# The Heat Shock Protein Gp96 –
# The Immune System's Swiss Army Knife

# Das Hitzeschockprotein Gp96 –
# Das Schweizer Taschenmesser des Immunsystems

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## Contents

# 1 A brief history of immunology

It is rather difficult to determine the beginning of the science of immunology. However, there seems to be a consensus between immunology textbooks that 1798 is the appropriate starting point. In this year, the English physician Edward Jenner published "An inquiry into the Causes and Effects of the Variolae Vaccinae", introducing for the first time the act of *vaccination* (from vacca, lat. for cow). The publication describes the vaccination of an eight-year old boy with material from a pustule of a milkmaid that had been infected by cowpox or vaccinia, a non-fatal disease for humans. Jenner demonstrated that after this initial inoculation the boy was protected or *immune* against subsequent challenges by the related human smallpox disease, which is fatal for humans in many cases. In this way Jenner introduced the practice of smallpox vaccination which finally led to the announcement of the eradication of smallpox by the World Health Organization in 1974. However, Jenner had not been aware of the existence of microorganisms which were later shown to be responsible for the cause of infectious diseases by Robert Koch who isolated *Mycobacterium tuberculosis*. The broader term *pathogen* describes a group of infectious agents including microorganisms like viruses, bacteria, pathogenic fungi as well as eukaryotic parasites.

In 1878 Louis Pasteur succeeded in growing cultures of bacteria later called *Pasteurella multocida*, which cause cholera in chicken but not in humans. He inoculated chicken with a culture that hat been lying around during summer holidays. The chicken got sick but recovered within a few days. When injecting fresh Pasteurella cultures into chicken only those that had received the previous inoculation recovered again, demonstrating that a culture containing weakened or *attenuated* bacteria was successful in vaccination against chicken cholera.

In the early 1880s a debate about the mechanisms of vaccination started and resulted in the proposal of two models of immunity: the humoral and the cellular theories of immunity. Followers of the first theory were convinced that immunity was mediated by soluble substances in body fluids (lat. humor) while others thought that immunity was primarily mediated by cells.

The first indication for the cellular theory of immunity came from the Russian zoologist Elie Metchnikoff in 1883 who described the phenomenon of phagocytosis by macrophages in transparent starfish larvae. He proposed that pathogens were cleared in the organism by

macrophages and that the function of antibody was only to prepare their phagocytosis. Metchnikoff received the Nobel Prize for his work in 1908 together with Paul Ehrlich. Metchnikoff's hypothesis was supported by observations of Almroth Wright and Stewart Douglas in 1903. They quantified phagocytosis of bacteria by washed, serum-free leukocytes and demonstrated that the phagocytic index (the number of ingested bacteria) was higher when the leukocytes were incubated in serum. They proposed that the serum contained *opsonins* (i.e. a humoral component) that enhanced the phagocytic activity of blood cells and referred to this phenomenon as *opsonization*. Metchnikoff and others had identified what was later attributed to the *innate immune system*, the unspecific uptake of pathogens by phagocytosis. They had to wait until 1942 to see the first proof of the involvement of lymphocytes in immune defense. Meanwhile, most experiments continued to deal with the identification of the humoral components of immunity.

Diphtheria, a disease caused by *Corynebacterium diphtheriae* leads to the formation of a leatherous membrane in the throat and to diphtheric lesions found in almost all organs although the bacterium never leaves the diphtheric membrane. In 1888, two students of Pasteur, Roux and Yersin, explained this paradox by demonstrating that filtered, bacteria-free supernatant of cultures of *C. diphtheriae* could also cause diphtheric lesions. They postulated that soluble substances they called *toxins* that were released by the bacteria accounted for the spread of the disease. Two years later, Emil von Behring and Shibasaburo Kitasato injected serum from animals resistant to diphtheria into normal animals and demonstrated that the vaccinated animals became resistant to the disease. They concluded that the serum of immune animals contained *antitoxins* (the serum containing antitoxin was called *antiserum*) neutralizing *specifically* the toxins of bacteria that had infected the animals from which the antitoxins were raised. In the following years several terms were introduced to describe soluble substances conferring immunity through body fluids: *Bacteriolysins,* because Richard Pfeiffer in 1894 described that antiserum also reacted with bacteria themselves and could lyse C. diphtheriae. *Agglutinins*, because in 1896 Gruber and Durham reported that antisera could agglutinate bacteria causing cholera and typhus. *Precipitins*, because Rudolf Kraus in 1897 discovered *immunoprecipation* by showing that bacterial filtrate became clouded with precipitate when mixed with antiserum from immune animals. In 1900, Karl Landsteiner introduced the German word "*Antikörper*" which was translated into *antibodies* and until 1930 accepted as a single group of substances accounting for all the reactions described

above, whereas the substances recognized by antibodies were named *antigens*. A different serum factor was discovered in 1893 by Hans Büchner who reported that certain antisera lost their lytic activity when heated to 58°C. However, this activity could be reconstituted by adding serum from non-immunized animals. He therefore concluded that serum contained specific, thermostable antibodies and non-specific, thermolabile *alexin*, a term which was later replaced by Jules Bordet by *complement*. Bordet received the Nobel Prize for his work on complement in 1919.

In 1900, Paul Ehrlich tried to explain the specific interaction between antigen and antibody by postulating that the antibody molecule is shaped to fit a particular antigen and only that antigen. A year later, Karl Landsteiner demonstrated that antibodies could not only be found against bacteria but also against cells from another individual. With the help of agglutination reactions between sera from distinct individuals he was able to identify three groups of antigens on erythrocytes, which he named blood groups A, B and C. (Later C was changed to O and a fourth group, AB, was discovered.) Landsteiner's discovery – for which he received the Nobel Prize in 1930 – was the first demonstration of the phenomenon of *alloreactivity*, an immunological reaction of natural antibodies against cells distinct from the cells of the own organism in their molecular surface pattern. Between 1901 and 1908 Carl Jensen, Leo Loeb and others established that tumors could be transplanted from one individual to another if certain genes matched which they called susceptibility genes. At that time, they were not aware that this could be attributed not only to tumors but to any transplant. The great significance in Landsteiner's and Jensen's work was that the inherited molecular pattern of cells became crucial for the determination of immune response. This notion turned out to become the basis of the study of *immunogenetics*.

In 1917, Landsteiner introduced *haptens*, small organic molecules that induced antibodies to themselves only if they were attached to a larger molecule like ovalbumin. By altering the attached haptens Landsteiner could demonstrate that only slight modifications in the chemical structure could dramatically change its reactivity with specific antibodies. In 1923, Michael Heidelberger and Oswald Avery showed evidence that not only proteins but also carbohydrates like bacterial polysaccharides could be antigens. The first purification of antibodies was accomplished by Felton and Bailey in 1926 by injecting these polysaccharides into horses and isolating the generated antibodies from the horse serum by precipitating them

with the polysaccharides. Chemical analysis of the precipitate showed that antibodies were proteins. In 1934, John Marrack proposed that antigen and antibody have more than one binding site so that a lattice of interlocking aggregate is formed between antigen and antibody molecules. This explained the phenomenon of immunoprecipitation. A breakthrough was achieved with the development of electrophoresis by Arne Tiselius in 1937. He showed that part of the ammonium sulfate-precipitated fraction of serum from immunized animals, which had been called *gamma globulin* (because they were thought to be related to hemoglobulin), gave a higher peak compared to this fraction of serum from non-immunized animals. Later, globulins with antibody activity were designated by Heremans as *immunoglobulins*.

In 1936, the term *immunogenetics* was introduced by Robert Irwin based on the works started with the discovery of blood groups by Landsteiner. In the same year Peter Gorer, who worked with inbred mouse strains and was interested in the genes controlling tumor growth, succeeded in identifying the four blood groups of the mouse designated I to IV. Group II turned out to be a group of genes determining the outcome of tissue transplantation and later, George Snell renamed this blood-group gene into *histocompatibility-2* or *H-2*. Gorer and Snell proved that histocompatibility genes control the rejection of tissue transplants by encoding different antigens between individuals. Much later, the term *major histocompatibility complex* (MHC) was introduced to describe similar genes in other species.

