File ID	161777
Filename	Chapter 1: General introduction

SOURCE (OR PART OF THE FOLLOWING SOURCE):

Type	Dissertation
Title	Molecular mechanisms underlying CD27-CD70 costimulation
Author	V. Peperzak
Faculty	Faculty of Science
Year	2010
Pages	161

FULL BIBLIOGRAPHIC DETAILS:
http://dare.uva.nl/record/328469

Chapter 1

General Introduction

GENERAL INTRODUCTION

We are in a constant state of war, threatened by both exogenous enemies in the form of killing pathogens, or enemies from within in the form of tumor cells. The key for survival of our species amongst an overwhelming amount and diversity of microorganisms, such as viruses, bacteria and parasites is a well functioning immune system. Essential for the immune system to function properly is the ability to distinguish between foreign agents, malignant cells and the organism's own healthy cells. The earliest written mention of immunity goes back to 430 BC during the plague of Athens when it was discovered that people who had recovered from the disease could nurse the sick without contracting the illness a second time (1). However, it was not until the 19th and 20th centuries before the concept developed into scientific theory.

Two different types of immune responses can be distinguished that are referred to as the innate and the adaptive immune system. Usually, physical barriers prevent pathogens like bacteria and viruses from entering an organism, but if a pathogen breaches these barriers, the innate immune system provides an immediate albeit non-specific response. The innate immune system is thought to constitute an evolutionarily old defense strategy and is found in all plants and animals. Major functions of the vertebrate innate immune system include the identification and removal of foreign substances, the activation of the complement cascade, recruitment of immune cells to sites of infection and activation of the adaptive immune system through antigen presentation (2,3).

The adaptive immune response

An acquired, or adaptive immune response develops during the lifetime of an individual as an adaptation of infection with that pathogen. This process creates immunological memory. Immunological memory created from a primary response to a specific pathogen provides an enhanced response to secondary encounters with that same specific pathogen. This process of acquired immunity is the basis of vaccination strategies (3).

Cells that make up the adaptive immune system comprise B and T lymphocytes. The specificity of the adaptive immune response is determined by antigen receptors on B and T cells, the B cell antigen receptor (BCR) and the T cell antigen receptor (TCR), respectively. As part of the adaptive immune system, B cells regulate humoral responses by secreting a soluble form of their antigen receptor referred to as immunoglobulins (Ig). These Ig, or antibodies, promote phagocytosis of pathogens and support complement mediated lysis of infected cells. Activation and maturation of B cells requires the activation of dendritic cells (DC) and the recruitment of antigen specific helper T (T_H) cells. This leads to the development of germinal centers (GC), where B cells mature and differentiate into efficient high-affinity antibody secreting cells (plasma cells) (4).

Naïve T cells are activated by antigen derived peptides in the context of major histocompatibility complexes (MHC), which are expressed on antigen presenting cells (APC). $CD4^+$ T cells recognize peptides derived from exogenous proteins in the context of MHC class II that are expressed solely on professional APC, which include DC, B cells and macrophages. $CD4^+$ T cells can differentiate into different T-helper (T_H) subsets with functions that are tailored to their respective roles during infection. As such, in the presence of the cytokines interleukin (IL)-12 and IFN-γ, $CD4^+$ T cells can differentiate into T_H1 cells that typically express IFN-γ and TNF-α and aid macrophages to kill pathogens. Additionally, T_H1 cells can promote the $CD8^+$ T cell response directly by providing the pro-survival cytokine IL-2 or by stimulating the maturation of DC to further

help the $CD8^+$ T cell response. When the cytokine IL-4 is present, $CD4^+$ T cells can differentiate into T_H2 cells that subsequently produce IL-4, IL-5 and IL-13 and promote humoral immune responses. Finally, when naïve $CD4^+$ T cells are activated in the presence of IL-6 and TGFβ, $CD4^+$ T cells will differentiate into T_H17 cells and express the cytokine IL-17 and in some cases also IL-21 and IL-22. The exact role for T_H17 cells is currently under investigation, but recent work has shown that these cells participate in certain infections with Gram-negative bacteria, fungi and parasites (5). In addition, $CD4^+$ T cells exist in the form of regulatory T cells (Treg). Treg can differentiate from naïve $CD4^+$ T cells in the periphery (inducible Treg) or develop from $CD4^+CD8^+$ double positive thymocytes (natural Treg). In contrast to T helper cells, Tregs suppress immune responses via secretion of IL-10 and TGFβ and by expression of inhibitory receptors such as CTLA-4 (6).

