Inhibitory and Regulatory Immune Synapses

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Contents

1 Introduction 64
2 Definition of Inhibitory Immune Synapses 64
3 Formation of Inhibitory Synapses 65
4 Balancing Synapses with Kinapses and Kinetic Proofreading at the Cellular Level 69
5 Unzipping the Synapse 70
6 Regulatory Synapses 70
7 The Regulatory NK-Cell Synapse 71
8 Triggering Cytokine Secretion Versus Cytolysis 71
References 73

Abstract Cell contact-dependent inhibition and regulation of immune responses play an essential role in balancing the need for rapid and efficient responses to a wide variety of pathological challenges, while at the same time maintaining self-tolerance. Much attention has been given to immune synapses that lead to the activation of, for example, cell-mediated cytotoxicity, and here we compare the supramolecular dynamics of synapses that lead to inhibition or regulatory functions. We focus on natural killer cells where such different synapses have been best studied. An emergent principle is that inhibition or regulatory responses are commonly achieved by selective recruitment of signalling proteins to the synapse and exclusion of membrane-proximal intracellular proteins needed for activation. We also discuss evidence that an inhibitory synapse triggers or maintains effector cells in a migratory configuration, which serves to break the synapse before the steps needed for effector cell activation can be completed. This model implies that the concept of kinetic-proofreading, previously used to describe activation of

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individual T-cell receptors, can also apply in determining the outcome of intercellular conjugation.

1 Introduction

Many of the key cell surface molecules involved in immune cell surveillance have been identified and an important new scientific frontier is to understand where and when each protein–protein interaction occurs to regulate cell functions. Thus, imaging has a major role to play in contemporary cell biology and one interesting theme to emerge is that immune cell communication is often accompanied by the segregation of proteins into micrometre-scale domains at an intercellular contact or immune synapse (IS) (Davis 2006). More recently, it has been shown that kinases, adaptors and antigen receptors accumulate at synapses within micron- or sub-micron-scaled structures termed microclusters. T- and B-cell receptor signalling, for example, is initiated in such microclusters (Bunnell et al. 2002; Campi et al. 2005; Yokosuka et al. 2005) and these signals are terminated as microclusters move from the periphery to the centre of the IS (Harwood and Batista 2008; Seminario and Bunnell 2008; Varma et al. 2006; Yokosuka et al. 2005). In natural killer (NK) cells, phosphorylation of inhibitory killer cell immunoglobulin (Ig)-like receptors (KIR) is restricted to microclusters (Treanor et al. 2006) implicating that inhibitory signalling is also restricted to microclusters. The emerging new paradigm is that interactions between immune cell kinases, adaptors and other proteins are at least in part controlled by the dynamics of supramolecular assemblies rather than isolated protein–protein interactions that are commonly depicted in textbook diagrams of immune receptor signalling pathways. While this has been widely discussed for immune cell activation, here we review what happens at an IS where inhibitory or regulatory signals dominate.

2 Definition of Inhibitory Immune Synapses

It is well established that inhibitory receptor functions are crucial to maintaining self-tolerance and control responses spatially and temporally while allowing rapid and efficient responses when appropriate (Long 1999). This is particularly evident from the data associating inhibitory receptor dysfunction, or genetic variations in inhibitory receptor expression, with susceptibility to a variety of diseases, including autoimmunity, viral infection and cancer (Chouaib et al. 2002; Pritchard and Smith 2003; Rajagopalan and Long 2005). Broadly, inhibitory receptors can be divided into two classes based on the presence or absence of cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs). Inhibition by ITIM-containing receptors is initiated by tyrosine phosphorylation and recruitment of Src homology 2 (SH2) domain-containing phosphatases SHP-1 and/or SHP-2 or the

SH2 domain-containing inositol phosphatase (SHIP) (Daeron et al. 2008; Long 2008). Engagement of inhibitory receptors changes the micrometre-scale organization of proteins at the IS, compared to activating interactions to form a so-called "inhibitory immune synapse" (Davis and Dustin 2004). An inhibitory synapse is not merely a transient intercellular contact at which little happens. Such transient interactions may occur when T cells briefly interact with target cells or APCs that lack a relevant peptide/MHC and they do not involve assembly of an IS. Rather, an inhibitory synapse can be defined as an intercellular contact at which the encounter causes proteins to segregate into micrometre-scale domains and where directed signalling serves to terminate or prevent immune cell activation.