The first demonstration that lymphocytes are involved in immunity was delivered by Karl Landsteiner and Merrill Chase in 1942. They were able to show that contact sensitivity, an allergic reaction in some patients who came in contact with a variety of substances, could not be transferred from a sensitized guinea pig to another by injection of serum. However, when they transferred lymphocytes from the sensitive animal, the recipient of the cells became as sensitive as the donor animal. This was the first demonstration that contact sensitivity was transferable by lymphocytes. In 1944 Peter Medawar and N. Avrion Mitchison formulated the immunological theory of *allograft rejection* demonstrating that the rejection of allogeneic transplants was also based on the transfer of lymphocytes and not serum.

At that time immunologists started believing in a strong connection between the humoral and cellular components of the immune system. In 1948, Astrid Fagraeus who studied spleens of immunized animals found that some lymphocytes transform into plasma cells which then

secrete antibodies. Based on her and following works of others F. Macfarlene Burnet introduced the clonal selection hypothesis in 1957 which was also formulated independently by David Talmage and Niels K. Jerne. Burnet proposed that an individual possessed a large collection of lymphocyte clones and that one clone could only produce one antibody and displayed this antibody on the surface (which was confirmed in 1941 by Albert Coones by *immunofluorescence* techniques). If antigen was bound by a clone, this antigen *selected* this clone to proliferate and become an antibody-producing plasma cell. This implied that an individual had to be tolerant to his self-molecules and that he had *acquired tolerance to self* at some early stage in his development, a notion that Burnet had already formulated in 1949 with Frank Fenner and was confirmed again by Medawar and others 1953 in mice. Burnet and Medawar received the Nobel Prize for their work on immunological tolerance in 1960, Niels Jerne in 1984.

The *bursa of Fabricius* is a tiny organ in the posterior digestive tract of birds. Although this gland was discovered in the 16$^{th}$ century, its function was elucidated in 1956 by Bruce Glick and Timothy Chang. They observed that after bursectomy of chickens these were no longer able to generate antibody responses and hence speculated that the avian bursa is essential for antibody production. However, skin grafts were still rejected by bursectomized animals demonstrating that the bursa was only involved in humoral immunity.
Another organ whose function was unknown for a long time was the *thymus*. In 1961, Jacques Miller and Robert Good performed thymectomy on animals at their birth and observed that graft rejection by these animals was significantly slower than in control animals. Hence, a year later Burnet and others proposed that humoral and cellular immunity were governed by different organs and speculated that two different kinds of lymphocytes, later named *B- and T-cells*, originating from the bursa and from the thymus, respectively, were responsible for the two arms of immunity observed. The first cooperation between B- and T-cells was demonstrated in the 1960s by Anthony Davies, Henry Claman, Graham Mitchell and Jacques Miller. Their experiments showed that certain T cells could help B cells in their differentiation to plasma cells. This also explained why thymectomy in some cases resulted in weakened antibody responses.

In 1958 Jean Dausset and in 1962 Jon van Rood and Rose Payne described genes which were similar in their behaviour to the H-2 complex in mice and Payne named these the *human*

*leukocyte antigens* (HLA), the MHC of humans. At the same time Gerald Edelman and Rodney Porter identified the structure of immunoglobulins, a discovery for which they both received the Nobel Prize in 1972. In the following years more attention was addressed to the H-2 and HLA complexes.

Between 1974 to 1976 Susumu Tonegawa gathered first evidence that the diversity of antibodies is based on the somatic recombination of immunoglobulin genes which he could identify (Hozumi and Tonegawa, 1976). Tonegawa received the Nobel Prize for his work in 1987. In 1975 George Köhler and Cesar Milstein were able to fuse spleen cells from an immunized mouse with myeloma tumor cells resulting in hybridoma cells from which finally a clone could be isolated that produced one antibody of predefined specificity. For the technique producing such monoclonal antibodies Köhler and Milstein received the Nobel Prize in 1984. In 1974 Ralph Steinman identified a novel cell type in lymphoid organs which he called dendritic cell because of the various protrusions and dendrites exhibited by these cells. It took more than 20 years until the importance of dendritic cells in the initiation and modulation of adaptive immune responses was demonstrated (Steinman et al., 1974).

In 1974 Peter Doherty and Rolf Zinkernagel made a very important discovery: they could establish cultures of T cells from mice that killed cells infected with the lymphocytic choriomeningitis virus (LCMV). However these T cells were not able to kill infected cells from another mouse strain. They showed conclusively that lymphocytes required to recognize simultaneously foreign antigen (from the virus) and self antigen (MHC molecules). Doherty and Zinkernagel received the Nobel Prize for identifying this *MHC restriction* in 1996.

In a series of experiments in 1984 Tak Mak (Yanagi et al., 1984) and Mark Davis (Hedrick et al., 1984) were able to identify the genes encoding the T-cell receptor, thus providing the missing antigen receptor on T cells. In 1987, Townsend and co-workers were able to identify the foreign antigen recognized by T cells as short peptides derived from viral proteins and presented on the MHC molecules. The structure of the MHC molecule was identified by X-ray crystallography in 1987 by Bjorkman, Wiley and Strominger and revealed a groove on the surface of the MHC class I molecule that contained "heterogenous antigenic material" (Bjorkman et al., 1987). It was immediately speculated that these antigens were derived from the protein pool of the cell and in this respect MHC molecules would serve as monitors

revealing all intracellular components to T cells. Finally, the antigenic material in the MHC groove could be identified as short peptides. The isolation of these peptides from the MHC molecule allowed to determine the nature of the MHC-bound antigens (Rotzschke et al., 1990; Falk et al., 1991). It was clear now to immunologists that *antigen processing* involved the cell's proteolytic machinery to degrade viral proteins into peptides that were subsequently transferred onto MHC molecules and then presented to T cells on the cell surface.

Today, the branches of cellular and humoral immunity are both classified as *adaptive immune responses*. A specific immune response, e.g. the rejection of grafts by lymphocytes as described by Medewar and others is considered as adaptive immunity because it occurs during the lifetime of an individual as an adaptation to the introduction of a foreign antigen and in many cases confers protective immunity to subsequent antigen encounters. On the other hand, processes like the somewhat unspecific uptake of pathogens by macrophages, first described by Metchnikoff, are considered to belong to the *innate immune system* which delivers components that seem non-specific but are immediately available for defence against pathogens without requirement of prior exposure. In the last few years the innate branch of immunity has received considerable attention from the community of immunologists. There are several reasons for the strong interest in innate immune events: first, so-called non-specific, innate immunity has turned out to be actually quite specific and is able to distinguish between non-self and self via pathogen-associated pattern recognition receptors. Second, it has been shown that the separation into adaptive and innate immunity is not so clearcut and that both branches of immunity cooperate on different levels, thus combining their strength to efficiently combat a wide range of pathogens.

# 2 Components of adaptive and innate immunity

All cells of the immune system derive from the pluripotent hematopoeietic stem cells in the bone marrow. They give rise to different lineages: the erythroblast, progenitor of erythrocytes; the megakaryocyte, progenitor of thrombocytes or platelets and to the common lymphoid and myeloid progenitor. The myeloid progenitor is the precursor of granulocytes, monocytes, macrophages, dendritic cells and mast cells. The lymphoid progenitor differentiates into two major lymphocytes: B cells (originating from the bursa of Fabricius in birds), T cells (developing in the thymus) and natural killer cells (NK cells).

