration to the B/T cells interaction zone within the lymph node and lowers the threshold of B cell activation. Thus, C3 plays the role of an natural adjuvant during B cell function (64, 65). Interestingly, CD21 is also involved in the actual shaping of the specificity of the B cell population itself. The source for protective natural antibodies in the immune system is a subset of B cells termed B-1 cells. B-1 cells are positively selected during development and CD21 signalling on B cells has been shown being important for the expansion and maintenance of these cells (66). Furthermore, CD21 contributes also to the maintenance of memory B cell responses. Indeed, it has been shown that CD21 plays a crucial role for the function of the follicular dendritic cells (FDCs) which are central to the initiation of the adaptive immune response. FDCs capture complement-opsonized antigen via CD21 and allow thus for long-term retention of the antigen in germinal centres and (re)presentation of the antigen to previously primed B cell (67). In line with the growing understanding that complement is also required for cell homeostasis and negative control of immunity, CD21 seems to also contribute to the negative selection of self-reactive B cells as mice lacking either C4 or CD21/CD35 fail to induce B cell anergy towards self-antigen (68). The complement-mediated mechanism preventing the production of autoantibodies is not fully understood but may operate through lowering the threshold of B cells for apoptosis induction during the negative selection process (69).

The work of several groups on the direct role of complement in the modulation of T cell responses over the last decade has led to two surprising findings. Firstly, direct complement receptor-mediated signalling is critically required for normal

induction of T helper type 1 (Th1) responses but appear to affect Th2 and Th17 responses only indirectly. Secondly, Th1 induction both in mice and humans requires complement activation fragments generated by the APCs and/or T cell itself and seems to be mostly independent of serum-derived complement (27, 70). The first *in vivo* evidence demonstrating an impact of local complement on T cell responses has been revealed using a transplant model. In these studies, C3-deficient kidneys were protected from T cell-mediated graft rejection when transplanted into a wild type (WT) host, despite a normal systemic complement compartment (71). Furthermore, T cells in chimeric mice with C3-deficient bone marrow-derived cells did not respond to alloantigenic stimuli with IFN-γ production (27, 72). Although the liver is generally viewed as the main source of systemically circulating complement, several groups have demonstrated over the years that immune and non-immune cells can locally produce complement components (73-76). In regards to T cell activation, subsequent studies using mouse models demonstrated that during the cognate interaction between T cells and APCs, T cell receptor (TCR) activation in conjunction with CD28 costimulation, induce complement production and secretion of the key components C3, C5, Factor (F) B, and FD in both cells. This is followed by extracellular C3 and C5 convertases formation and the generation of the complement activation fragments C3b, C3a, C3a-desArg and C5a and C5a-desArg in the T cell-APC synaptic space. Simultaneously, the APC-T cell interaction in mice also upregulates the C3aR and C5aR expression on both cells which then allows these receptors to bind the extracellularly generated anaphylatoxins and to initiate C3aR and C5aR-driven signals for the specific production of IFN-γ and Th1 induction (27, 70, 72). This model of locally and autorine functioning complement aligns with the findings that APCs from C1q-, C3-, FB- and FD-deficient mice exhibit a less

activated phenotype and have a reduced capacity to stimulate antigen specific T cells (53, 70, 77, 78) while T cells and APCs from *Daf*$^{-/-}$ mice produce locally increased anaphylatoxins (because of unrestrained local C3 and C5 convertase activity) and have, thus, hyperactive Th1 responses (27, 72, 79).

As mentioned above, although it is well-understood that complement also impacts on Th2 and Th17 responses, these effector responses appear to be regulated by 'complement-instructed' epithelial cells and APCs, respectively, rather than by T cell-expressed complement receptors. Furthermore, data generated about this subject are currently mostly derived from mouse models (80). For example, C3aR and C5aR signalling cyclic adenosine monophosphate (cAMP), extracellular signal-regulated kinases (ERK), nuclear factor kappa-light-chain enhancer of activated B cells (NF-kB) stimulate dendritic cells to secrete pro-inflammatory cytokines such as IL-12, IL-23, IL-6 and TGF-β which are instrumental in mediating either Th2 or Th17 responses (81-83). C5a particularly affects the generation and modulation of proinflammatory T cell effector responses of the Th17 type by regulating IL-6 and IL-1-β produced by DCs and/or macrophages (84). Interestingly, while, TLR2 and C5aR induced signals synergize in mouse DCs to induce IL-12p70 production, C5aR-deficient DCs from the spleen generate more IL-6 and IL-23 compared to C5aR-sufficient DCs when stimulated with the TLR ligand Pam3Cys (85). This strongly indicates that the specific impact of the anaphylatoxins on Th17-instructing cytokine production is dependent on the type of APC sensing the C5a signal and the TLR that is concurrently stimulated. Aligning with this notion, Lajoie and colleagues found that C5a inhibits house dust mite-induced IL-23 production from bone-marrow derived DCs in an asthma model, while C5aR-deficient bone marrow-derived DCs are fail to produce IL-1β, IL-6 or IL-23 upon combined

ovalbumin (OVA) and lipopolysaccharide (LPS) activation (86). A similar role for the anaphylatoxins in the regulation of human Th17 is supported by two recent observations: *Candida albicans* triggers IL-6 secretion in human peripheral blood mononuclear cells (PBMC) in a C5a-dependent manner (87) and C3aR engagement induces IL-1β production in human monocytes through the induction of the NLPR3 inflammasome. Such C3a-instructed monocytes subsequently induce strong Th17 response in activated human $CD4^+$T cells while leaving Th1 and Th2 responses unaltered (41). Although our understanding of the 'complement-T cell relationship' is in its early hours and the exact underlying mechanism through which complement impacts on T cell immunity are clearly far from being defined, the idea that complement forms a critical bridge between innate and adaptive immunity is now becoming firmly integrated into mainstream immunology.

The role of intracellular complement activation in human Th1 induction.

The intracellular C3 system

Although the importance of anaphylatoxin receptor signalling - at minimum on the APC level - for the normal induction of Th1 responses in mice has been conclusively demonstrated in several studies using pertinent disease models and C3aR and/or C5aR1 knock out animals, the complement-mediated pathways regulating Th1 immunity in humans are quite distinct. In humans, combined signalling by the C3b/C4b-binding complement regulator CD46 and the C3aR, stimulated in an autocrine fashion by cell derived complement fragments, has been established as an absolute pre-requisite for Th1 induction, and more specifically production of the pro-inflammatory cytokine IFN-γ (figure 2a) (7, 22, 88, 89). Accordingly, patients that are

deficient in CD46 cannot mount normal Th1 responses and suffer from life-long infections. In some cases, CD46-deficient patients even develop common variable immune deficiency (CVID) and require monthly immune globulin (IgG) infusions. Similarly, patients that are deficient in serum C3, also have incidences of recurrent infections in early childhood, however, C3-deficient patients seem to acquire immune protection with age as their infections cease to occur during adulthood (7, 13, 15, 89). CD46 was initially discovered as a ubiquitously expressed complement regulator that functions as a cofactor for the serine protease FI during the proteolytic inactivation of C3b and C4b deposited on host tissue (90). It became quickly clear that CD46 also transduces signals upon activation and can regulate macrophage and T cell function (22, 88, 91). Importantly, although mice have a gene (*Cd46, Mcp*) that has homology to the human *CD46* gene, mice (and also rats and rabbits) only express mCD46 in immune-privileged tissues such as the testis, the brain and the eye (45, 92, 93). Furthermore, while human CD46 is expressed in different isoforms due to splicing from a single gene and the distinct intracellular domains have clear signalling capacities (94) (see below), the mouse CD46 protein is only expressed in a single isoform and its intracellular domain does not contain any known signalling motifs (95). While the rodent-specific protein Crry/p65 has cofactor activity for mFI in the cleavage of mC3b and is indeed expressed on mouse lymphocytes, its activation favours rather Th2 induction via IL-4 secretion (96) and *Crry*$^{-/-}$ mice have increased, instead of defective Th1 responses (97). Thus, the current mechanistic models of complement-driven effector cell responses in mice do not integrate autocrine C3b-mediated signals.