Such inhibitory immune synapses were first proposed for NK cells conjugated to EBV-transformed B cells that were protected from lysis by expression of class I MHC protein recognized by inhibitory NK-cell receptors (Davis et al. 1999). It is broadly accepted that expression of class I MHC protein facilitates self-tolerance by NK cells and that conversely, viral-infected or tumour cells can become susceptible to lysis by NK cells via decreased expression of self class I MHC protein (Karre et al. 1986). Commonly referred to as the "missing self-hypothesis" (Ljunggren and Karre 1990), this provides a conceptual framework in which the importance of an inhibitory IS is well-documented.

Recently, ligation of inhibitory receptors has been shown to assemble distinct synaptic structures in many other immune cell interactions, including those involving T cells, B cells and macrophages (Dietrich et al. 2001; Fourmentraux-Neves et al. 2008; Guerra et al. 2002; Henel et al. 2006; Schneider et al. 2008; Sohn et al. 2008; Tsai and Discher 2008). For example, subpopulations of T cells express inhibitory receptors of the KIR family, the C-type lectin-like heterodimer CD94/NKG2A, the Ig-like transcript (ILT) 2 or members of the CD28:B7 Ig superfamily, such as CTLA-4 (CD154) (Peggs et al. 2008; Ugolini and Vivier 2000). Engagement of these receptors negatively regulates signalling through the T-cell antigen receptor (Chouaib et al. 2002; Peggs et al. 2008; McMahon and Raulet 2001; Snyder et al. 2002; Ugolini and Vivier 2000; van Bergen et al. 2004) and influences the supramolecular organization of the IS (Dietrich et al. 2001; Fourmentraux-Neves et al. 2008; Guerra et al. 2002; Henel et al. 2006; Schneider et al. 2008). The B cell inhibitory receptor FcγRIIB similarly disrupts formation of an activating synapse in response to membrane bound antigen (Sohn et al. 2008). The formation of a phagocytic contact between macrophages and red blood cells is inhibited by binding of the inhibitory receptor SIRPα (CD172a) to its ligand CD47 (Tsai and Discher 2008). These data extend the concept of the inhibitory synapse to other cellular interactions and demonstrate its broad relevance.

3 Formation of Inhibitory Synapses

The inhibitory NK-cell IS is the best studied inhibitory IS and may be considered "prototypic" (see Fig. 1 for a summary of molecular processes at the inhibitory NK-cell IS). Inhibition of NK-cell activity through engagement of inhibitory