## 2.1 Antibodies and B cells

The primary function of B cells is to produce antibodies. The IgG antibody is a 150 kD-sized glycoprotein that consists of two heavy chains (50 kD) and two light chains (25 kD). Two types of light chains termed lambda (l) and kappa (k) are found, their ratio is 20:1 in mice and 2:1 in humans. There are five types of heavy chains – μ, δ, γ, α and ε – that define the isotype of the antibody, which are IgM, IgD, IgG, IgA and IgE, respectively. For IgG, several subclasses – IgG1, IgG2a, IgG2b, IgG3 and IgG4 – exist in humans. In their soluble forms IgM usually occurs as a pentamer and IgA as a dimer, all others as monomers. Antibodies can be cleaved by the protease papain into two Fab fragments which recognize the antigen specifically with their variable region and one Fc fragment which can be recognized by Fc receptors on antigen presenting cells. Various functions can be attributed to the different isotypes of antibodies. While IgG and IgM are the most abundant antibodies found in the blood stream and are responsible for pathogen neutralization by opsonization, IgA antibodies are rather found on epithelial surfaces, saliva and breast milk to provide a first line of defense against pathogens that are to enter the body. IgE antibodies play a large role in allergy because they sensitize mast cells to release histamine and are thought to act against parasites. B cells develop in the bone marrow with the help of stromal cells which provide a large array of adhesion molecules as well as growth factors. The stages of B-cell development are defined by the sequential rearrangement and expression of heavy- and light-chain immunoglobulin genes. The early pro-B cell joins the D and J gene segments of the heavy chain and expresses various cell-adhesion molecules and the integrin VLA-4 which binds to VCAM-1 to keep attached to stromal cells. These interactions promote the binding of stem-cell factor (SCF) on the stromal cells to the receptor tyrosine kinase Kit on the surface of the

pro-B cell which induces its proliferation and maturation into the late pro-B cell. The latter is classified by joining the V segment of the heavy chain to the D-J segments and expresses the IL-7 receptor and CD25 which is part of the IL-2 receptor, both required for further development. This leads to the expression of an intact μ chain that associates with a surrogate light chain to the pre-B-cell receptor, the hallmark of the pre-B cell. Signaling downstream the pre-B-cell receptor starts the rearrangement of the genes of the light chain which finally leads to the assembly of a complete IgM molecule on the surface (the B-cell receptor) of the now called immature B cell. Surface molecules like CD45R, in mice known as B220, and CD19 which are part of the signaling complex are expressed throughout all development stages and are popular markers for study of B cells. Once a B cell expresses IgM, it undergoes a selection process to prevent the generation of antibodies to self antigens. Immature B cells that bind self antigens have different fates depending on the nature of the antigen: a multivalent self molecule like MHC on the surface usually induces clonal deletion of the B cell by apoptosis. In some cases the B cell can escape its destruction by editing its receptor into a new, non-self-reactive form. Immature B cells that encounter soluble self molecules either become anergic or ignorant. B cells that do not show any self reaction leave the bone marrow and migrate into the peripheral lymphoid tissues as mature, naive B cells.
The B-cell receptor (BCR) only bears a very short intracellular domain which is not capable of transmitting signals into the cell. For this purpose, the BCR always occurs together with two immunglobulin-like proteins, Igα and Igβ, which are also required for the cell surface expression of the BCR. Signalling through the BCR complex depends on sequences in Igα and Igβ originally named *Reth motifs* and now called immunoreceptor tyrosine-based activation motifs (*ITAMs*) which are also present in a large variety of other lymphocyte receptors. ITAMs are composed of two tyrosine residues neighboured by leucin and valin and separated by another 7-10 amino acids. Antigens that cross-link at least two B-cell receptors allow receptor-associated kinases like Blk, Fyn and Lyn to phosphorylate the ITAMs and let Syk bind to the phosphorylated ITAMs. Because of the clustering of at least two BCRs by cross-linking Syk molecules become bound in close proximity and can activate each other by transphosphorylation initiating a multi-featured signaling cascade. This cascade involves the activation of various kinases like MAP kinases and the release of transcription factors NF-kB, NFAT and AP-1 into the nucleus to induce specific gene transcription leading to proliferation and differentiation processes. The B-cell antigen receptor signaling is positively regulated by

a co-receptor complex of the cell-surface molecules CD19, CD21 (complement receptor 2, CR2) and CD81 (TAPA-1).

In order to prime naïve B cells, i.e. to activate the B cells and induce their proliferation and differentiation into an antibody-secreting plasma cell, at least three signals are required. The first is delivered by the antigen that cross-links the B-cell receptor. The second signal is delivered by a T helper cell receptor that recognizes specifically degraded peptide fragements presented on MHC class II molecules on the B-cell surface of the antigen. The third signal is also delivered by the T helper cell via ligation of CD40 on the B cell surface by CD40 ligand (CD40L) on the T cell surface. In order to deliver the latter two signals the T helper cell has to be primed itself in an earlier step. Priming of T cells is discussed below. The ligation of CD40L on the T helper cell on the other side also stimulates the helper cell itself and leads to the secretion of cytokines like IL-4 which give an additional impulse to the proliferation and differentiation of B cells. B cells encounter T cells while they migrate through the T-cell zone of secondary lymphoid organs like the lymph node. Antigen binding traps B cells in the T-cell zone and gives rise to a *primary focus* of antigen-specific B- and T-cells. In the *first phase* of the humoral immune response lymphocytes will proliferate for several days in the primary focus until some of the proliferating B cells differentiate into antibody-synthesizing *plasma cells*. Plasma cells are characterized by their increased cytoplasm dominated by multiple layers of endoplasmic reticulum; they down-regulate expression of MHC class II molecules, surface immunoglobulins and some B-cell specific markers like CD20 and in a later stage, also CD19 while they up-regulate the plasma cell marker CD38. These early events ensure the prompt secretion of often low-affinity IgM antibodies that serve as immediate protection to the body. In the *second phase* of the humoral immune response some of the proliferating B cells in the primary focus cells migrate into primary lymphoid follicules where they proliferate and form a *germinal center*. Germinal centers consist of a dark zone of proliferating centroblasts and a light zone of smaller centrocytes which contact *follicular dendritic cells* (FDCs, distinct from dendritic cells discussed below) which attract B cells into the follicles by secreting the B-lymphocyte chemokine (BLC) recognized by the chemokine receptor CXCR5 constitutively expressed on B cells. In the germinal centers B cells undergo *somatic hypermutation* which alters the V regions, *affinity maturation* which selects for the survival of B cells with high affinity immunoglobulins and *isotype switching*, which allows the affinity-selected B cells to produce different isotypes of antibodies. These selected B cells will either differentiate into antibody-secreting plasma cells or memory B cells. Memory B

cells can respond to their specific antigen 10- to 100-fold faster and produce antibodies of higher affinity than unprimed, naïve B cells.
In some cases B-cell priming does not require the help of specific T cells. B cells can also respond to thymus-independent (TI) antigens (hence the name as the response is independent of the thymus-borne T cells). Two types of thymus-independent antigens are known: TI-1 antigens contain an intrinsic activity that can directly induce a polyclonal B-cell activation, e.g. lipopolysaccharide (LPS), a cell wall constituent of gram-negative bacteria. TI-2 antigens consist of bacterial capsular polysaccharides with highly repetitive structures. TI-1 antigens can activate both immature and mature B cells while TI-2 antigens are only capable to activate mature B cells and even inactivate immature B cells. LPS activates B cells polyclonally via Toll-like receptor 4 and induces an non-specific antibody response which mainly involves secretion of IgM molecules. Toll-like receptors will be discussed below.

## 2.2 T cells

The progeny of the common lymphoid progenitor that is destined to become a T lymphocyte leaves the bone marrow and migrates into the thymus. The thymus is divided into an outer cortical region, the *cortex* and an inner region called *medulla*. During their development the thymocytes migrate slowly from the cortex where they interact with epithelial cells to the medulla where they additionally encounter dendritic cells and macrophages. In a young adult mouse $5x10^7$ new cells are generated every day in the thymus but only 2 to 4% leave the thymus as mature T cells; the rest dies via apoptosis and subsequent phagocytosis by macrophages. The development of thymocytes is marked by the status of the T-cell receptor genes and the expression of the TCR and of co-receptors. The first-stage 'double-negative' thymocytes (because they do not express CD4 and CD8) with unarranged receptor genes give rise to the $\gamma{:}\delta$ *T cells* (20%), the α:β-TCR expressing *NK T cells* co-expressing NK1.1 (20%) and $\alpha{:}\beta$ *T cells* (60%).