Another unexpected and likely paradigm-shifting observation has as yet only been made in the 'human system': Complement activation is, surprisingly, not confined to

the extracellular space, but occurs intracellularly and is required for homeostatic T cell survival and the induction of Th1 effector function (figure 2) (7, 8). Importantly, engaging intracellular complement receptors induces signalling pathways distinct from those triggered by the same receptors when expressed on the cell surface (7, 14, 89). This implies that the location of complement activation dictates the functional outcome of complement activation and the existence of a novel concept in which complement can induce 'inside-in' and 'outside-in' signalling (14) (see below). Specifically, in resting human $CD4^+$ T cells, activation of C3 occurs intracellularly continuously via cathepsin L (CTSL)-mediated cleavage, and C3a generated via this 'pathway' stimulates intracellular C3aR signalling on lysosomes, thereby sustaining homeostatic T cell survival (figure 2b) (see below). Upon sensing of danger, in the case of T cells, TCR activation, this intracellularly generated C3a and C3b rapidly translocate to the cell surface, where they engage cell surface C3aR and CD46, respectively, events together driving IFNγ production. Although the biologic function(s) of intracellular complement activation is, at this point, mostly studied in human $CD4^+$ T cells, it occurs in all cells analyzed so far – and hence is likely of broad physiological relevance (7). Importantly, this intracellular C3 system regulates the magnitude of Th1 responses and its dysregulation contributes to human autoimmune disease: $CD4^+$ T cells isolated from the inflamed joints of patients with juvenile idiopathic arthritis (JIA) have significantly increased intracellular C3 activation that drives their hyperactive Th1 responses. Excitingly, the pathogenic C3 activation levels are amendable to pharmacological intervention as activation of the patient's T cells in the presence of a cell-permeable CTSL inhibitor fully normalized intracellular C3a generation and Th1 responses in culture (7). The latter data though have been generated *in vitro* and fast translation into *in vivo* application is currently

not realistic as CTSL has many additional functions (98) and any therapeutic would need to be carefully targeted to not only specific cells but likely also to specific cellular sub-compartments. Nonetheless, the discovery of 'targetable' CTSL as C3 activating protease further funnels into our understanding that complement-activating proteases in general may be of more physiological and therapeutic importance than previously thought. Although intracellular C3 activation occurs in a broad range of cells, the activating protease(s) most likely differ from one cell type to another. While the lysosomal protease CTSL also processes C3 in human monocytes, C3 activation in human epithelial cells is CTSL-independent (7). Thus, each cell type may possibly rely on a different intracellular C3 activating machinery. In fact, convertase-independent activation of complement in serum or on the cell surface has been described early on (21, 99, 100), and one can envision that this plays an important role for the control and activation of the immune responses at microenvironmental level by providing a mode for more rapid processing of the complement compounds. For example, cathepsin D (CTSD) has been shown to cleave C5 to generate active C5a *in vitro* (20) and various proteases from the coagulation pathway including Factors Xa and XI are well known to be potent C3 and C5 activators (19, 99, 101). Most of these proteases are produced by the liver and pancreas and therefore might have a preponderant role as sentinel proteases patrolling the host tissues and digestive track. On the other hand, immune cells such as mast cells can produce various proteases as granzyme B, tryptase, carboxypeptidases which can act more locally and timely (21, 102, 103) – thus, this 'systemic versus local versus intracellular' scheme for complement function may also extend to the complement-activating proteases.

The intracellular C5 system

After the discovery of the intracellular functional C3 activation system, it was natural to inquire if T and other immune cells contain also an intracellular C5 system and whether this system may contribute to cell effector function(s). Indeed, non-activated human $CD4^+$ T lymphocytes contain intracellular stores of C5 and produced low levels of C5a in the resting state (89). TCR activation, and particularly in conjunction with CD46 costimulation, increases the amounts of intracellular C5a, which is associated with secretion of portions of this intracellularly generated C5a to the cell surface (figure 2a). Human $CD4^+$ T cells also demonstrate a specific C5aR expression pattern: while the C5aR1 is exclusively expressed intracellularly in resting and activated cells, the C5aR2 can be found both intracellularly and on the cell surface (89). A subsequent assessment of functional consequences of intracellular C5 activation revealed that it surprisingly drives the assembly of the NLRP3 inflammasome in T cells. Inflammasomes are multiprotein complexes consisting of caspase 1, PYCARD, and NALP and assemble upon PAMP or DAMP sensing via their upstream PPRs (104). The exact composition of an inflammasome depends on the inducing signal which initiates inflammasome assembly, e.g. dsRNA derived from pathogens will trigger one inflammasome composition whereas self-derived danger, such as cholesterol crystals will generate a different variant (5, 105). The NLRP3 inflammasome activates caspase-1 which in turn then processes the proenzyme forms of the key host pro-inflammatory cytokines Interleukin 1β (IL-1β) and Interleukin 18 (IL-18) into their active forms. NLRP3 inflammasome function requires a priming signal 1 (which induces *NLRP3* and *IL1B* gene transcription) and a signal 2 that induces functional inflammasome assembly (105). The NLRP3 inflammasome is present in myeloid innate immune cells and in several non-immune cell types such

as microglia, endothelial and epithelial cells (106-108). However, canonical NLRP3 inflammasome activity had previously not been described in lymphoid adaptive immune cells – thus, the presence of a (C5-driven) NLRP3 inflammasome in T cells was a surprising observation. Mechanistically, intracellular engagement by the C5aR1 upon T cell stimulation increases *IL1B* gene expression (while CD46 simultaneously drives *NLRP3* gene expression) and induces the generation of mitochondrial reactive oxygen species (ROS) (109) and via this provides signals 1 and 2 for NLRP3 inflammasome activation (figure 2a). NLRP3 inflammasome-driven caspase-1 activation leads to IL-1β secretion, which subsequently promotes specifically interferon (IFN)-γ production and Th1 differentiation (but not Th2 and Th17 induction) in an autocrine fashion. The biological relevance of complement-induced IL-1β production by T cells is supported by the finding that T cells from patients with distinct gain-of-function mutations in NLRP3, and that suffer from cryopyrin-associated periodic syndrome (CAPS), have a hyperactive Th1 response that can be re-set *in vitro* to normal levels with a specific NLRP3 inhibitor, MCC950 (89, 110, 111). Furthermore, utilization of T cells from mice that are *Nlrp3*-deficient showed that normal Nlrp3 inflammasome activity in T cells in not only required for optimal protective IFN-γ immunity during viral infection but also controls the balance of Th1 versus Th17 responses during intestinal inflammation (89, 112).