$CD8^+$ T cells recognize peptides derived from endogenous proteins in the context of MHC class I that is expressed on all nucleated cells (7). Alternatively, $CD8^+$ T cells can also recognize peptides derived from cell-exogenous sources in a process referred to as cross-presentation (8). This process requires a professional APC expressing MHC class I. Differentiated effector $CD8^+$ T cells, the so called cytotoxic T lymphocytes (CTL), are capable of killing virus infected cells or tumor cells via the expression of the cytotoxins perforin and granzyme. An alternative killing mechanism that can also be used by $CD4^+$ T cells proceeds by expression of the apoptosis promoting Fas (CD95) ligand on the T cell surface (3).

T cell activation

During the interaction between a T cell and an APC, a specialized molecular structure at the contact site is formed. Several key molecules important for T cell activation are reorganized in this structure which is referred to as the immunological synapse (IS). Costimulatory molecules, signaling molecules and certain adaptor proteins can be recruited to the immunological synapse and these are critical in deciding the fate of the contacted T cell. (9).

At first, a T cell contacts an APC by virtue of interactions of the adhesion molecule leukocyte function-associated antigen-1 (LFA-1) expressed by T cells with its ligand intercellular adhesion molecule-1 (ICAM-1) present on APC. Subsequently, the TCR and CD4 or CD8 molecules interact with MHC molecules on APC (10). It has been observed that this results in a TCR-rich area surrounding a central area of LFA-1 at the contact site. Engaged TCRs are present in microclusters to which the CD8 or CD4 co-receptors and various co-stimulatory molecules are recruited (11-13). CD4 or CD8 molecules deliver Lck, bound to their cytoplasmic tail, to the TCR-associated CD3 chains (14,15). Lck engages the Src family kinase Fyn and together these kinases phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs), which are present in CD3 molecules and in the CD3ζ chain associated protein of 70 kDa (ZAP-70). ZAP-70 phosphorylates tyrosine residues of the adaptor protein linker of activation for T cells (LAT) and Src-homology-2-domain-containing leukocyte protein of 76 kDa (SLP-76), serving as a platform for recruitment of several signaling molecules, such as phospholipase C γ (PLCγ) (16). Subsequently, PLCγ generates inositoltrisphosphate (IP_3) and diacylglycerol (DAG) from the membrane lipid phosphatidylinositolbisphosphate (PIP_2). IP_3 promotes the release of Ca^{2+} from intracellular stores, thereby activating nuclear factor of activated T cells (NFAT)-mediated transcription (11,17).

Besides the aggregation of TCR signaling components to the IS, the recruitment and clustering of co-stimulatory molecules in the

IS can facilitate their function. For example, engagement of the Ig-like co-stimulatory receptor CD28 outside the IS results in stabilization of IL-2 mRNA, but not in increased IL-2 transcription that is observed when CD28 is engaged in the IS. As a consequence, IL-2 secretion is higher when CD28 engagement occurs in the IS where the signaling component PKCθ, required for IL-2 transcription, is more abundant (18). Furthermore, movement of the Golgi apparatus towards the IS suggests that the secretion of molecules is also polarized in the direction of the IS (19). Indeed, naïve $CD4^+$ T cells polarize IL-2 expression to the site of highest TCR concentration (20). Moreover, the IL-2 receptor and the IFN-γ receptor localize to the IS and can be activated instantaneously by IL-2 and IFN-γ before these cytokines are able to leave the IS (21).