Little is known about γ:δ T cells residing in the lymphoid and intraepithelial tissue. The intraepithelial γ:δ T cells are thought to recognize ligands directly without the need of presentation on MHC molecules when a cell is infected. Candidate ligands are heat shock proteins, non-classical MHC molecules, nucleotides and phospholipids. γ:δ T cell Knockout mice show exaggerated responses to pathogens and autoimmunity suggesting that γ:δ T cells, which can secrete cytokines in their activated state, have an immunomodulatory function.

NK T cells are activated as part of an early response to infections and are thought to recognize antigen rather on CD1 than on MHC class I or II molecules.

The first step in development of non-NK α:β T cells is the expression of the adhesion molecule CD44 and subsequently, the α chain of the IL-2 receptor CD25. In these cells rearrangment of the TCR β chain genes starts. Cells that make a productive β-chain arrangement lose their CD25 expression again, the β chain is paired with the monomorphic germline α chain resulting in the pre-T cell receptor complexed with the signalling molecule CD3. This assembly leads to proliferation, arrest of further β chain arrangements and the expression of CD4 and CD8 ('double-positive' thymocytes). Proliferation ceases and with the start of the α-chain locus rearrangements low levels of the first T cell receptor are expressed. Only T cells recognizing self MHC molecules with their TCRs survive (a process called *positive selection*), express high amounts of their TCRs and move to the medulla of the thymus. Here they have to undergo the process of *negative selection* where all T cells that recognize self antigens are eliminated. At this point one of the co-receptors is no longer expressed and the T cells leave the thymus as single-positive, mature and naive CD4 or CD8 T cells. The time between the entry of the T-cell progenitor into the thymus and the release as mature T cell is estimated to be around 3 weeks in the mouse.

Naïve T cells circulate in the blood stream and through the secondary lymphoid organs. T cells enter the lymph nodes via the high endothelial venules (HEV), which secrete the secondary lymphoid tissue chemokine (SLC) binding to CCR7 on T cells and express a large array of adhesion molecules facilitating the homing of lymphocytes, e.g. GlyCAM-1 and CD34 binding to L-selectins on the surface of naïve T cells. Once a T cell has migrated in to the lymph node, cell-cell interactions are mainly initiated via integrins, which include intercellular adhesion molecules (ICAMs) and lymphocyte function-associated antigen 1 (LFA-1). Interactions of T cells with antigen presenting cells (APCs) include following interacting pairs, respectively: CD2/LFA-3, LFA-1/ICAM-1, LFA-1/ICAM2 and ICAM-3 with the lectin DC-SIGN exclusively expressed on dendritic cells. Establishing this initial association of APC and T cell is the prerequisite for the antigen-specific priming of T cells. According to the current dogma naïve T cells require at least two signals for priming: the first signal results from the specific interaction of the TCR with the peptide in the context of an MHC molecule. The second signal required is the co-stimulatory signal. The best

characterized co-stimulus is the ligation of CD28 on T cells with the B7 molecules including B7.1 (CD80) and B7.2 (CD86) on antigen-presenting cells. But the 'APC-T cell dialogue' goes much further. It involves upregulation of signaling molecules on both sides resulting in a *bi-directional* interaction. For instance, activated CD4-positive T cells upregulate CD40L which interacts with CD40 on APCs, and leads to further expression of B7 and MHC molecules on the surface. A second example of the dialogue: activated T cells also express 4-1BB (CD137) which ligates 4-1BB Ligand (4-1BBL, CD137L) expressed on activated dendritic cells. Another molecule expressed on activated T cells is CTLA-4 (CD152) which, like CD28, also binds to B7 molecules but has an inhibitory effect on T cells and in this way modulates the T cell response. CTLA-4 knockout mice develop a fatal disorder characterized by massive, uncontrolled T cell proliferation. Activated T cells also express the inducible co-stimulator (ICOS) which ligates LICOS on all activated APCs.

A naïve T cell that is activated or in other words *primed* in this way reenters the cell cycle and starts proliferating two or three times a day for several days. At the same time T cells start secreting IL-2 and induce the synthesis of the IL-2 receptor α chain (CD25) which associates with the pre-existing β and γ chain sensitizing the T cell enourmously to its produced IL-2. After 4-5 days of proliferation activated T cells have differentiated into armed effector T cells specialized in their function.

Naïve CD4 T cells are differentiated into helper T cells of the $T_H1$ or $T_H2$ type which differ in their cytokine profile: $T_H1$ T cells are identified by their production of Interferon-γ (IFN-γ) and their preference of inducing cellular immunity. They pre-dominantly activate macrophages (which present the T helper antigens on their MHC class II molecules) by upregulating CD40L and delivering cytokines to the macrophages. $T_H2$ T cells preferentially activate B cells by secretion of IL-4 and IL-5 and elevated expression of CD40L, so they preferably induce humoral immunity. It is difficult to understand the processes that determine whether a proliferating CD4 T cell will become a $T_H1$ or $T_H2$ cell. Factors that are involved in this important decision are the cytokine profiles that immune cells exhibit as a reaction to infection, the nature and amount of co-stimulatory molecules and the nature of the antigen. Helper T cells deliver their effector function upon recognition of their specific antigen on MHC class II molecules.

Naïve CD8 T cells become cytotoxic T lymphocytes (CTLs) that are capable of destroying cells when they recognize their cognate antigen in the context of an MHC class I molecule. It is thought that their main function is to eliminate virus-infected cells to limit further synthesis and assembly of virus particles. CTLs have several effector molecules in their combat arsenal: *perforin* which creates holes in the target cell membrane, *granzymes* which are proteases that act intracellulary in the target cells to trigger apoptosis, and *Fas ligand* (CD95L) which triggers the death receptor *Fas* (CD95) also inducing apoptosis in the target cell.

When a naïve T cell recognizes its antigen in the periphery without a second, co-stimulatory signal the T cell is inactivated and called anergic. Such T cells are not able to produce IL-2 which prevents them from proliferating and differentiating into effector cells. This ensures to establish tolerance to self molecules in case of T cells that were not negatively selected because their cognate antigen was not expressed in the thymus at all or at the time of their thymic maturation. It seems important that for priming of immune responses the same APC delivers both the antigen and the co-stimulatory signal at the same time to the naïve T cell. Otherwise, self-reactive T cells could easily be primed in the periphery by recognizing self-antigens on tissue cells and receiving co-stimulus by nearby or distantly located activated APCs. However, there is still considerable debate regarding this issue and it has also been argued, that indeed a co-stimulatory milieau delivered by cytokines and co-stimuli of bystander APCs is sufficient to prime a naïve T cell that recognizes tissue antigens. Although the first model seems more reasonable for the control of immunity, the latter one explains why autoimmune diseases are probably often intiated by infections that might lead to the generation of such co-stimulatory milieus.

Recent focus has been set on specialized CD4 T cells that co-express CD25 constitutively. Such $CD4^+CD25^+$ are considered as *regulatory T cells* because they have been shown to inhibit T cell responses and thus may be required to maintain tolerance. $CD25^+$ regulatory T cells comprise 5-15% of all CD4 T cells and are generated in the thymus. They have been recently identified in humans and it is now possible to maintain and expand human regulatory T cells in vitro (Shevach, 2001). The mechanism of suppression by $CD25^+CD4^+$ T cells is still not fully clear. Some data hints into the direction that it occurs via membrane-bound TGF-β (Nakamura et al., 2001).