In sum, T cells contain intracellular C3 and C5 activation systems and the regulated crosstalk between intracellularly activated complement components, the complosome, (14) and the NLRP3 inflammasome emerges as fundamental to normal IFN-γ production in human $CD4^+$ T cells. The unexpected finding that established innate immune pathways previously not thought to be operative in adaptive immune cells are critical to the initiation of Th1 immunity expands our

current knowledge about the evolution and function of the immune system substantially. Importantly, and similar to the intracellular C3 system, intracellular C5 activation is not confined to T cells but also present in other immune cells, and we observed that intracellular C5aR1 activation in human monocytes is required for appropriate cellular responses to self-derived DAMP signals which indicates that this system is also important during the initiation of sterile inflammation (unpublished data). Thus, while serum-circulating complement constitutes a vital sentinel system for direct pathogen sensing and removal, the complosome emerges as critical regulator of (adaptive) immunity via crosstalk with other intracellular effector systems. The recent observations that the NLRP3 protein functions as transcription factor regulating *Gata3* gene expression in the nucleus of mouse $CD4^+$ T cells (113) and that secreted NLRP3 inflammasome amplifies as extracellular danger signal the inflammatory response of macrophages (114) fits into the emerging concept that these old PRR systems likely have additional and yet to be discovered functions at novel locations.

The role of complement in T cell homeostasis

Complement in the maintenance of the resting T cell pool

Our perception of complement as mostly pro-inflammatory effector system is now changing slowly as a body of work shows that the complement system also plays prominent roles in the negative control of immune cell effector responses, and, thus, general immune homeostasis (9, 14, 115). For example, the intracellular 'tonic' generation of C3a via CTSL cleavage is critically required for T cells to survive in the resting and circulating state. C3a binds to the C3aR expressed on lysosomes and

sustains low-levels of mTOR required for such survival (figure 2b). Neither the exact signalling pathways driven by the lysosomal C3aR1 mediating this function nor the modes of regulation of intracellular CTSL-mediated C3 activation are currently defined (7). This unexpected finding, however, also initiated further assessment of the intracellular 'C3 system' in patients with serum C3 deficiency because T cells from these individuals are unable to mount *in vitro* Th1 responses but have no survival defect *in vitro* and *in vivo.* This led to the discovery that all immune cells from patients with systemic C3-deficiency generate sufficient intracellular C3a from the mutated C3 protein to survive, but fail to secrete the C3 and activation fragments (7). Thus, homeostatic pro-survival signals are normally generated, while autocrine cell surface activation of complement receptor pathways required for T cell effector responses cannot be induced (7). We have noted a similar 'situation' for immune cells from patients with serum C5-deficiency: T cells and monocytes from these individuals generate intracellular C5a and engage intracellular C5aR1 pathways – but cannot secrete C5 and its activation products (unpublished data). The autocrine C5 system is also required for homeostatic survival of APCs and T cells in mice as studies performed with *C5ar1*$^{-/-}$ mice showed that circulating immune cells in non-challenged animals have a decreased lifespan (27) because of failure in sustaining the phosphatidylinositol 3-kinase (PI3k) - protein kinase B (Akt) - mammalian target of rapamycin (mTOR) pathway, PI(3)K–Akt–mTOR pathway, which inhibits apoptosis (27, 116-118). Whether C5ar1-mediated survival signals in mouse immune cells require intracellular C5ar1 activation has so far not been assessed. Nonetheless, these data support that immune cell-generated anaphylatoxins emerge as important drivers of cell homeostasis and survival and advocate that – although serum C3 and

C5 deficiencies associated with recurrent infections clearly exist (119, 120) – intracellular complete absence of C3 and C5 may not occur in humans.

Importantly, the C3b portion of activated C3 is also critical to human T cell function. It has been shown that CD46-mediated signals regulate the expression of the IL-7 receptor (CD127/CD132) (121, 122), which provides an important survival signal for circulating non-activated T cells through STAT5-mediated activation of Akt and sustained expression of anti-apoptotic B-cell lymphoma 2 (BCL-2) (123-125). Importantly, although the expression of the IL-7 receptor is normal on quiescent cells from CD46-deficient patients (which explains their normal peripheral T cell counts), upon activation, their T cells fail to down-regulate CD127, which leads to the disruption of signalling through the general common γ-chain cytokine family (121).

Another important function for CD46 on resting human T cells is that of a 'homeostatic brake' (121) – and this function for CD46 is mediated via a crosstalk between CD46 and the Notch system. Indeed, the Notch protein family member Jagged-1 has been recently identified as a new physiological ligand for CD46, with a binding site located in the *N*-terminal part of CD46 (the first two so-called short consensus repeat (SCR) domains) (121). In the resting state, CD46 sequesters Jagged1 and via this prevents a 'inadequate' Jagged1-Notch1 interaction that would lead to $CD4^+$ T cell activation (figure 2b). Upon TCR engagement, however, T cell-derived C3b will engage CD46 (but in SCRs 3 and 4, thus a domain different from the Jagged1 binding site), which induces CD46 signalling but also shedding of CD46 from the T cell membrane via metalloproteinases (121, 126). This then 'frees' Jagged1 and allows for a concurrent Jagged1-Notch1 interaction that is also required to full Th1 induction (121). The importance of the functional crosstalk between CD46 and Notch is exemplified by the observation that patients with

mutations in Jagged1 have, similar to CD46-defiecient patients, also a defect in Th1 induction and suffer from recurrent upper chest viral infections (127). As discussed above, such contributions for immune cell-expressed C3b have so far not been defined in the mouse, further underpinning the differences in complement-mediated signals impacting on T cell regulation between these species.

Complement in Th1 contraction and Treg induction

Aside from its prominent role in Th1 induction, CD46 turned out to be also a critical component of the 'Th1 contraction programme', thus, in the shut down of IFN-γ secretion by $CD4^+$ T cells. This occurs, when CD46-mediated signals integrate signals from the IL-2R that respond to increases in high environmental IL-2 generated during the expansion of productive Th1 responses. This CD46-IL-2R crosstalk induces the coproduction of the immunosuppressive cytokine IL-10 in Th1 cells and with that a shift in the effector response toward a (self)regulatory T cell phenotype (88), with the cells finally 'collapsing' into IL-10 single producing T cells. This CD46-driven switch of Th1 cells (from IFN-γ^+ IL-10^- to IFN-γ^+ IL-10^+ and then to IFN-γ^- IL-10^+) is associated with the upregulation of the ICER CREM transcriptional regulator of the IL-2 gene and suppression of *IL2* expression (13). IL-10 co-producing Th1 cells themselves proliferate strongly despite their production of this usually anti-proliferative cytokine, but suppress the responses of bystander $CD4^+$, $CD8^+$ and γδ T cells via IL-10 secretion to a similar extent as natural regulatory T cells (nTregs) and other sets of induced regulatory T cells (iTregs) (88, 128). In addition, these cells are capable of mediating contact dependent cytotoxicity toward activated T cells via the expression of granzyme B and perforin (129). It is thought that this CD46-driven molecular switch regulates the 'natural life-cycle' of Th1 cells

with the purpose of keeping immune responses under tight control and preventing the local over-production of IFN-γ that would usually lead to tissue pathologies. In support of this model is the observation that dysregulations in this molecular switch towards a self-regulatory phenotype has indeed been identified as one strongly contributing factor to the hyperactive Th1 response in T cells from patients with RA and MS (13, 130). T cells from these patients, when stimulated *in vitro* demonstrated disturbed IL-10 switching and produced up to 10x more IFN-γ than IL-10 when compared to T cells from healthy individuals. The ability to prevent or induce this CD46-driven switch in Th1 cells at will, would possibly constitute a therapeutic means to stop or reduce chronic infection and cancer or autoimmunity and organ rejection, respectively. However, while we have now a reasonable understanding about the CD46-driven signalling pathways inducing Th1 responses (see below), little is known about the CD46 and IL-2R crosstalk that is vital in switching a Th1 cell into the regulatory IL-10 program. A better mechanistic understanding of this switch though is a declared goal of our laboratory and as the signal transducer and activator of transcription (STAT) and the Janus kinase (JAK) protein families are key mediators of cytokine receptor signalling pathways (131), inquiring for a potential connection between CD46 and STATs and JAKs may be a suitable strategy.