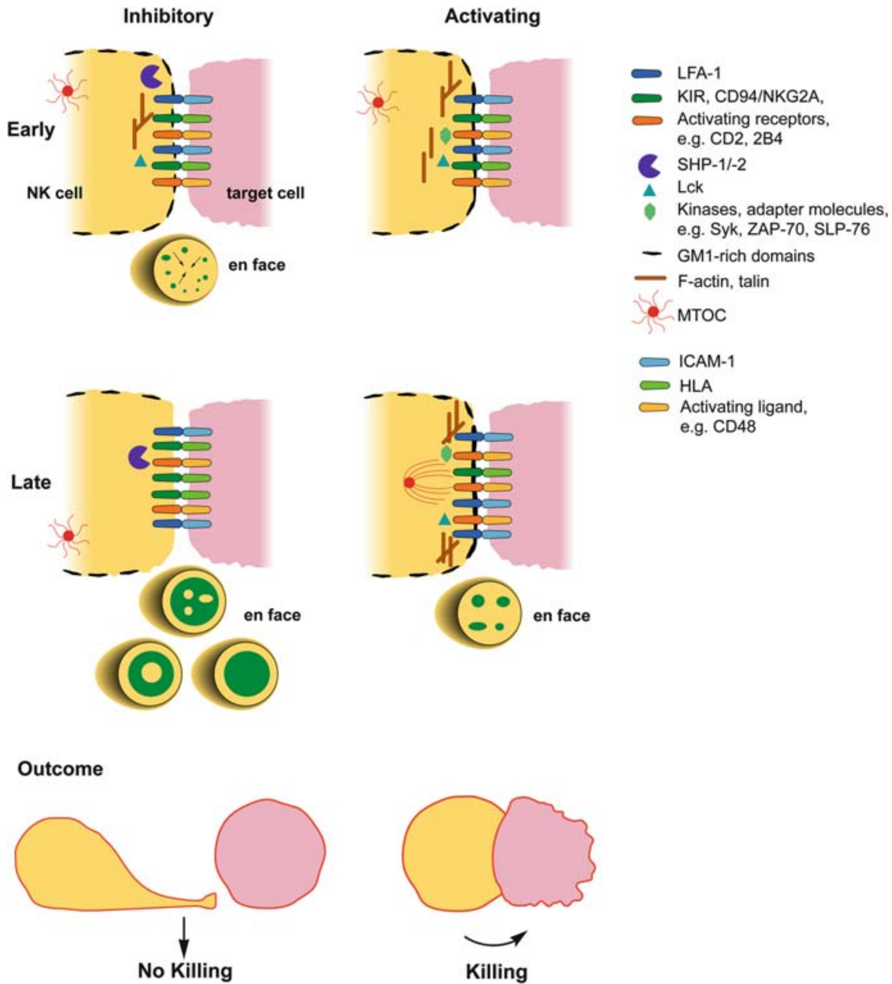

Fig. 1 Comparison of the inhibitory and cytolytic NK-cell IS. Contacts between NK cells and target cells that are protected from NK cell attack result in the formation of an inhibitory synapse with recruitment of inhibitory receptors bound to their respective MHC ligands (Davis et al. 1999; Eriksson et al. 1999b). Initially, inhibitory receptors appear in small clusters that move from the periphery to the centre of the IS to form larger aggregates during IS maturation (Oddos et al. 2008). Concomitantly, the phosphatases SHP-1 and SHP-2 and the kinase Lck accumulate. In the early inhibitory IS, cytoskeletal proteins talin and F-actin and GM1-rich microdomains are detectable (Masilamani et al. 2006; Treanor et al. 2006; Vyas et al. 2002, 2004). Lck, F-actin, talin and GM1-rich microdomains are excluded from the inhibitory IS at later time points, while SHP-1 and SHP-2 remain (Vyas et al. 2002, 2004). Inhibitory receptors segregate from integrins and arrange in different patterns across the synapse being either homogeneously distributed, ring shaped, or containing multiple exclusions (Almeida and Davis 2006; Carlin et al. 2001; Davis et al. 1999; Vyas et al. 2004). Activating receptors, e.g., CD2 and 2B4, are present within the inhibitory IS (Schleinitz et al. 2008). Finally, the NK cell detaches from the target and moves away. Target cells that display reduced expression of class I MHC protein or increased expression of activating NK cell