## 2.3 Pathways of antigen processing

Antigen processing is the generation of T-cell ligands, i.e. the degradation of proteins into peptides that bind to MHC molecules and are presented to and recognized by T cells, which defines these peptides as *T-cell epitopes*. Pathogens can exist and multiply at two different locations: intracellularly and extracellularly. Therefore the immune system has developed two types of antigen processing pathways to combat pathogens: One pathway that is responsible for degradation of intracellular antigens in the cytosol, thus degrading viral proteins, and loading these antigens onto MHC class I molecules recognized by $CD8^+$ T cells. The other pathway results in degradation of antigens that have been taken up from the extracellular fluid into endosomes, thus degrading bacterial and fungal proteins and loading these onto MHC class II molecules for presentation to $CD4^+$ T cells. Antigen processing takes place in all cells that possess MHC molecules, but immune responses are usually induced by *antigen presenting cells* only: B cells that take up antigen by their immunoglobulin receptor and macrophages and dendritic cells that take up antigen via micro- and macropinocytosis ("cellular drinking and eating") and receptor-mediated endocytosis.

### *2.3.1 MHC molecules*

The two classes of Major Histocompatiblity molecules have distinct subunit structures but similar three-dimensional structures. The MHC class I molecule is a heterodimer of a membrane-spanning α chain (43 kD) bound non-covalently to β2-microglobulin (12 kD). The α chain folds into three domains called α1, α2 and α3. The α1 and α2 domains form a β sheet surrounded by two alpha helixes forming a peptide-binding cleft closed at both ends; the α3 domain and β2-microglobulin show similarities to immunoglobulin C domains. The MHC class II molecule is a heterodimer between two membrane-spanning chains, α (34 kD) and β (29 kD) which both consist of two domains each. The α1 and β1 domains form the peptide-binding cleft which is open at both ends. Consequently, there is a difference in the peptides that bind to MHC class I and II molecules. MHC class I peptides are short (8 to 10 amino acids) and contain certain *anchor residues*, conserved amino acids at positions in the peptide that are important for interaction with the MHC molecule. The amino acids at and the the position of the anchor residues vary depending on the MHC allele. Therefore, one can find a *binding motif* for every MHC molecule (Falk et al., 1991; Rammensee, 1995). Peptides that bind to MHC class II molecules vary in length. They are usually 13 amino acids long, but can

be much longer and do also contain anchor residues. In both cases, for MHC class I and II, binding to peptides is a critical factor for folding and assembly of MHC molecules. Although several hundred allelic variants have been found in humans and mice, every individual carries only up to six different class I and twelve different class II alleles. This *MHC polyphormism* makes it highly probable that in a large population always some individuals will have matching MHC molecules for initiation of immune responses against newly arisen pathogens.

### *2.3.2 MHC class I processing*

For the degradation of cytosolic proteins into peptides the immune system uses a pre-existing and evolutionary rather old degradation machinery called the *proteasome*. The eukaryotic 20S proteasome is a 770 kD protein complex consisting of 14 α and 14 β units forming four rings of 7 subunits of the total dimension 15 x 11 nm. The outer α rings are proteolytically inactive, the inner β rings contain three proteolytic subunits: β1, β2 and β5 (Delta, MECL-1, MB1) which are all three threonin proteases. The crystal structure of the yeast *20S proteasome* (Groll et al., 1997) shows that the four stacked rings form a barrel-like structure with two open ends and three inner cavities where the degradation of the substrate presumably takes place. Addition of interferon-γ induces the synthesis of alternative proteolytic subunits called iβ1, iβ2 and iβ5, which now form the so-called *immunoproteasome*. The proteolytic specificity of the immunoproteasome differs signifantly from those of the regular proteasome (Toes et al., 2001). However, it is not fully clear whether a shift of the intracellular equilibrium toward the immunoproteasomal form is of advantage for the generation an immune response. The 20S proteasome can be extended at the outer rings by one or two 19S caps (or PA700 regulators), the newly formed complex is termed *26S proteasome* and can recognize substrates that have been tagged for degradation with ubiquitin (Hershko and Ciechanover, 1998). Association of the 19S caps also results in a change of proteolytic specificity (Emmerich et al., 2000). Additionally, the 19S caps can be substituted by PA28 (11S regulator), if induced by interferon-γ. Studies of the proteasomal degradation products by mass spectroscopy reveal that in many cases the proteasome generates the correct C terminus of the epitope and leaves the processing of the N terminus to other proteases.
A fraction of peptides that have been generated by the proteasome can escape further degradation to amino acids by translocation into the endoplasmic reticulum (ER). This specific and ATP-dependent transport is facilitated by the *transporter associated with antigen*

*processing (TAP)*, a heterodimer of the two ATP-binding cassette (ABC) proteins TAP1 and TAP2. Specificity of the TAP complex has been defined by ER peptide translocation experiments: human TAP prefers peptides with a hydrophobic or a basic C terminus while the mouse TAP prefers only peptides with a hydrophobic C terminus (Powis et al., 1996).
In humans, newly synthesized MHC class I α chains assemble in the ER together with the chaperone *calnexin*. This complex binds β2-microglobulin which results in a dissociation from calnexin and association to the chaperone molecules *calreticulin* and *Erp57*. This whole complex associates to the ER lumenal part of TAP via *tapasin*. The MHC class I molecule is retained in the ER until peptides bind to it and complete its folding. Once a peptide has bound to the MHC molecule the MHC:peptide complex is transported to the cell surface via the Golgi apparatus.

### 2.3.3 MHC class II processing

The α and β chains of the MHC class II molecule are also synthesized in the endoplasmic reticulum but the peptide binding takes place in the endosomal pathway. To prevent peptides from binding to MHC class II molecules inside the ER, class II molecules are blocked by the *invariant chain* (Ii), which forms trimers with each subunit binding to one MHC class II heterodimer. The assembly of the MHC class II chains is assisted by the chaperone *calnexin*. Once the assembly of a MHC class II:invariant chain trimer is completed, the whole complex is targeted to late endosomes by the invariant chain. In these low-pH compartments acid proteases like Cathepsin S (or Cathepsin L in thymic cortical epithelial cells) cleave the invariant chain so that a truncated form of Ii, called *CLIP* (class II-associateed invariant-chain peptide) remains associated to the MHC class II molecule. It has also been demonstrated that the MHC II:Ii complex can first be transported to the cell surface, from where it can be re-internalized into endosomes. Compartments in which Ii is cleaved are not cleary defined and have numerous names like *MIIC* or *CIIV* (Neefjes, 1999). Pathogens that have been taken up by macropinocytosis or receptor-mediated endocytosis (e.g. by the B-cell receptor) are also internalized into the endosomal pathway where their proteins are degraded by acid proteases. The generated peptides cannot bind to MHC class II molecules that are occupied by CLIP. To release CLIP, the non-classical MHC class II-like molecule *HLA-DM* in humans and *H-2M* in mice which is found predominantly in MIIC compartments is able to stabilize empty MHC class II molecules and thus catalyzes the exchange of CLIP by other peptides. HLA-DM also selects for stably bound peptides, a process termed *peptide editing*: it will continously bind

and rebind to MHC class II molecules removing weakly bind peptides and allowing other peptides to replace them. HLA-DM is negatively regulated by another non-classical MHC-like molecule called *HLA-DO*. The expression of the HLA-DO β chain, in contrast to HLA-DM, is not increased by IFN-γ. Thus, during an inflammation the increased expression of HLA-DM is able to overcome that of HLA-DO.

### *2.3.4 Cross-presentation*

The strict separation of the two classical antigen processing pathways makes sense. Cells that are virus-infected usually display viral antigen on their MHC class I molecules and can be eliminated by primed cytotoxic T cells. But cells that take up viral debris by endocytosis should be spared by CTLs.

On the other side, T cells can only be primed by professional antigen-presenting cells, who deliver co-stimulatory signals to naïve T cells. This requires, for instance, that all viruses infect antigen presenting cells. Indeed, most viruses do infect dendritic cells but some viruses exist that do only infect peripheral tissue cells, e.g. the human Papillomavirus (HPV) which infects epithelial cells exclusively (McMurray et al., 2001). At the same time, natural HPV CTL epitopes have been identified (Ressing et al., 1995). Thus, antigen must have been transferred from infected epithelial cells to antigen presenting cells, taken up by these APCs and processed for presentation on MHC class I molecules. This would imply the existence of an antigen processing pathway in which antigen that has been taken up from the extracellular fluid is processed and transferred onto MHC class I molecules. Such a pathway breaks the strict separation of MHC class I and II processing and is termed *cross-presentation*.