Costimulation

The fate of activated T cells is determined by the collective contribution of different co-stimulatory and inhibitory molecules upon initial engagement of the TCR. Engagement of the TCR alone on naïve T cells results in anergy. To prevent this, a second, so-called co-stimulatory signal is generally key for clonal expansion and differentiation of a naïve T cell into an effector cell. While triggering of the TCR promotes entry into the G1 phase of the cell cycle, CD28 stimulation by its ligands CD80 and CD86 is necessary for the T cell to enter the S phase and complete subsequent steps leading to division of the T cell. It has been suggested that CD28 functions as a signal amplifier for the TCR when TCR input is low (22). Stimulation of CD28 increases IL-2 expression, enhances T cell division and can also mediate the survival of T cells by two different manners. First, CD28 can bind phosphoinositide 3-kinase (PI-3K), which leads to the activation of Akt and consequent induction of the anti-apoptotic Bcl-2 family member Bcl-x_L (23,24). Alternatively, CD28 induced Akt can promote the activity of mammalian target of rapamycin (mTOR) which improves glucose and amino acid uptake and promotes the efficiency of protein synthesis during rapid expansion of T cells where an increase in metabolism is crucial for the cell to survive these stressful conditions (25-27).

In addition to the effects of CD28-CD80/86 interactions, members of the Tumor Necrosis Factor Receptor (TNFR) family are emerging as key mediators of survival signaling in T cells (28,29). Well studied TNFR family members include OX40, 4-1BB, HVEM, CD30, GITR and CD27. When engaged by their respective ligands, these TNF receptors bind to TNF receptor-associated factors (TRAFs) and can have co-stimulatory effects on T cells (30,31). All of these six TNF receptors have been shown to recruit TRAF family member 2 (TRAF2), which induces activation of the transcription factor complex nuclear factor κB (NF-κB) (29,32). Transcription of several well known anti-apoptotic genes is mediated by activated NF-κB, such as cellular inhibitors of apoptosis (cIAP1 and -2), Bcl-x_L and c-Flip (33). Additionally, TRAF2 aggregation can result in the activation of mitogen activated protein (MAP) 3 or MAP4 kinases, thereby linking to the c-Jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAPK cascades (31,34).

The contribution of CD27, 4-1BB and OX40 to the survival of primed T cells has been compared in several studies (35). Using mice genetically deficient for 4-1BB, OX40, or their ligands, has revealed that the impact of OX40 is most prominent on the survival of primed $CD4^+$ T cells, while the impact of 4-1BB is more pronounced on $CD8^+$ T cell survival (36-41). In contrast, CD27 promotes the survival of both $CD4^+$ as well as $CD8^+$ T cells during the primary immune response, which has been investigated intensively using $CD27^{-/-}$ mice in a

model of influenza virus infection (42,43). These data were confirmed by reverse approaches, in which WT $CD8^+$ T cell responses were examined and soluble recombinant CD70 was infused, CD27-CD70 signaling was blocked by inhibitory anti-CD70 mAb, or CD70 was expressed transgenically on B cells or DC (44-49). Additionally, the CD27-CD70 interaction promotes the formation of $CD8^+$ memory T cells (35). When comparing the role for 4-1BB, OX40 and CD27 side-by-side in a model of influenza virus infection, it has been shown that each receptor makes unique contributions to the immune response. Specifically, CD27 expressed on naïve T cells promotes the accumulation of influenza virus-specific T cells during clonal expansion, whereas 4-1BB, induced upon activation, supports the subsequent survival of effector T cells. On the other hand, OX40 does not appear to contribute to the accumulation of $CD8^+$ effector T cells during the clonal expansion or effector phases, but promotes the formation of $CD8^+$ memory T cells (35).