receptors is essential to provide protection of "self" (Ljunggren and Karre 1990) because, in contrast to T- and B-cells, NK-cell activation does not depend on antigen receptors and is independent of prior sensitization or priming. Inhibitory receptors expressed on human NK cells include the ITIM-containing class I MHC protein binding KIRs and CD94/NKG2A (Lanier 2005). These inhibitory receptors and their ligands rapidly cluster at an inhibitory IS (Davis et al. 1999; Dietrich et al. 2001; Egen and Allison 2002; Eriksson et al. 1999b; Fourmentraux-Neves et al. 2008; Henel et al. 2006; Standeven et al. 2004). Interestingly, clustering of KIR and its class I MHC protein ligands is largely a spontaneous process triggered by binding alone. Accumulation of these proteins does not require receptor signalling or ligation of adhesion molecules (Fassett et al. 2001; Faure et al. 2003) and is largely independent of actin reorganization or ATP-driven cellular processes (Almeida and Davis 2006; Carlin et al. 2001; Davis et al. 1999; Standeven et al. 2004). Indeed, insect cell transfectants expressing class I MHC protein, considered to not express any other ligands for NK cells, can trigger efficient clustering and tyrosine phosphorylation of KIR at the NK-cell IS (Faure et al. 2003). Intriguingly, clustering and phosphorylation of KIR does require the presence of divalent cations such as Zn^{2+} (Davis et al. 1999; Fan et al. 2000; Fassett et al. 2001; Rajagopalan and Long 1998; Rajagopalan et al. 1995; Vales-Gomez et al. 2001), although the molecular basis for this remains unclear. It still remains to be established if efficient spontaneous clustering of inhibitory NK-cell receptors and ligands is essential for their function but it is tempting to speculate that the rapid spontaneous clustering of inhibitory receptors and ligands is important in keeping NK-cell responses tightly controlled. In contrast, however, recruitment of the inhibitory receptor CTLA-4 to the T-cell IS is proportional to the strength of the TCR stimulus (Egen and Allison 2002). This difference may reflect an interesting distinction in the functions and/or mechanisms by which these different inhibitory receptors operate but this requires further investigation.

The supramolecular organization of class I MHC protein across an inhibitory NK-cell IS can form a single cluster, a ring, or a cluster containing multiple regions where class I MHC protein is excluded (Almeida and Davis 2006; Carlin et al. 2001; Oddos et al. 2008). These configurations are dynamic and interchangeable (Almeida and Davis 2006). However, these patterns are less clear for peripheral blood NK-cell clones compared to larger immortal NK-cell lines and thus, it seems unlikely that class I MHC protein being organized in a single or multiple foci, for example, has any direct influence on the outcome of the cell–cell interaction (Almeida and Davis 2006). Instead, recent evidence points to the extent of co-localisation or segregation

Fig. 1 (continued) ligands will activate NK cell cytotoxicity. Early stages of the cytolytic IS are characterized by actin reorganization, the accumulation of GM1-rich microdomains and the recruitment of kinases and adapter molecules including Lck, Syk, ZAP-70 and SLP-76 (Orange et al. 2003; Vyas et al. 2001, 2002, 2004). During maturation, f-actin reorganizes to form a ring in the periphery of the IS, while the MTOC and lytic granules polarize towards the centre (Culley et al. 2009; McCann et al. 2003; Orange et al. 2003; Orange et al. 2002; Vyas et al. 2001). Inhibitory receptors can still be present in cytolytic IS but cluster in multifocal patterns (Almeida and Davis 2006; Schleinitz et al. 2008)

between different receptor/ligand pairs within the organized IS being the important issue. For example, the level of expression of HLA-C on target cells determined its supramolecular organization and the extent of segregation from ICAM-1 (CD54) at the NK-cell IS (Almeida and Davis 2006). Strikingly, for individual peripheral blood NK clones, specific thresholds in the level of target cell HLA-C needed to cause segregation of HLA-C from ICAM-1 at the IS, directly correlated with the threshold needed to functionally inhibit cytotoxicity (Almeida and Davis 2006). Thus, the organization of HLA-C at the IS, determined by its level of expression, may directly influence NK-cell inhibition by regulating the proximity of activating and inhibitory receptors. This would be consistent with earlier studies, using mAb cross-linking, demonstrating that co-clustering of activating and inhibitory receptors was required for inhibition (Blery et al. 1997).