Similar to the C3b-CD46 interaction that directs Th1 induction but also contraction, T cell C5-derived activation fragment C5a not only support the Th1 response via ROS generation and NLRP3 inflammasome assembly – C5a also contributes to the negative control of human Th1 responses. As mentioned above, activated T cells use intracellularly generated C5a to drive signals via the exclusively intracellularly expressed C5aR1 but they simultaneously secrete a proportion of C5a to the cell surface. Here, the C5a (or C5a-desArg) engages the surface expressed

C5aR2 and this interaction exerts negative control over C5-driven NLRP3 inflammasome activation, autocrine IL-1β secretion and hence the levels of IFN-γ produced by human $CD4^+$ T cells (figure 2a) (89, 111). The exact mechanisms by which C5aR2 negatively regulates NLRP3 inflammasome activity are currently not delineated but could include suppressive effects on intracellular C5aR1 signalling but also yet to be defined direct effects on inflammasome assembly. Thus, a balanced and concurrent engagement of C5aR1 Th1 driving signals versus C5aR2 'dampening' signals also contributes to the tight control of human Th1 responses through an inflammasome axis. Our observation that T cell-derived IL-1β is needed for normal IFN-γ production *in vitro* and *in vivo* (89) was initially surprising, given that APCs provide usually rather large amounts of IL-1β during the cognate APC-T cell interaction. We suggest, however, that APC-derived IL-1β supports initial Th1 priming, but that successful 'imprinting' and then maintenance of the Th1 phenotype during differentiation and migration into tissues relies on autocrine NLRP3 activity. The likely reason for the firm control of IL-1β secretion via the C5 system, on the other hand, is that IL-1β is a strong suppressor of IL-10 production (132) and would likely block CD46-indced IL-10 switching if produced at uncontrolled levels. In line with this notion, specific blockade of C5aR2 signalling during T cell activation or addition of rIL-1β the IFN-γ:IL-10 ratio in $CD4^+$ T cells and, importantly, T cells from CAPS patients (which produce increased IL1-β) have significantly reduced IFN-γ to IL-10 switching (89).

Of note, the protease cathepsin G has recently been shown to cleave and inactivate the C5aR1 (133). Although this novel activity of cathepsin G was discovered on the surface of neutrophils, T cells and monocytes also express

cathepsin G and one can envision that this event may also occur within cells where it may constitute a mechanism of intracellular C5aR1 signalling control.

Given the participation of the intracellular C3 and C5 activation fragments in the induction and regulation of Th1 responses, it will now be interesting to explore whether IFN-γ secretion by $CD8^+$ T cells (54), natural killer T (NKT) cells, and/or innate lymphoid type 1 (ILC1) cells also involves the activity of the complosome. Aligning with such scheme that complement activation fragments are initially needed for protective T cell responses, recent studies demonstrate that lack of C3aR and C5aR1 signalling on human and mouse $CD4^+$T cells induces the default generation of $Foxp3^+$ Tregs with strong suppressive capacities (82, 83). It should be noted though that the expression of the anaphylatoxin receptors by mouse T cells is still controversial in the field with some groups observing the expression of these receptors on activated cells while others fail to detect them in either resting or stimulated cells (134). The reasons for these discrepancies are not resolved but could be rooted in distinct experimental approaches and model systems employed by the different groups or also in the sensitivity levels of the reagents used for the detection of the anaphylatoxin receptors. The C3aR, C5aR1 and C5aR2 expression patterns in human T cells, however, are now better defined and here, a picture emerges in which CD46 (or rather a controlled deviation in its function) is also connected with nTreg function: Human nTregs express all complement components required for proinflammatory cytokine production and generate intracellular C3a (7) and C5a (unpublished data), however these cells have disengaged the signalling capacity of CD46 that usually drives IFN-γ (see below) enabling this particular T cell sub-population to remain in an anti-inflammatory and suppressive state. Thus, CD46-deficient patients have perfectly normal numbers of fully functional nTreg cells

(7). The understanding that complement is also critical to the negative control of Th1 immunity aligns well with a recent change of thinking in the field and the growing recognition of complement as a true mediator of general homeostasis. For example, regulated (local) complement activation is needed for normal tissue and organ development and also for tissue repair after injury or insult (for an excellent review on this subject, please see (9)) and targeting complement therapeutically should take this 'spatial-temporal' action of complement into consideration.

Complement's functional crosstalk with cell physiological effector systems

Work performed on understanding the CD46-mediated signalling pathways regulating Th1 induction and contraction have led to the additional discovery that the complement system also plays a central role in basic physiological pathways of the cell and particularly those of metabolic nature. For example, CD46 co-stimulation during T cell activation drives the nutrient influx and specific metabolic reprogramming that accompanies Th1 cell induction (8, 135). T cell differentiation into effector cells induces significant changes in cellular metabolic pathway utilization. Among the hallmarks of such metabolic 'remodelling' that occurs in activated T cells is the up-regulation of aerobic glycolysis (Warburg effect), which is needed for cell growth, proliferation and acquisition of effector function. Glycolytic metabolites are essential for biomolecular synthesis in dividing cells (136) and T cell stimulation also enhances mitochondrial biogenesis and oxidative phosphorylation (OXPHOS), as well as the uptake of glucose and amino acids (AAs) (137, 138). The metabolic-checkpoint kinase mTOR senses and integrates incoming environmental nutrient signals, which then trigger glycolysis, OXPHOS and lipid synthesis, and via this support proliferation and differentiation of resting T cells into effector cells (139-

141). CD46 is expressed in different isoforms that arise from differential splicing of a single gene in $CD4^+$ T cells and these isoforms vary in the expression of their cytoplasmic tails, termed CYT-1 and CYT-2. Both domains can transduce intracellular signals in several cell types (126, 142-144) and non-stimulated T cells express predominantly the CYT-2-bearing isoforms (13, 145). Upon TCR stimulation, the CD46-CYT-1 isoforms are strongly upregulated (through a mechanism that is currently not understood) and engaged via T cell autocrine C3b production. The CD46-CYT-1 isoforms then induce the increased expression of genes coding for the glucose (GLUT1) and amino acid channels (LAT1), *SLC2A1* and *SLC7A5* respectively, and thereby mediate the nutrient influx needed to meet the T cell's increased requirement for 'food' upon activation (figure 2a). Simultaneously, CD46-CYT-1 also upregulates the late endosomal/lysosomal adaptor, MAPK and MTOR activator 5 (LAMTOR5), which is an important scaffolding protein required for the assembly of the nutrient-sensing complex mechanistic target of rapamycin complex 1 (mTORC1) and mTOR at the lysosomes (8). T cells from CD46-deficient patients fail to induce these critical metabolic events upon activation. Thus, although these metabolic pathways are initiated in mouse $CD4^+$ T cells by TCR and CD28 costimulation (146), human T cells show an absolute requirement of CD46-CYT-1-mediated signals for the induction of the glycolytic levels needed for IFN-γ secretion (135) and CD28 costimulation alone is not sufficient for successful Th1 induction (8). Furthermore, CD46 is – similar to receptors of the Notch family - processed on the surface via metalloproteinases and intracellularly by γ-secretase (147, 148) and we have recently demonstrated that the nuclear translocation of the cleaved intracellular tails of CD46 are required for increased glycolysis and OXPHOS induction in activated T cells (8).