In early experiments Michael Bevan demonstrated that minor histocompatibility antigens were capable of being transferred from the cells expressing these antigens to APCs of another host different in the MHC haplotype. Priming involving this type of antigen transfer was termed *cross-priming* to differentiate it from direct T cell activation by the cells expressing the minor antigens (Bevan, 1976b; Bevan, 1976a). According to this definition, cross-presentation can involve either class I- or class II-restricted cell-associated antigens, but usually the term is only used with class I-restricted antigens. Cross-presentation can either lead to cross-priming or *cross-tolerance*, depending on the effect on the T cells that interact with the cross-presenting APC. The phenomenon of cross-tolerance is best reviewed by (Heath and Carbone, 2001).

There has been considerable debate about the nature of the cross-presenting APC. There are various in vitro studies that show dendritic cells (Norbury et al., 1995), macrophages (Norbury et al., 1997) and B cells (Ke and Kapp, 1996) being capable of class I-restricted presentation of exogenous antigen. However, only few have studied the exogenous presentation of cellular material, i.e. cross-presentation. Most of the latter studies have dealt with the uptake of apoptotic bodies by dendritic cells (Albert et al., 1998b) and macrophages (Bellone et al., 1997) and the subsequent cross-presentation of antigens contained in these bodies. Recent work indicates that only bone-marrow derived or splenic dendritic cells can cross-present cellular antigen and it has been claimed that macrophage preparations that have been used for cross-presenatation experiments contained contaminations of dendritic cells (Sauter et al., 2000).

## 2.4 Dendritic cells

Dendritic cells (DCs) were discovered 1974 by Ralph Steinman and named so because of their prominent protrusions and the dendrites they exhibit. At that time Steinman was not aware that more than 20 years later dendritic cells would be considered by many as the most fascinating antigen presenting cell.

Dendritic cells can take up antigen via micro- and macropinocytosis and, more importantly, via receptor-mediated endocytosis very efficiently and present degraded peptides of the antigen on MHC class I and II molecules. Thus, they are thought to differ from macrophages and B cells which are able to present endocytosed antigen on MHC class II but usually not MHC class I molecules. Moreover, through the careful regulation of co-stimulatory activity they are able to stimulate naïve T cells and to control the quality of the immune response, dricing naïve lymphocytes into distinct classes of effectors. In addition, they play a critical role in innate immunity. DCs can respond to compenents of pathogens by releasing pro-inflammatory cytokines like IL-12, TNF-$\alpha$ as well as both type I and II interferons. They can also activate NK and NK T cells that rapidly kill select targets and produce important cytokines.

The extraordinary quality of dendritic cells is mainly based on specializations that enable DCs to capture and present antigen in a manner distinct from other antigen presenting cells. Dendritic cells in culture can occur in at least two different states: immature and mature. Immature DCs like Langerhans cells that reside right under the surface of the skin, are specialized in antigen uptake and express relatively low levels of MHC class I and II and co-

stimulatory molecules. Thus immature DCs can take up antigen but do not present it efficiently to T cells. On the other hand, mature DCs down-regulate a whole array of surface receptors necessary for endocytosis of antigen and they are also extremely inefficient in macropinocytosis. But they exhibit a high amount of MHC class II molecules, co-stimulatory activity like CD80 and CD86 and T-cell adhesion molecules like CD48 and CD58 on their cell surface. Additionally, mature DCs release pro- as well as anti-inflammatory cytokines and chemokines into the surrounding extracellular fluid. Thus, mature DCs are professionals in antigen presentation and priming of T cell responses but not uptake of antigen. Immature DCs can be transformed into mature ones by a large variety of signals that usually originate from microbial components like lipopolysaccharide (LPS) or bacterial DNA. Thus, the antigen presenting and T-cell priming capabilities corresponds nicely with the presense of infectious pathogens. Transformation of immature into mature dendritic cells is accompanied by a dramatic cytoplasmic reorganization which can be characterized for instance by the redistribution of MHC class II molecules from the late endosomes and lysosomes to the cell surface. The family of *Toll-like receptors* (TLRs), originally identified as components of innate immunity, seems to play an important role in the transformation process. TLRs are crucial for the signaling of bacterial and viral components like LPS, CpG DNA (bacterial DNA containing unmethylated CpG motifs) and double-stranded RNA to the dendritic cells and probably bind these substances on the cell surface or in some cases intracellulary. Toll-like receptors and their signaling pathways are discussed in more detail below.

### *2.4.1 Cross-priming by dendritic cells*

One of the most striking features of dendritic cells is the ability to cross-present antigen that has been taken up from the extracellular fluid on MHC class II *and* I molecules and thus, to cross-prime cytotoxic T cell responses to antigen from other cells. This capability is unique in dendritic cells as recently shown by several works.

Bhardwaj and co-workers have demonstrated that cross-presentation of antigen that is contained in apoptotic bodies (vesicles that result from cell destruction through apoptosis) can be taken up by macrophages and dendritic cells via the scavenger receptor CD36 and the integrin $\alpha_v\beta_3$. DCs are specific for the expression of the integrin $\alpha_v\beta_5$ which is required for cross-presentation of antigen onto MHC class I molecules (Albert et al., 1998a). Hence, in this example the extraordinary ability of DCs to cross-present in contrast to macrophages lies in the exclusive expression of certain cell surface receptors.

Amigorena and co-workers have shown that dendritic cells but not macrophages or B cells are able to cross-present antigen that has been taken up via Fcγ receptors. (Fc receptors are expressed by antigen presenting cells and play a critical role in complement activation following opsonization of bacteria by specific antibodies and the uptake of bacteria via the Fc part of the opsonizing antibodies.) Amigorena could attribute this exclusive ability of dendritic cells to a specialized antigen processing pathway existent only in dendritic cells. Antigen that had been taken up by Fc receptors into endosomes and lysosomes could be selectively transferred into the cytosol and was subsequently integrated in the regular MHC class I processing pathway involving activity of the proteasome and TAP(Rodriguez et al., 1999). Thus, dendritic cells on the top of possessing specialized receptors also bear special translocation systems to break the normally strict separation of MHC class I and II processing.

### *2.4.2 Subsets of dendritic cells*

All dendritic cells originate from the bone marrow. Originally, dendritic cells were considered to be of myeloid origin only (Banchereau et al., 2000). Such **myeloid-derived DCs** can be generated *in vitro* from monocytes or from bone-marrow stem cells with the help of cytokine and growth factors according to protocols established by Ralph Steinman's group (Inaba et al., 1992; Bender et al., 1996). This subset of DCs has been generally called "dendritic cell" until 2000/2001 and all chapters in this thesis refer to myeloid-derived DCs generated *in vitro*. *In vivo* differentiation of monocytes into dendritic cells upon migration to the lymph nodes has been reported (Randolph et al., 1999).

Dendritic cells also occur in the thymus where they are thought essential in negative selection of self-reactive T cells. However it was unclear, whether thymic DCs differentiate in the thymus from a precursor cells or whether they enter the thymus preformed. A population of early $CD4^{low}$ T-cell precursors was found to have the ability to differentiate into B cells, T cells and dendritic cells, but not myeloid or erythroid cells, when transferred from the adult mouse thymus into another animal intravenously (Ardavin et al., 1993). This was the first evidence for the existence of **lymphoid-derived DCs**, also called $CD8^+$ DCs because they express CD8α in the mouse. Similar results were obtained with human bone-marrow lymphoid-committed precursors (Galy et al., 1995). Very recently, the group of Michael Bevan has observed that $CD8^+$ DCs are able to cross-prime while $CD8^-$ DCs are not (den Haan et al., 2000). However, the concept of $CD8^+$ lymphoid-derived and $CD8^-$ myeloid-

derived DCs has been challenged by demonstrating that both $CD8^-$ and $CD8^+$ DCs can be generated from $CD4^{low}$ lymphoid-comitted precursors (Martin et al., 2000) as well as from myeloid precursors (Traver et al., 2000). A third subset, human **plasmacytoid dendritic cells**, were proposed to be lymphoid-derived because they were derived from $CD4^+CD11c^-CD3^+$ plasmacytoid T cells with IL-3 and CD40 ligand but not GM-CSF for their differentiation (Grouard et al., 1997). Very recently, plasmacytoid DCs have also been described in the mouse (Asselin-Paturel et al., 2001).