What causes the segregation of different receptor/ligand pairs across the inhibitory NK-cell IS remains unproven. In the "kinetic-segregation model" for T cell receptor triggering it has been proposed that proteins can be organized according to the size of their extracellular domains (Davis and van der Merwe 2006). Accordingly at the inhibitory NK-cell IS, it has been demonstrated that larger proteins, e.g., CD43, are excluded from the IS (McCann et al. 2003). Moreover, the size of KIR-MHC protein is significantly smaller than that of LFA-1 (CD11a/C18)-ICAM-1, which is consistent with their segregation being driven by size differences (Davis 2002; Davis and van der Merwe 1996, 2006; McCann et al. 2002; Springer 1990). Such a model would also explain why the extent of segregation between these proteins was greater when their expression levels were increased (Almeida and Davis 2006). However, a prediction of this model would be that the size of the synaptic cleft would match the size of different proteins where they clustered. In contrast, at least after fixation for examination by electron microscopy, the size of the synaptic cleft varies considerably and apparently randomly, seemingly able to accommodate a range of protein sizes in close proximity (McCann et al. 2003). Thus, it is important to study in more detail whether the size of proteins influences their organization at the NK-cell IS and if so, it must be clarified whether this affects protein segregation at the level of microclusters and/or larger-scale segregation across the synapse.

While some receptor/ligand pairs can accumulate spontaneously, active receptor signalling also plays a crucial role in the organization of inhibitory synapses. Functional ITIM tyrosines and the catalytic activity of SHP-1 are required for disruption of the actin cytoskeleton and exclusion of GM1-rich microdomains from NK-cell or cytotoxic T-cell synapses (Fassett et al. 2001; Guerra et al. 2002; Lou et al. 2000; Masilamani et al. 2006). Proteins associated with GM1-rich microdomains play an essential role in the initial phosphorylation of activating NK-cell receptors (Inoue et al. 2002; Watzl and Long 2003). Thus, one way in which inhibitory NK-cell receptors can be effective, is by blocking the actin-cytoskeleton dependent recruitment of signalling proteins within GM1-rich microdomains to the IS (Endt et al. 2007; Fassett et al. 2001; Fourmentraux-Neves et al. 2008; Guerra et al. 2002; Lou et al. 2000; Masilamani et al. 2006; Sanni et al. 2004; Sohn et al. 2008; Tsai and Discher 2008; Vyas et al. 2002; Vyas et al. 2004; Watzl

and Long 2003). Consistent with this model, activating receptors CD2 and 2B4 (CD244) are not inhibited from being recruited to an inhibitory NK-cell IS (Schleinitz et al. 2008), but rather are likely to be impaired in their ability to signal there. Similarly in B cells, for example, the inhibitory receptor FcγRIIB blocks association of the BCR with lipid raft-like domains and also prevents subsequent accumulation of BCR-enriched microclusters in the centre of the synapse (Sohn et al. 2008). In T cells, the inhibitory receptor CTLA-4 inhibits formation of ZAP-70 microclusters (Schneider et al. 2008). Taken together, this suggests a common principle in that inhibitory synapses still accumulate activating receptors and ligands, but specifically exclude the membrane-proximal intracellular proteins needed for activation.

4 Balancing Synapses with Kinapses and Kinetic Proofreading at the Cellular Level

The term kinapse has recently been proposed to describe junctions involving moving T cells that allow signals to be integrated (Dustin 2008a, b). Kinapses lack the degree of stability characteristic for synapses and cell polarity is maintained in the direction of cell movement, rather than being orientated to face the intercellular contact. It is well established that ligation of the TCR delivers a stop signal to T cells (Dustin et al. 1997) that precedes synapse formation (Lee et al. 2002). NK cells similarly crawl over the surface of target cells, notably with higher motility for inhibitory contacts (Burshtyn et al. 2000; Davis et al. 1999; Eriksson et al. 1999a). Likewise, ligation of activating NK-cell receptors provides a stop signal that results in symmetrical spreading of NK cells over their targets, while ligation of inhibitory receptors provides a reverse-stop signal that breaks the symmetry of spreading and encourages NK-cell migration (Culley et al. 2009). Similarly, the inhibitory receptor CTLA-4 reverses the TCR-mediated stop signal (Schneider et al. 2006). Thus, an inhibitory synapse may be considered as a transient symmetrical synapse driving an effector cell to revert to its migratory kinapse configuration. PKCθ and WASp were found to favour T cells forming a kinapse or synapse, respectively (Sims et al. 2007). It would be interesting to determine if inhibitory NK-cell IS exploit these pathways in driving cells to a kinapse configuration. Indeed, inhibitory signals in NK cells are known to directly regulate cytoskeletal processes involving WASp (Krzewski et al. 2006) and Vav (Stebbins et al. 2003).