Congruent with its role in Th1 'shut down', CD46-mediated signals also induce the switch from a high glycolytic state back to steady-state glycolysis levels in $CD4^+$ T cells and, via this, subsequently IL-10 coproduction and finally Th1 contraction. This switch from high to low glycolysis is driven by CD46-CYT-2-expressing isoforms, which become again the predominant CD46 isoforms in contracting T cells (figure 2b) (8). CD46-CYT-1 also increases OXPHOS levels in activated T cells. As the induction of an OXPHOS 'burst' in activated T cells is a prerequisite for normal memory cell generation, it may be worthy to assess whether intracellular and/or autocrine complement also contributes to the development of central and/or effector memory pools. Furthermore, the contributions of CD46 to key metabolic pathways in T cells may explain the unusual 'propensity' of autocrine-functioning complement to directly regulate Th1 lineage induction with relatively little direct impact on Th2 and Th17 responses as $CD4^+$ T cell subsets have distinct metabolic requirements. For example, effector T cells demand high levels of glycolysis, whereas Treg cells are more dependent on OXPHOS. Furthermore, mTORC1 activity is required for Th1 and Th17 cell responses, whereas mTORC2 drives Th2 cell function (140, 149, 150). Since it has been shown that IFN-γ production requires a particularly high induction of glycolysis (135), it is feasible that CD46-mediated signals can specifically meet this demand. Of note, only complete absence of CD46 led to absence of Th17 cell responses (8), further suggesting metabolic threshold differences between induction of Th1 and Th17 cell effector populations. And finally, as T cells from CD46-deficient patients proliferate normally, these differences likely relate to non-bioenergetic aspects of subset-specific metabolic reprogramming (151).

Aside from directly impacting on metabolic pathways, CD46 also contributes to cell physiology via its impact on the assembly of key cytokine and growth factor

receptors on T cells during activation. CD46 regulates the expression of CD127 and CD132 on these cells (121) which are components of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (the IL-2R family), most of which are involved in normal lymphocyte function. IL-2-mediated signalling through the high-affinity IL-2R - composed of CD25 (also known as IL-2RA), CD122 (also known IL-2RB) and CD132 (also known as common gamma chain IL2RG) - is required for Th1 cell induction. Thus, CD46 partakes also in an important cytokine network that directs T cell responses by integrating environmental nutrient cues and subsequent modulation of catabolic and anabolic pathways. In mice, C3aR and C5aR1-mediated signals can regulate the expression of the IL-12Rβ chain (81, 152) but a direct impact on the regulation of the IL-2R family has not been observed.

The 'C5 system' also contributes to the modulation of key physiological pathways in T cells via the induction of oxygen metabolism: As detailed above, intracellular C5aR1 activation in human $CD4^+$ T cells through intracellularly generated C5a induces there generation of mitochondrial ROS (89). ROS is now established to play indispensable roles as signalling molecules in various redox-sensitive pathways – also in the modern adaptive immune system with ROS generation within T cells being required for successful Th1 induction (153). Furthermore, the ability of the C5aR1 to induce assembly of the NLRP3 inflammasome will highly likely also impact on basic cell pathways aside from the 'simple' induction of pro-inflammatory IL-1β secretion. Although complement, the TLRs, and the inflammasomes were initially discovered as pathogen sensors, it is now understood that the ability of these systems to recognize imbalances in normal cell metabolic processes and their capability to evoke appropriate reactive responses is of equal importance to cell and tissue homeostasis (154). For example, NLRP3

inflammasome priming and activation are strongly driven by increased glucose influx, heightened glycolysis and increased ATP production, as demonstrated in several human cells types (41, 155), all events generally required for cell activation, proliferation and effector function (156). Importantly, metabolic by-products can also inhibit NLRP3 inflammasome activation and particularly increased AMP generation leads to activation of the nutrient sensor AMP-dependent protein kinase (AMPK) which subsequently inhibits NLRP3 inflammasome function. This is mediated by the ability of AMPK to switch the cell from energy-consuming processes such as glycolysis to oxidative metabolism associated with anti-inflammatory and quiescent states and favours mitochondrial biogenesis and reduction in NLRP3 activation (157, 158). Thus, the regulated crosstalk between complement, the inflammasome and growth factor receptors dictate the metabolic state of a cell during the induction of T cell effector function but also during the contracting and quiescent phases of the T cell life cycle. Furthermore, although this complement-inflammasome-metabolism axis (111) has been discovered in T cells, it is highly likely that this system also operates in other immune cells.

What's next? - Complement and the three 'M's'

We are now at a juncture in complement research were we clearly need to adjust our old view on complement: Away from a mostly innate, liver-derived and pro-inflammatory system to a revised view in which complement is a major bridge between innate and adaptive immunity, operates within cells (and even in specialised intracellular subcompartments), cross-engages with other effector systems to regulate basic cellular pathways and actively partakes in cell and tissue

homeostasis (9, 14). Based on a range of studies, recent and less recent, it is becoming acknowledged that complement is critical for the initiation of the general inflammatory reaction upon pathogen breach, the subsequent instruction of normal APC maturation and function with subsequent priming of B and T cell in the lymph nodes, and finally in the induction of appropriate effector T cell responses in the tissue (Figure 3). The new and unexpected roles of complement give now rise to a wealth of questions that need to be answered. One direction of research would focus on moving further into the inside of cells and ask, for example, 'What is the complosome composition in other immune cells?", 'What are the complosome functions?", 'How is it regulated?", and 'How do intra- and extracellular complement functions intersect?'. Our unpublished data suggest that core components such as C3 and C5 as well anaphylatoxin receptors are present in all cells but that the intracellular expression of complement regulators may differ, and there is also indication that the complosome regulates histone modifications (unpublished data). However, other areas, where our knowledge about complement is still remarkably limited, pertain to its place in the multitude of networks that orchestrate and define immunity on a whole organism level through life such as cell Migration, Memory development and the interaction with the Microbiome. Although complement has pioneered the research on the mechanisms underlying cell migration with the discovery of C5a as major chemoattractant (159-162), relatively little is known about the exact contributions of complement during extravasation, movement in and out of the lymph nodes and tissues (Figure 3) and the maintenance of tissue-resident immune cells. Cell migration and tissue occupancy are regulated by an extensive network of chemokine and integrin receptors and it is known that complement regulates important rolling and adhesion receptors including ICAM-1, VCAM and P-

selectin on the endothelium (163) as well as CXCR4 on immune cells (164). It would, however, not be surprising if the functional crosstalk between complement and integrins and between complement and the extended chemokine receptor repertoire is much more extensive than initially thought and contributes to the 'body-wide' orchestration of immune cell movements (Figure 3).