## 2.5 Toll-like receptors

Immunity can be categorized into two branches: adaptive and innate immunity. Adaptive immunity is mediated by T and B cells that clonally expand in recognition of foreign antigens resulting in effector and memory cells. Innate immunity was initially thought to be a rather nonspecific immune response characterized by uptake of pathogens by macrophages and granulocytes. Today, after having gathered more knowledge about the components and functions of innate immunity, the definition of the innate branch of the immune system has broadened. All direct pathogen-specific responses of immune cells to pathogens or substances originating from pathogens are considered as innate immune responses: this includes the release of cytokines, chemokines, toxic oxygen-derived products, toxic nitrogen oxides, antimicrobial peptides, enzymes (e.g. lysozyme). It also includes the complement system that labels pathogens for uptake and destruction and the surveillance by granulocytes and natural killer (NK) cells. These components represent the first line of immune defence and in some cases are responsible for the initiation of an inflammatory response which allows the adaptive immune system to recruit its cells to the site, where the pathogens reside. Most strikingly, innate immunity from a modern point of view, although not being able to respond to the individual pathogen, is not non-specific at all. It can distiguish between self and non-self by recognizing pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs). Among these receptors is the *mannose receptor*, mainly expressed on macrophages, that binds to conserved sugar molecule motifs on bacteria and some viruses (Aderem and Underhill, 1999). Another set of PRRs are the *scavenger receptors*, e.g. CD36 and CD91, that recognize certain anionic polymers and acetylated low-density lipoproteins (Febbraio et al., 2001; Herz and Strickland, 2001).

As described above, antigen presenting cells respond to bacterial components like lipopolysaccharide (LPS) by secreting cytokines, chemokines and up-regulating co-

stimulatory molecules. The pattern-recognition receptors that are used for these processes belong to the large group of *Toll-like receptors*.

Toll-like receptors were discovered by C. Janeway and co-workers as a transmembrane protein family (Medzhitov et al., 1997) related to the *Toll* proteins first identified by C. Nüsslein-Volhard's group in drosophila (Anderson et al., 1985). In insects, Toll and related proteins play an essential role in development and antifungal immunity (Lemaitre et al., 1996). Toll-like receptors (TLRs) share similarity in the cytoplasmic domain with Toll proteins and the IL-1 receptor, thus this domain is called Toll-IL-1R or TIR domain (O'Neill, 2000).
Until now, nine members of the TLR family have been identified, designated TLR1 to TLR9. Toll-like receptors can be classified based on their expression pattern in ubiquitous (TLR1), restricted (TLR2, TLR4) and specific (TLR3). Surface expression of TLRs is usually very low, measured to a few thousand molecules per cell in monocytes and 10-fold less in dendritic cells as shown for TLR1-5 (Visintin et al., 2001b).
Triggering of toll-like receptors on antigen presenting cells intiates a multi-level anti-microbial response including release of cytokines and chemokines, increase of MHC class I and II surface expression, co-stimulatory activity, antigen presenting and T-cell stimulatory capacity.

### *2.5.1 Toll-like receptor 4*

TLR4 is the best-studied toll-like receptor and recognizes the Lipid A component of lipopolysaccharide (LPS) from gram-negative bacteria. The role of TLR4 was identified in the mouse strain C3H/HeJ which is hyporesponsive to LPS due to a missense point mutation within the TLR4 gene region encoding the cytoplasmatic domain, thus C3H/HeJ APCs only express non-functional TLR4 on their surface (Poltorak et al., 1998). Similarly, in humans LPS hyporesponsiveness has been found to be associated with mutations in the TLR4 locus (Arbour et al., 2000). For LPS signaling additional molecules are required: a complex of LPS and the serum protein LBP (LPS-binding protein) has to bind to surface-expressed CD14. However, recently evidence has been provided for a CD14-independent LPS signalling. Interestingly, in this model LPS mediates its signal via a cluster of heat shock proteins 70 (Hsp70) and 90 (Hsp90), chemokine receptor 4 and growth differentiation factor 5 (Triantafilou et al., 2001). Another molecule that is strictly required for LPS signaling, is the

secreted large polymeric protein MD-2 which physically associates with the extracellular domain of TLR4 (Shimazu et al., 1999; Visintin et al., 2001a). Other molecules besides LPS from gram-negative bacteria that have been found to signal via TLR4 are the plant-derived anti-tumor drug Taxol (Kawasaki et al., 2000), host-derived fibronectin (Okamura et al., 2001) and the heat shock protein 60 (Hsp60, see below).

### *2.5.2 Toll-like receptor 2*

Ligands of the toll-like receptor 2 are lipoproteins from some gram-positive and -negative bacteria (Aliprantis et al., 1999; Brightbill et al., 1999). The immunostimulatory components of these lipoproteins are lipids that are covalently linked to N-terminal cyteines. Additionally, TLR2 mediates innate immune responses to various pathogen-associated ligands including LPS from gram-positive bacteria like Leptospirae (Werts et al., 2001), mycobacterial lipoarabinomannan (Means et al., 1999), lipoteichoic acid and peptidoglycan (Schwandner et al., 1999), GPI anchors from the parasite Trypanosoma cruzi (Campos et al., 2001) and yeast-derived zymosan (Underhill et al., 1999). In the latter work, it was also demonstrated that toll-like receptor 2 was specifically recruited into phagosomes. For a long time it has been unclear whether LPS signalling from gram-negative bacteria is also dependent on TLR2. However, many groups agree today that the LPS preparations used in the experiments identifying TLR2-dependent LPS signaling (Yang et al., 1998; Kirschning et al., 1998) probably contained contaminations of LPS from gram-positive bacteria or other pathogen-derived components. The demonstration that commercial LPS preparations that have been re-purified by phenol extraction do no longer signal via toll-like receptor 2 (Hirschfeld et al., 2000) is in line with the last argument.

Interestingly, ligand specificity and signal transducing ability for TLR2 activity is determined by heterodimeric interactions with two other toll-like receptors: the cytoplasmic domains of TLR1 and TLR6 can pair with those of TLR2 (Ozinsky et al., 2000). This work also shows that TLR6 is recruited to phagosomes. It has been demonstrated that co-expression of TLR2 and TLR6 is required for signaling by mycoplasmal macrophage-activating lipopeptide 2 (MALP-2) (Takeuchi et al., 2001).

### *2.5.3 Ligands of other toll-like receptors*

Following TLR2 and 4 the **toll-like receptor 9** has been studied best. TLR9 recognizes bacterial DNA that contains certain oligonucleotides with unmethylated CpG dinucleotides.

Unmethylated CpG DNA (or often just called CpG DNA) is able to stimulate murine (Hemmi et al., 2000) and human (Takeshita et al., 2001) lymphocytes, monocytes and dendritic cells, whereas eurkaryotic DNA is not. CpG DNA has very strong immunostimulatory properties: dendritic cells incubated with CpG Oligodinucleotides (ODN) show massive $T_H1$-like cytokine responses with preference for IL-12 and IL-18, a strong up-regulation of co-stimulatory molecules and in case of B cells, mitogenic activity driving B cells into proliferation. CpG motifs are species-specific (Bauer et al., 2001), so CpG ODNs with different motifs have to be used to stimulate human and mouse APCs.
Very recently, the ligands of TLR5 and TLR3 have been identified. **Toll-like receptor 5** recognizes flagellin, a protein monomer from bacterial flagella, which extends from the membrane of gram-negative and -positive bacteria and allows these pathogens to propel in fluids (Hayashi et al., 2001). **Toll-like receptor 3** is the receptor for double-stranded RNA (dsRNA), a molecular pattern associated with viral infection (Alexopoulou et al., 2001). Many Viruses produce dsRNA at some point during their replication. It has been demonstrated that dsRNA can induce maturation of dendritic cells resulting in enhanced T cell stimulatory capacity (Cella et al., 1999).