It is well established that cytolytic NK cell synapses go through sequential steps that lead to the directed release of lytic granules (Davis 2002, 2009; Davis and Dustin 2004; Krzewski and Strominger 2008; Orange 2008; Wulfing et al 2003). Thus, the process of cellular activation can be considered as directly analogous to the model of kinetic-proofreading for triggering individual TCR signals (McKeithan 1995). Specifically, there are a number of steps that two cells in contact must go through before lytic granules are released or other effector functions

realized. These include a multitude of cellular processes, such as calcium flux, integrin-mediated tight adhesion, MTOC reorientation, translocation of granules to the synapse and many others. These steps introduce a series of time delays from initial intercellular contact until the effector function is realized, e.g. lytic granules are released. Thus, an inhibitory synapse serves to shorten the half-life of the intercellular conjugate and break the IS before these steps can be completed, preventing effector functions.

5 Unzipping the Synapse

There has been extensive research on the assembly of the IS yet relatively little attention has been given to its disassembly. Thousands of individual protein–protein interactions exist across the IS such that the disassembly of this contact cannot be trivial. Perhaps most acutely, when inhibition dominates the outcome of surveillance, e.g. at the inhibitory NK-cell IS, protein–protein interactions accumulated at the synapse must be rapidly removed or broken so that NK cells can readily move on to survey other target cells. Similarly, for cytolytic interactions involving CTL or NK cells, it is unclear how the effector cells efficiently move away from dead or dying target cells. Efficient disassembly of the synapse is important to allow cells to move between target cells and must be necessary, for example, for CTL or NK cells to sequentially kill several target cells (Bhat and Watzl 2007; Martz 1976). It has been demonstrated that some receptors are endocytosed from the IS upon ligation, e.g., the T-cell receptor (TCR) (Cemerski et al. 2008; Lee et al. 2003), but it has not been directly tested whether or not these events are important in the disassembly of the IS. Indeed, it is unclear how many protein-protein interactions would need to be removed from an IS to allow cells to move apart. Alternatively, it can be envisaged that exocytosis of the synaptic membrane from the target cells or APCs could contribute to the disassembly of the IS. This could relate to the common process of intercellular transfer of surface proteins between immune cells that can occur by several specific mechanisms (Davis 2007; LeMaoult et al. 2007). More broadly, the extent to which specific signalling events control termination of the synapse has been little studied. It is well understood that inside-out signalling leads to a high-affinity conformation of LFA-1 that in turn contributes to intercellular conjugation and assembly of the IS (Luo et al. 2007). However, it has been far less-studied whether or not specific signals could return LFA-1 to a lower affinity state and contribute to the disassembly of the synapse.

6 Regulatory Synapses

In addition to the autonomous interaction of NK cells with infected or transformed cells, research has expanded in recent years to study the cross-talk between NK cells and other immune cells, including monocytes, macrophages, dendritic cells

and T cells. These interactions can augment or initiate NK-cell responses to pathological challenges and can also shape adaptive immune responses, e.g., by triggering DC maturation (Andoniou et al. 2008; Fernandez et al. 1999; Moretta et al. 2006; Newman and Riley 2007; Raulet 2004; Strowig et al. 2008). Contact-dependent reciprocal stimulation plays an important role during these interactions and several studies have therefore investigated the organization of these intercellular contacts termed regulatory synapses (Borg et al. 2004; Brilot et al. 2007; Nedvetzki et al. 2007; Pallandre et al. 2008).