A role for complement in the development of normal B cell memory has been demonstrated previously, although the underlying complement-driven molecular mechanisms have not been delineated yet in detail (67). Given that intracellular C3 activation occurs in B cells (7), it may be worthy to assess if the complosome could be a contributing component. Indeed, the observation that complement is important in the T cell metabolic reprogramming pathways (and possibly histone remodelling) that are ultimately also connected to T cell memory development, raises the possibility that the complosome activation state, or a specific complosome signature (for example, a particular CD46 CYT-1/CYT-2 expression profile in naïve versus memory cells), may be a component of both B and T cell memory-induction and -function. Importantly, this concept may not be restricted to adaptive immune cells, but may also extend to innate immune cells: Previously, the inability of innate immunity to mount immunological memory was considered as key difference to adaptive immunity. However, this paradigm has recently been challenged with the delivery of evidence for innate immune memory that leads to increased responses to secondary infections in natural killer T cells and monocytes and macrophages (165, 166). The evolutionary close connection of complement with innate immune cell function thus makes it an intuitive question to ask whether complement may play a role in innate immune memory. Of course, a close look at complement towards the 'opposing end' of immunological memory, the pathways directing the initial immune

cell lineage development in the thymus or bone marrow, is also worth consideration. C5a has recently been shown to promote human embryonic pluripotency (167) but a role for complement in thymic selection – either in the immune cell precursors or the 'instructing' thymic epithelial cells - remains unexplored.

The observation that complement is operative within cells and drives metabolism suggests that we may want to take a fresh look at the role of complement in infections with pathogenic microbes. The importance of CD46 as an immune-modulatory protein has not gone unnoticed by a brigade of pathogens with a range of important human pathogenic bacteria and viruses using CD46 as cell entry receptor (168). We previously favoured the hypothesis that this is due to the fact that CD46 drives the secretion of IL-10 that would generate an immunosuppressed and 'infection-conducive' microenvironment. However, since particularly viruses rely on an activated high-glycolytic state of the host cell to ensure their successful replication and virion generation, the interplay between CD46 and CD46-binding pathogens may therefore, in addition, have a previously unappreciated 'metabolic dimension'. Similarly, the existence of the complosome in conjunction with the recent unexpected observation that intracellular pathogens trigger mitochondrial anti-viral signalling (MAVS) responses in a C3-dependent manner (169) imply that the complosome contributes to the control of intracellular bacteria/pathogens. Thus, deviations in complosome function will likely also alter the course of infections. Finally, the gut *microbiota* is critical for maintaining the host energy balance via regulation of dietary fat absorption and management through intestinal epithelial cells (170). Given the role of complement in the maintenance of intestinal epithelial cell integrity (144) and in the regulation of cell metabolism, we predict a functional relationship of biological

relevance between the complement system and the microbiome – which is currently a research area that remains unexplored.

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FIGURE LEGENDS

Figure 1: Activation and regulation of complement in serum. Liver derived serum circulating complement is on the front lines of host defense against invading pathogens. The pathways leading to complement activation on the pathogen cell surface membrane are traditionally divided into three separate pathways defined by their mode of pathogen recognition. The classical pathway molecule C1q binds to surface bound complement fixing antibody whereas the lectin pathway molecules MBL, ficolins and collectin 11 bind to carbohydrate moieties. Through the formation of C3 convertases (C4bC2a for the classical and lectin pathways and C3bBb for the alternative pathway) all three pathways result in the formation of the opsonin C3b

and the anaphylatoxin C3a. The subsequent formation of the C5 convertase (C4bC2aC3b for the classical and lectin pathways and C3bBbC3b for the alternative pathway) leads to generation of the membrane attack complex (MAC) initiating molecule C5b and the anaphylatoxin C5a. The anaphylatoxins play an important role in promoting chemotaxis, inflammation and cellular activation. Complement activation must be tightly controlled to prevent damage to host tissue. Regulators of complement activation exist both in the fluid phase and bound to the host cell surface: C1 inhibitor (C1-inh) inhibits the proteases C1r and C1s, and mannan-binding lectin associated serine protease-2 (MASP2). C3b and C4b are inactivated by the serine protease Factor I and one of several cofactor proteins (surface-bound CD46 and complement receptor 1 (CR1) or fluid phase Factor H and C4b-binding protein (C4bBP). C3 convertases are disassembled by regulators possessing decay accelerating properties such as surface bound CD55 and CR1 and fluid phase C4BP and Factor H. The membrane attack complex is regulated by CD59, which prevents MAC formation by preventing C9 polymerization. Vitronectin also inhibits MAC formation by preventing the partially assembled MAC from inserting into the cell membrane.

Figure 2: The role of Complement in Th1 regulation. (A) The induction of T helper Th1 responses, initiated by TCR activation and CD28 co-stimulatiom, induces the shuttle of intracellular C3a and C3b storages to the cell surface and allows for CD46 (via C3b) and C3aR (via C3a) engagement (1). Binding of C3b to CD46 initiates γ-secretase-mediated cleavage and translocation of CD46 cytoplasmic tail 1 into the nucleus where it may act in concerts with transcription activators or repressors to regulate CD46 target gene expression (denoted by a questions mark) (2). This

CD46-tail 1 nuclear translocation triggers up-regulation of the genes encoding the IL-2R α-chain for optimal IL-2R assembly, the glucose transporter GLUT1 (*SLC2A1*) and the amino acid channel LAT1 (*SLC7A5*), thus leading to an increased influx of glucose and amino acids into the cells. In addition, CD46-mediated signals up-regulates the expression of LAMTOR5 which is part of the Ragulator complex and is involved in amino acid sensing and activation of mTORC1 (2). C3aR1 stimulation from the cell surface supports Th1 induction via supporting CD46-mediated mTORC1 activation. Hence, both C3aR- and CD46-mediated events stimulate the high glycolysis and OXPHOS levels required for IFN-γ secretion. At the same time, CD46 engagement induces gene expression of *NLRP3* and *IL1B* to instruct the NLRP3 inflammasome as well as increased intracellular cleavage of C5 into C5a and C5b by a yet unknown proteolytic mechanism. Intracellular engagement of C5aR1 by C5a induces ROS production which in turn activates the NLRP3 inflammasome and subsequent production of mature IL1β production (but not IL-18) (3) – which in turn sustains Th1 induction. (B) The role of complement in T cell homeostasis (left side) and 'effector phase' contraction (right side). On resting $CD4^+$ T cells, CD46 constitutively binds Jagged1 thereby limiting potential T cell-activating Jagged1 and Notch1 interactions (as the CD46 and Jagged1 interaction is of higher affinity compared to the Jagged1 and Notch1 interaction). Furthermore, CD46-mediated signals regulate the expression of the IL-7 receptor which provides an important homeostatic survival signal for circulating non-activated T cells. 'Tonic' intracellular C3a generation via cathepsin L cleavage leads to low-level mTOR activation, which is indispensable for homeostatic T cell survival. The crosstalk between CD46 and IL-2R mediated signals and signals via the C5aR2 also regulate the Th1 contraction phase. Specifically, in the presence of high concentrations of environmental IL-2

(generated during Th1 expansion) CD46 induces a switch from IFN-γ production to IL-10 secretion and Th1 contraction is initiated. In parallel, the local levels of secreted C5a (or C5a-desArg) increase during the Th1 response and C5a engages the surface-expressed C5aR2, an interaction that exerts negative control over intracellular C5aR1-driven NLRP3 inflammasome activation and IL-1β secretion, and hence the levels of IFN-γ produced by human $CD4^+$ T cells.

Figure 3: The evolving view on complement in inflammation. (A) (1) Pathogen breach activates complement and leads to generation of anaphylatoxins (An, C3a and C5a) and opsonins (Op, C3b and C4b) which will engage the complement receptors (CRs) expressed on immune cells and mediates the acute inflammatory response, (2) tags pathogens or other noxious antigens for removal by phagocytes, and (3) promotes migration and activation of immunocompetent cells. In addition, (4) complement increases the phagocytic activity of APCs and initiates (5) their maturation and migration to the draining lymph nodes. (B) In the lymph node, APCs prime naïve B cells and T cells into effector B cells and T cells which involves several complement receptors, notably CR2, CD21. Following activation, B cells and T cells then egress into the peripheral circulation. Based on the evidence that complement regulates important rolling and adhesion receptors, we anticipate that complement play a role in the migration and the extravasion of immune cells in and out of the lymph nodes and tissues. (C). Complement also plays an important role in the induction and sustenance of Th1, Th2, Th17 and natural regulatory T cell responses once the cells returned to the site of infection and/or inflamed tissues. The key words within 'dashed boxes' indicate areas that we anticipate will also, in the future, demonstrate functional impact by complement.