CD27

Hematopoietic stem cells (HSC) reside in the adult bone marrow and can be subdivided into long-term repopulating HSC (LT-HSC) and short-term repopulating HSC (ST-HSC). It is thought that the LT-HSC are upstream of ST-HSC which can differentiate into either common myeloid progenitors (CMP) or common lymphoid progenitors (CLP), of which the latter can further differentiate into B and T cells (50). Stimulation of CD27, which is highly expressed on ST-HSC, but not on LT-HSC (51,52), inhibits leukocyte differentiation and may serve as a feedback mechanism that regulates hematopoiesis during conditions of chronic infection (53). On CLP, CD27 expression marks cells that differentiate into T cells, whereas CLP that lose CD27 membrane expression differentiate into B cells (54). CD27 remains expressed on lymphoid progenitors when leaving the bone marrow until arrival in the thymus (55,56). Early thymocyte progenitor cells, phenotyped as CD4 and CD8 double negative and $CD44^+CD25^-$ (DN1), express high levels of CD27, but this rapidly decreases throughout the $CD44^+CD25^+$ (DN2) and $CD44^-CD25^+$ (DN3) stage of thymic development. In the DN3 stage two populations can be distinguished, one with low CD27 expression (DN3a) and one with high CD27 expression (DN3b). Since DN3b thymocytes are much more efficient in generating CD4 and CD8 double positive (DP) progeny, it is suggested that CD27 expression might be a crucial checkpoint for progression of DN3 cells to the DP stage (57). Instead of differentiating into DP cells expressing a TCR with an α and β chain ($\alpha\beta$-TCR), DN T cells in the thymus that express a re-arranged TCR gamma and delta chain ($\gamma\delta$-T cells) can also differentiate into mature T cells (58). CD27 expression discriminates between two functionally different cellular subsets of $\gamma\delta$-T cells. CD27 positive $\gamma\delta$-T cells up-regulate the lymphotoxin beta receptor (LTβR) and produce IFN-γ upon stimulation, while CD27 negative $\gamma\delta$-T cells are potent producers of IL-17 (59).

Initially, CD27 was described as a T cell specific antigen of 55 kDa (60-62). Addition of antibodies directed against CD27 to T cell cultures with phytohemagglutinin (PHA) or anti-CD3 antibodies, results in enhanced T cell clonal expansion *in vitro* (60). These initial findings were the first to suggest that CD27 is an important molecule for T cell activation. CD27 is a Type I transmembrane receptor that shares with other TNFR family members an extracellular domain with cysteine repeats, which give these receptors the conformation to bind their TNF-like ligands (63,64). Human T cells can express a soluble form of CD27 (65) which is cleaved from the full length CD27 molecule by, most likely, a membrane-linked protease (66). In addition to expression on T cells, CD27 is also expressed on human (67) and murine (68) NK cells. In

humans, CD27 is expressed on activated (69) and memory (70) B cells, while in mice CD27 is transiently expressed upon activation during the germinal center (GC) reaction (71). Only a small percentage of murine memory B cells express CD27 and, in contrast to the human situation, is therefore not considered as a marker for memory B cells (71) (paragraphs summarized in Figure 1).

CD27 is expressed on most naïve conventional αβ-T cells in the periphery and can be further induced upon stimulation via the T cell receptor (TCR) as a result of *de novo* gene transcription. Several days after stimulation, CD27 membrane expression is down-regulated to the level of naïve T cells (65,72). In humans, CD27 represents a marker for naïve and memory $CD4^+$ and $CD8^+$ T cells. Effector T cells lose CD27 expression which is suggested to be a resultant of CD70-CD27 interactions (73,74,64).

Expression of CD70, the ligand for CD27, is highly transient and tightly controlled (75). In naïve resting humans or mice, expression of CD70 is almost totally absent, with an exception of the thymic epithelium where CD70 expression can be found on medullary thymic epithelial cells (mTEC) (76,77). In the context of an immune response, CD70 can also be expressed by DC, macrophages, B and T cells (78,79). CD70 expression in human (80) and murine (76) APC is induced in response to Toll-like receptor (TLR) and/or CD40 stimulation. Since CD70 is normally retained intracellularly in MHCII compartments (MIIC) in non-stimulated cells (81), expression of CD70 on the cell surface does not solely depend on *de novo* transcription (82,83), but also depends on the tightly controlled intracellular transport from MIIC to the IS (81). The importance of the regulated expression of CD70 became clear after analysis of mice with transgenic (Tg) CD70 expression. B cell specific CD70 transgenic mice (CD19-CD70) develop highly increased numbers of effector $CD4^+$ and $CD8^+$ T cells under steady state conditions, eventually leading to IFN-γ mediated depletion of B cells (84). Additionally, like in B cell specific transgenic mice (84), DC specific CD70 transgenic mice (CD11c-CD70) revealed a conversion of naïve $CD4^+$ and $CD8^+$ T cells into effector T cells in the absence of inflammatory conditions (49). In a study using B cell specific CD70 Tg mice, CD70 itself was found to convey signals into B cells via PI3K and MEK pathways, thereby regulating expansion and differentiation of these cells (85). Since CD70 has always been seen as a ligand that initiates signaling by CD27, this event was referred to as 'reverse signaling', conforming to observations for other TNF like ligands.