7 The Regulatory NK-Cell Synapse

Regulatory NK-cell synapses are long lasting and accumulate activating receptors, cytokine receptors and adhesion molecules (Borg et al. 2004; Brilot et al. 2007; Nedvetzki et al. 2007; Semino et al. 2005). Figure 2 summarizes current knowledge of the molecular arrangements at such regulatory NK-cell synapses. In contrast to an inhibitory IS, cytoskeletal components, f-actin, fascin and talin as well as GM1-rich microdomains all accumulate at the regulatory IS (Borg et al. 2004; Brilot et al. 2007; Nedvetzki et al. 2007; Semino et al. 2005). Inhibitory receptors do still cluster at such IS and accumulate adjacent to clusters of cytokine receptors, surrounded by a ring of LFA-1 and talin (Brilot et al. 2007). Cytokines and cytokine receptors accumulate at the regulatory NK-cell IS (Borg et al. 2004; Brilot et al. 2007; Semino et al. 2005). IL-18 is directionally secreted across the synapse between NK cells and immature DCs (iDCs) and stimulates secretion of HMGB1 by NK cells, which in turn is necessary to induce DC maturation (Semino et al. 2005). Mature DCs (mDCs) polarize preassembled stores of IL-12 towards the NK-cell IS (Borg et al. 2004) and NK cells accumulate the high affinity subunit of the IL-15 receptor, IL-15Rα, at the IS (Brilot et al. 2007). Polarization of cytokines towards the regulatory NK-cell IS implicates an importance of synapse formation for directed cytokine secretion. This is likely to be important in a wide range of immune cell interactions. For example, co-culture experiments using a transwell membrane recently determined that IL-18 is delivered to NK cells in a contact dependent manner by Kupffer (liver macrophage-like) cells (Tu et al. 2008). As determined for T cells, a general principle is that some cytokines and chemokines are secreted multi-directionally from effector cells, to have a broad impact on inflammation, while others are secreted directionally via the IS where specific intercellular communication is required (Brilot et al. 2007).

8 Triggering Cytokine Secretion Versus Cytolysis

There is evidence that immature DCs are susceptible to lysis by NK cells and acquire protection from lysis by maturation (Moretta et al. 2006; Strowig et al. 2008). Killing of iDCs can be triggered via the natural cytotoxicity receptor NKp30