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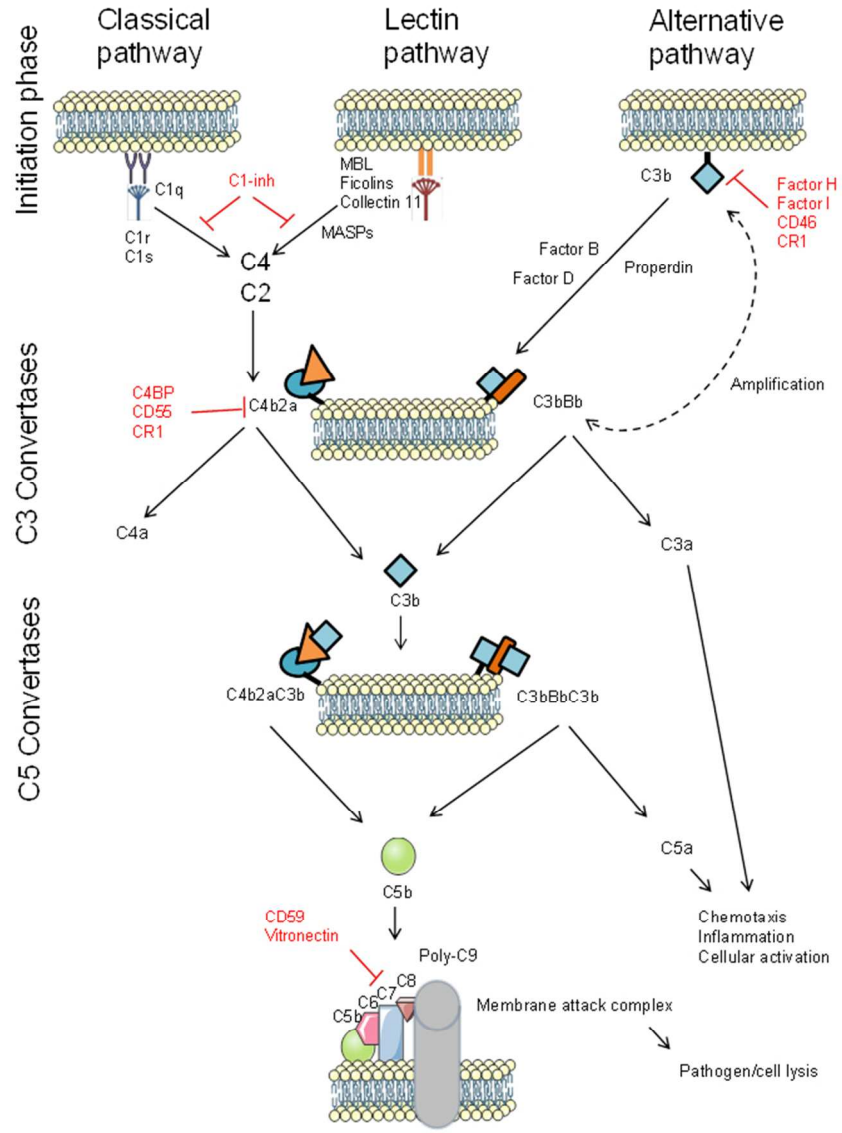
Figure 1



Activation and regulation of complement in serum. Liver derived serum circulating complement is on the front lines of host defense against invading pathogens. The pathways leading to complement activation on the pathogen cell surface membrane are traditionally divided into three separate pathways defined by their mode of pathogen recognition. The classical pathway molecule C1q binds to surface bound complement fixing antibody whereas the lectin pathway molecules MBL, ficolins and collectin 11 bind to carbohydrate moieties. Through the formation of C3 convertases (C4bC2a for the classical and lectin pathways and C3bBb for the alternative pathway) all three pathways result in the formation of the opsonin C3b and the anaphylatoxin C3a. The subsequent formation of the C5 convertase (C4bC2aC3b for the classical and lectin pathways and C3bBbC3b for the alternative pathway) leads to generation of the membrane attack complex (MAC) initiating molecule C5b and the anaphylatoxin C5a. The anaphylatoxins play an important role in promoting chemotaxis, inflammation and cellular activation. Complement activation must be tightly controlled to prevent damage to host tissue. Regulators of complement activation exist both in the fluid phase and bound to the host cell surface: C1 inhibitor (C1-inh) inhibits the proteases C1r and C1s, and

mannan-binding lectin associated serine protease-2 (MASP2). C3b and C4b are inactivated by the serine protease Factor I and one of several cofactor proteins (surface-bound CD46 and complement receptor 1 (CR1) or fluid phase Factor H and C4b-binding protein (C4bBP). C3 convertases are disassembled by regulators possessing decay accelerating properties such as surface bound CD55 and CR1 and fluid phase C4BP and Factor H. The membrane attack complex is regulated by CD59, which prevents MAC formation by preventing C9 polymerization. Vitronectin also inhibits MAC formation by preventing the partially assembled MAC from inserting into the cell membrane.
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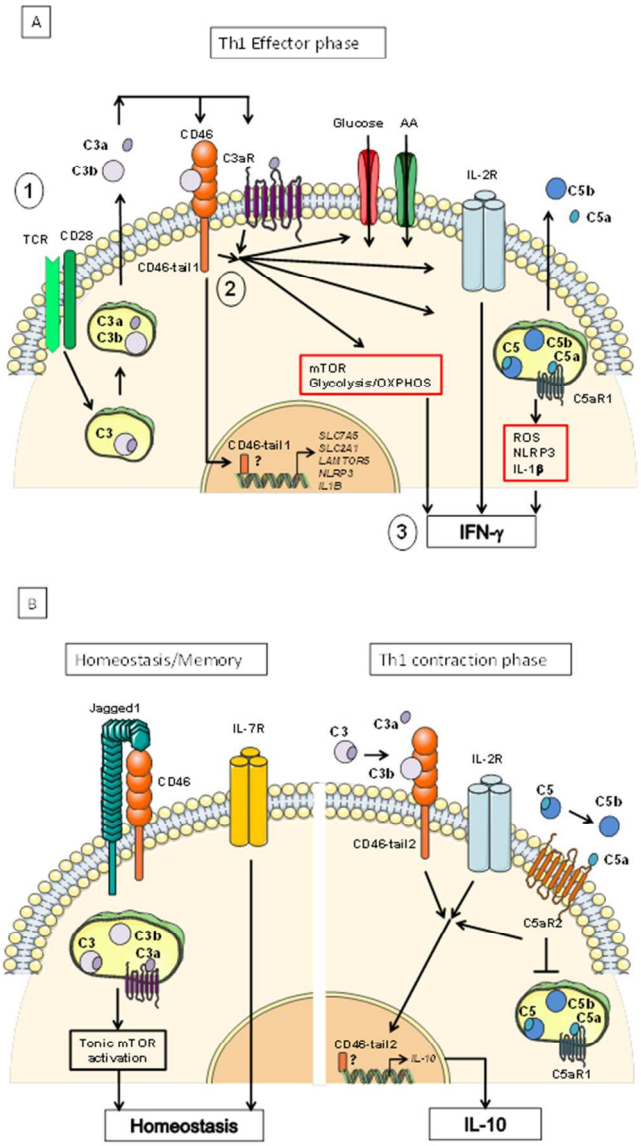
Figure 2