CD27 mediated signaling

After CD70 engagement, CD27 recruits both TRAF2 and TRAF5, thereby activating NF-κB inducing kinase (NIK) that degrades inhibitor of κB (IκB) proteins resulting in NF-κB mediated gene transcription (86,87). CD27 can signal both via the canonical as well as the non-canonical NF-κB pathway (88). Furthermore, CD27 induced aggregation of TRAF2 also promotes gene transcription via the JNK pathway (88-90). However, the transcriptional targets downstream of CD27 are largely unknown.

CD27 signaling is generally regarded to enhance the secretion of IFN-γ by a range of lymphocytes. For NK (91) and γδ-T cells (59), CD27-expressing subsets are known to produce higher quantities of IFN-γ than CD27-negative subsets, while ligation of CD27 on $CD4^+$ and $CD8^+$ T cells is known to enhance IFN-γ production (84). In humans, activation of CD27 on $CD4^+$ T cells has also been shown to mediate IFN-γ production, as well as up-regulate the expression of IL-12 receptor β chain (92). Therefore, CD27 stimulation is suggested to support T_H1 type responses (92).

CD27 has also been strongly implicated in stimulating pro-survival pathways in T cells

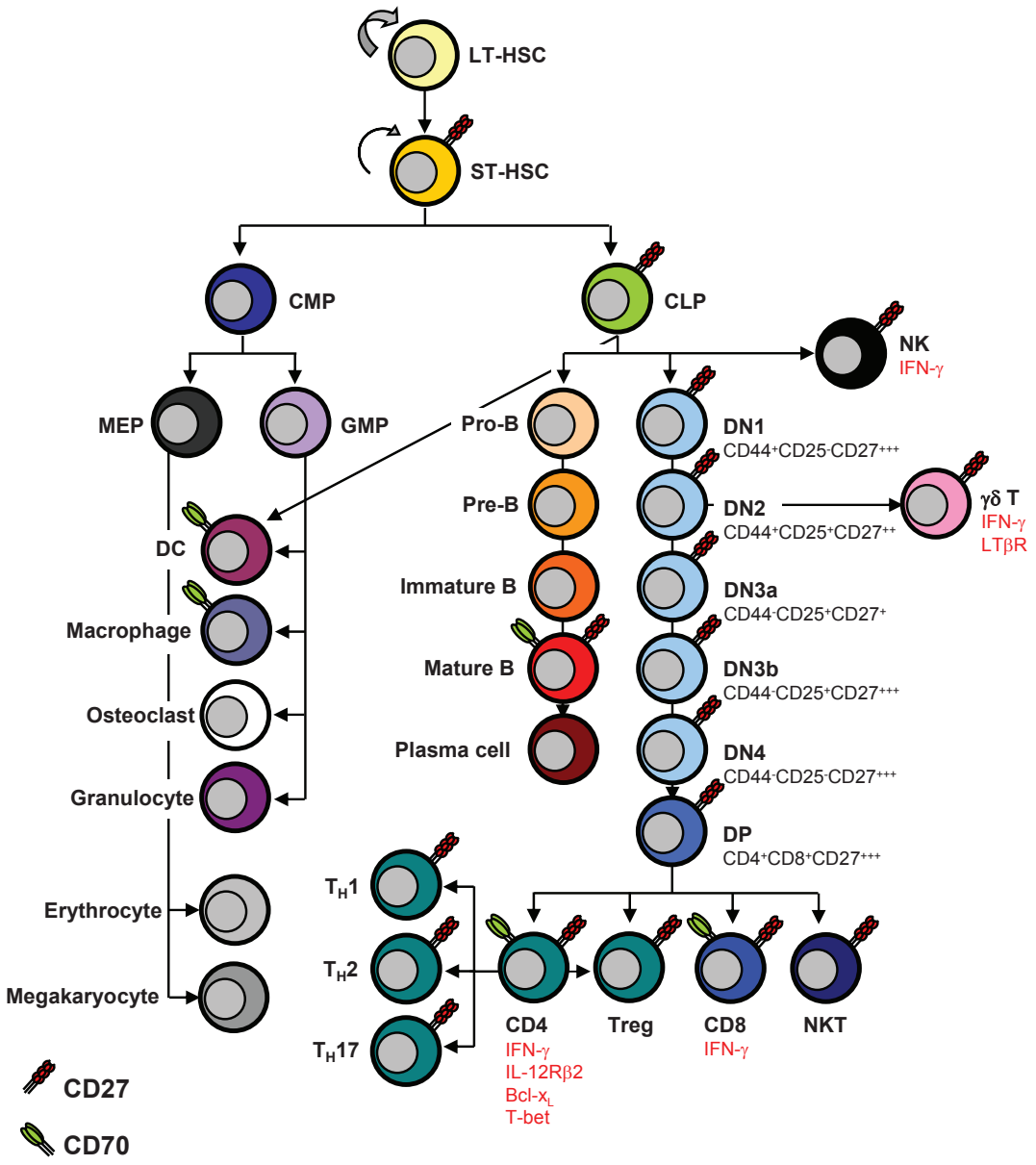

Figure 1. CD27 and CD70 expression during hematopoiesis.
Cell surface expression of CD27 is shown on different cell types during hematopoiesis. For CD27, presence on the cell surface is observed under steady state conditions. In contrast, CD70 is only expressed transiently on the cell surface of the indicated cell types during an immune response. In addition, molecules reported to be induced by CD27 signaling are depicted in red.

(43). Specifically, the anti-apoptotic Bcl-2 family member Bcl-x_L has been shown to be up-regulated in human $CD4^+$ T cells (92). TNF receptors OX40 and 4-1BB also mediate T cell survival via mechanisms that appear to involve the up-regulation of Bcl-2 family members (93-95). Paradoxically, a