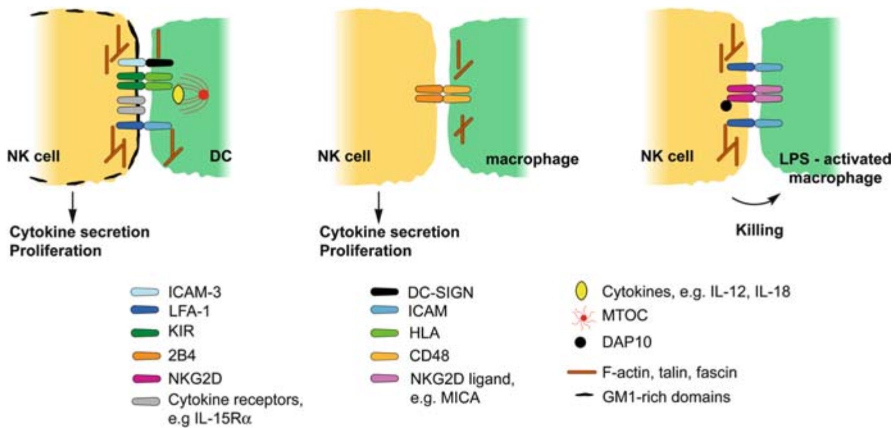

Fig. 2 The regulatory NK-cell IS. The interaction of NK cells with DCs or macrophages can induce NK cell proliferation or cytokine secretion, without triggering cytotoxicity. The resulting IS has therefore been termed regulatory (Borg et al. 2004; Brilot et al. 2007; Nedvetzki et al. 2007; Pallandre et al. 2008). DCs polarize the MTOC and cytokines including IL-12 and IL-18 towards the IS (Borg et al. 2004). In NK cells, the IL-15Rα subunit is recruited to the contact site where it segregates from inhibitory NK-cell receptors (Brilot et al. 2007). Furthermore, adhesion molecules and their ligands, e.g. LFA-1, DC-SIGN and ICAM-3, polarize towards the cell interface (Borg et al. 2004; Brilot et al. 2007). Both, NK cells and DCs show reorganization of the actin-cytoskeleton, with cytoskeletal proteins including f-actin, talin and fascin accumulating at the synapse (Borg et al. 2004; Brilot et al. 2007). Additionally, GM1-rich microdomains enrich within the NK cell membrane in the contact area (Borg et al. 2004). Macrophages stimulate NK cell proliferation and cytokine secretion and prime NK cell cytotoxicity against susceptible target cells. This is largely dependent on the engagement of the 2B4 receptor, which is recruited to the centre of such regulatory synapses. In these contacts F-actin accumulates at the synapse from within macrophages, but not NK cells. Macrophages exposed to a high dose of LPS upregulate ligands for the NK cell receptor NKG2D and are subsequently killed by NK cells. These cytolytic NK cell–macrophage synapses accumulate NKG2D and the signalling adapters DAP10 and CD3ζ at the centre of the IS, while ICAM-1 locates to the periphery. F-actin accumulates at the contact area from within the NK cells, but not in the macrophages (Nedvetzki et al. 2007)

(CD337) (Ferlazzo et al. 2002; Spaggiari et al. 2001) while increased expression of class I MHC protein during DC maturation provides protection from NK-cell lysis (Carbone et al. 1999; Ferlazzo et al. 2002). Whether macrophages induce a cytolytic or regulatory NK-cell IS depends on the activation state of the macrophage in terms of the strength of TLR-4 stimulation (Nedvetzki et al. 2007). Macrophages that are stimulated with a low dose of LPS form regulatory synapses, characterized by the recruitment of the NK-cell receptor 2B4, while stimulation with a high dose of LPS induces upregulation of NKG2D (CD314) ligands on macrophages that triggers NKG2D-mediated killing (Nedvetzki et al. 2007).

It is particularly intriguing that 2B4 is recruited to the regulatory NK-cell-macrophage IS and is important for triggering cytokine secretion (Nedvetzki et al. 2007), while at other synapses, e.g., during interactions with tumour cell targets, 2B4 ligation triggers cytotoxicity (Bhat et al. 2006). Mechanisms must be in place to determine the function of 2B4-mediated activation. One possibility is that

this depends on the synergy with other activating receptors. Indeed, only specific combinations of activating receptors can induce granule exocytosis in human NK cells (Bryceson et al. 2006). Receptors not specifically associated with NK-cell activation can be important in determining the NK cell response. For example, the chemokine CX3CL1 influences the distribution of KIR at the NK cell–DC synapse and is able to prevent phosphorylation of its ITIMs (Pallandre et al. 2008). Of particular interest, 2B4 has been shown to directly interact with a variety of signalling molecules (Eissmann et al. 2005) and to mediate activation of granule exocytosis or cytokine secretion (Kubin et al. 1999; Nakajima et al. 1999; Tangye et al. 1999), as well as inhibition (Parolini et al. 2000; Sivori et al. 2002). Thus, the availability of downstream signalling proteins at the IS may determine the outcome of 2B4 stimulation in NK cells. In this case, regulatory synapses would function by selectively recruiting certain membrane-proximal adaptors or kinases, analogous to the restricted recruitment of signalling proteins seen at inhibitory synapses.

Overall, it is clear that there is an important role for the IS in balancing activation, regulation and inhibition of immune responses. Much of what has been achieved so far is a direct result of the inter-disciplinary approach that immunologists have taken relatively recently to probe molecular recognition by individual cells. The continuing development and application of new techniques that allow intercellular communication to be probed with superior spatial and temporal resolution will enable scientists to resolve many of the outstanding issues highlighted throughout this review. Already, numerous recent super-resolution imaging techniques have the potential to directly report the spatial and temporal relationships of the key molecules (Fernandez-Suarez and Ting 2008). Perhaps most exciting is that as we continue to probe immune cell recognition with superior resolution, unexpected signalling and integration mechanisms that were not apparent at conventional diffraction-limited resolution will surely be revealed.

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