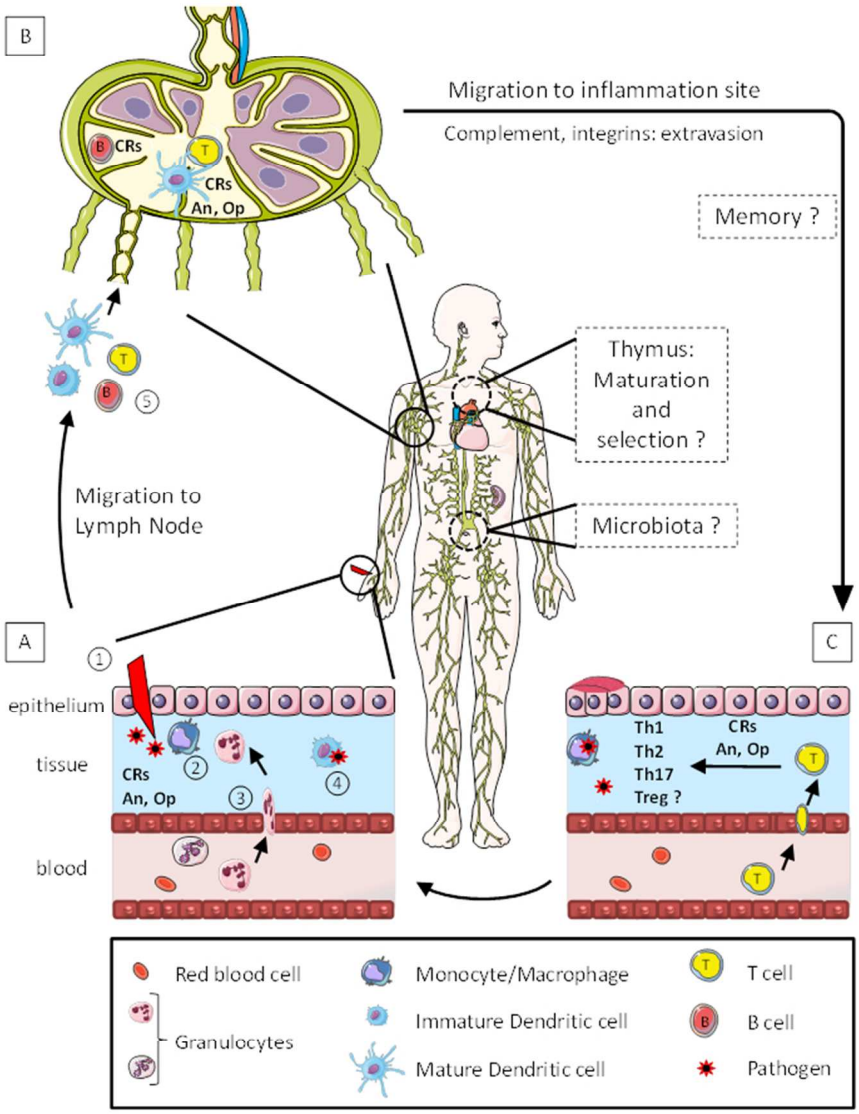


The role of Complement in Th1 regulation. (A) The induction of T helper Th1 responses, initiated by TCR activation and CD28 co-stimulatiom, induces the shuttle of intracellular C3a and C3b storages to the cell surface and allows for CD46 (via C3b) and C3aR (via C3a) engagement (1). Binding of C3b to CD46 initiates γ-secretase-mediated cleavage and translocation of CD46 cytoplasmic tail 1 into the nucleus where it may act in concerts with transcription activators or repressors to regulate CD46 target gene expression (denoted by a questions mark) (2). This CD46-tail 1 nuclear translocation triggers up-regulation of the genes encoding the IL-2R α-chain for optimal IL-2R assembly, the glucose transporter GLUT1 (SLC2A1) and the amino acid channel LAT1 (SLC7A5), thus leading to an increased influx of glucose and amino acids into the cells. In addition, CD46-mediated signals up-regulates the expression of LAMTOR5 which is part of the Ragulator complex and is involved in amino acid sensing and activation of mTORC1 (2). C3aR1 stimulation from the cell surface supports Th1 induction via supporting CD46-mediated mTORC1 activation. Hence, both C3aR- and CD46-mediated events stimulate the high glycolysis and OXPHOS levels required for IFN-γ secretion. At the same time, CD46 engagement induces gene expression of NLRP3 and IL1B to instruct the

NLRP3 inflammasome as well as increased intracellular cleavage of C5 into C5a and C5b by a yet unknown proteolytic mechanism. Intracellular engagement of C5aR1 by C5a induces ROS production which in turn activates the NLRP3 inflammasome and subsequent production of mature IL1β production (but not IL-18) (3) – which in turn sustains Th1 induction. (B) The role of complement in T cell homeostasis (left side) and 'effector phase' contraction (right side). On resting CD4+ T cells, CD46 constitutively binds Jagged1 thereby limiting potential T cell-activating Jagged1 and Notch1 interactions (as the CD46 and Jagged1 interaction is of higher affinity compared to the Jagged1 and Notch1 interaction). Furthermore, CD46-mediated signals regulate the expression of the IL-7 receptor which provides an important homeostatic survival signal for circulating non-activated T cells. 'Tonic' intracellular C3a generation via cathepsin L cleavage leads to low-level mTOR activation, which is indispensable for homeostatic T cell survival. The crosstalk between CD46 and IL-2R mediated signals and signals via the C5aR2 also regulate the Th1 contraction phase. Specifically, in the presence of high concentrations of environmental IL-2 (generated during Th1 expansion) CD46 induces a switch from IFN-γ production to IL-10 secretion and Th1 contraction is initiated. In parallel, the local levels of secreted C5a (or C5a-desArg) increase during the Th1 response and C5a engages the surface-expressed C5aR2, an interaction that exerts negative control over intracellular C5aR1-driven NLRP3 inflammasome activation and IL-1β secretion, and hence the levels of IFN-γ produced by human CD4+ T cells.

190x254mm (96 x 96 DPI)

Figure 3



The evolving view on complement in inflammation. (A) (1) Pathogen breach activates complement and leads to generation of anaphylatoxins (An, C3a and C5a) and opsonins (Op, C3b and C4b) which will engage the complement receptors (CRs) expressed on immune cells and mediates the acute inflammatory response, (2) tags pathogens or other noxious antigens for removal by phagocytes, and (3) promotes migration and activation of immunocompetent cells. In addition, (4) complement increases the phagocytic activity of APCs and initiates (5) their maturation and migration to the draining lymph nodes. (B) In the lymph node, APCs prime naïve B cells and T cells into effector B cells and T cells which involves several complement receptors, notably CR2, CD21. Following activation, B cells and T cells then egress into the peripheral circulation. Based on the evidence that complement regulates important rolling and adhesion receptors, we anticipate that complement play a role in the migration and the extravasion of immune cells in and out of the lymph nodes and tissues. (C). Complement also plays an important role in the induction and sustenance of Th1, Th2, Th17 and natural regulatory T cell responses once the cells returned to the site of infection and/or inflamed tissues. The key words within 'dashed boxes' indicate areas that we anticipate will also, in the future,

demonstrate functional impact by complement.
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