few studies implicated Siva in the signaling pathway downstream of CD27 (96,97). On the one hand Siva was shown to inhibit Bcl-x_L and mediate caspase dependent apoptosis in T cells (98,99). However, on the other hand, a recent publication argues that Siva strongly inhibited p53-dependent gene expression and apoptosis (100). Therefore, a contribution of Siva to CD27 function remains disputable. At the start of this thesis work, it was unknown exactly which survival pathways are involved in CD27 signaling.

Scope of this thesis

This thesis addresses the underlying mechanism of the function of CD27 during T cell responses. We use genome wide microarray analyses to explore the transcriptional targets of CD27 in T cells, followed by retroviral reconstitution of these genes in CD27-deficient T cells to examine their function. First, we reconsidered the role of CD27 in T cell development when gene array revealed an upregulation of its close relative HVEM in naïve $CD27^{-/-}$ T cells. (Chapter 2). The mechanisms of CD27-mediated $CD8^+$ effector T cell accumulation are examined in this thesis during the primary immune response against influenza virus infection (Chapters 3, 4 and 5). The timing and regulation of CD27 stimulation is crucial, while CD27 triggering can be especially beneficial during ongoing T cell responses, continuous stimulation of CD27 in steady state conditions can be extremely harmful (84,49). We examined when and to what extend CD27 stimulation on $CD4^+$ T cells may contribute to $CD8^+$ T cell responses (Chapter 6). Furthermore, we investigated the mechanism of CD70 trafficking to MHC class II compartments, which is necessary for recruitment to the cell surface and signal to CD27 on the engaged T cell (Chapter 7). Finally, a general discussion of the preceding chapters and their relation to published literature is presented in Chapter 8.

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