### *2.5.4 Toll-like receptor downstream signaling*

Activation of toll proteins in drosophila is believed to occur via an endogenous peptide rather than the microbial product itself (Levashina et al., 1999). However, in mammalian cells a direct contact seems very likely due to species-specificity of the interaction between LPS and TLR4. Transfection of cells of one species with TLR4 from another species has shown that the response to LPS is determined by the introduced TLR and not by the host cells. Thus, the authors of this work conclude, TLR4 interprets the chemical structure of LPS which requires a close proximity of TLR and the ligand (Poltorak et al., 2000). Direct interaction of LPS and TLR4 has also been demonstrated: LPS cross-links to TLR4 and MD-2 only in the presence of CD14 (da Silva et al., 2001).
The first event after the triggering of TLR4 is the recruitment of the adapter molecule MyD88, a serine-threonin kinase that binds to the TIR domain of TLRs and IL-1 receptor. This results in a phosphorylation of IRAK, which subsequently dissociates from the receptor complex and associates with TRAF6 (TNF receptor-activated factor 6). From here on, two different pathways are followed: on the one hand activation of JNK (c-Jun NH2-terminal kinase) and the p38 MAP (mitogen-activated protein) kinase family. Phosphorylation of JNK

leads to the activation of the transcription factor AP-1 which translocates into the nucleus to induce gene expression. On the other hand the IKK complex is activated, which leads to the degradation of IκB after phosphorylation and ubiquitinylation. IκB degradation liberates the Rel family transcription factor NF-κB and allows it to be translocated into the nucleus where it can induce target gene expression. (Fallon et al., 2001; Akira et al., 2001) Consequently, cytokine responses in macrophages from MyD88 Knockout mice to LPS from gram-negative bacteria were completely impaired (Kawai et al., 1999). Interestingly, activation of NF-κB and the MAP kinases was not abolished in MyD88$^{-/-}$ mice, though there was a considerable delay in comparison to wild type mice. This suggests that a MyD88-independent TLR4 signaling pathway exists, but its nature still has to clarified.

### *2.5.5 The danger model of innate immune response*

Increasing evidence suggests that innate immune responses can also be initiated in the absence of pathogens. This kind of response was coined "danger model of immune response" by Polly Matzinger (Matzinger, 1998; Gallucci and Matzinger, 2001). Danger signals could be generated by cell stress or trauma, for instance in the case of a wound or in case of viral infections leading to cell death called *necrosis*, which is known to be associated with inflammatory states, in contrast to *apoptosis* (programmed cell death), which is a usual reaction in developmental processes. For instance, it has been demonstrated that necrotic lysates but not apoptotic bodies generated from tumor cells are able to activate immature dendritic cells (Sauter et al., 2000).

Hsp60 signalling has been found to be dependent on TLR4 (Ohashi et al., 2000) and additionally TLR2 (Vabulas et al., 2001). Interestingly, Hsp60 is not only associated with pathogens like Chlamydiae pnemoniae, but is also a constitutively expressed chaperone in all eukaryotic cells. This latter work shows, that pathogen-associated pattern recognition receptors like the TLR family have extended their function to the sensoring of host proteins.

Thus, heat shock proteins, might be attractive candidates for signaling danger to the immune system utilizing the pre-existing toll-like receptor system which was originally thought to be directed against pathogens exclusively.

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## Chapter 2: Introduction, Part II

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# The Role of Heat Shock Proteins and their Receptors in the Activation of the Immune System

Minireview

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Heat shock proteins (HSPs) have been described as potent tumor vaccines in animal models and are currently studied in clinical trials. The underlying immune response relies on immunogenic peptides that the HSPs have acquired intracellularly by interfering with the classical antigen processing pathways. There have been numerous reports shedding light on how HSPs are able to gain this function and a number of important requirements for HSP-mediated specific immunity have been described. First, the ability of HSPs to bind immunogenic peptides. Second, the acquisition of HSPs by specialized antigen presenting cells with efficient antigen processing pathways capable of inducing cellular immune responses. Third, the existence of specific receptors on the surfaces of antigen presenting cells, allowing efficient and rapid uptake of HSP-peptide complexes from the extracellular fluid. And fourth, the ability of heat shock proteins to activate antigen presenting cells, enabling the latter to prime cytotoxic T cell responses against the peptides associated to HSPs.

## Introduction

All organisms respond to high temperature and a variety of other stresses like anoxia, heavy metals or glucose starvation by inducing the expression of a group of highly conserved proteins called the heat shock proteins (HSPs) which confer elevated stress tolerance to the cells they are expressed in (Lindquist, 1986). They fulfill this vital function by preventing the aggregation of partially denatured proteins in the stressed cell and by initiating their refolding or proteolytic degradation if the unfolding was irreversible. However, many HSPs are essential for life not only under heat shock conditions, but are constitutively present inside the cell accounting for a major portion of total cell protein. Under normal conditions they act as molecular chaperones, a class of proteins which assist in correct protein folding and subunit assembly (Bukau et al., 2000).

HSPs are divided into 6 subfamilies according to their molecular weight: small HSP, HSP40, HSP60, HSP70, HSP90 and HSP100. Due to the limitation of this review we will focus on the HSP families with shown immunological relevance (Table 1). The most intensely studied family of 70 kDa heat shock proteins is present in all major cellular compartments of eukaryotic cells. Hsp70 (Hsp72) and Hsc70 (Hsp73), the stress-inducible and constitutively expressed forms, respectively, are found in the cytosol. BIP (Grp78) represents the ER-resident form of HSP70, Grp75 the mitochondrial form. Similarly, Hsp90 is the HSP90 family member in the cytosol while Gp96 (Grp94) is its counterpart in the ER lumen. Both proteins have been shown to form homodimers via a C-terminal assembly domain. Hsp60, a large multimeric complex of 2 stacked heptameric rings, resides in the mitochondrion.

The HSP families differ in the way they contribute to proper protein folding, subunit assembly and cellular integrity. While the members of the HSP70 family bind as monomers to hydrophobic regions of nascent polypeptide chains or denatured proteins and keep them in a folding competent state, Hsp60 assists partially folded proteins in an ATP-dependent manner to reach their native state inside its large central channel (Bukau and Horwich, 1998). Finally, the 90 kDa heat shock proteins are believed to interact with substrate only in late stages of folding. The activity of a growing list of proteins – most prominent the steroid hormone receptors – is dependent on cytosolic Hsp90 although it is not generally involved in *de novo* protein folding (Buchner, 1999).

## Mechanistical insights into HSP substrate binding

For the 70 kDa heat shock proteins it is known now for several years that ATP is an important regulator of substrate binding. ATP, bound to the conserved N-terminal domain of Hsp70, induces a conformational change in the molecule which allows rapid binding and release of substrate. The substrate can be trapped by hydrolysis of ATP to ADP and its release is again dependent on ADP/ATP exchange (Pierpaoli et al., 1997). Our understanding of the role of ATP in the mechanism of substrate binding to HSP90 chaperones received a major impulse from X-ray crystallographic studies of the N-terminal Hsp90 domain in association with ATP (Prodromou et al., 1997). Although ATP binds with very low affinity to Hsp90, ATP binding and hydrolysis has been shown to be crucial for Hsp90 function *in vivo* (Obermann et al., 1998). Recently, it has been observed that ATP binding induces a transient association of the two N-terminal domains resulting in the formation of a 'molecular clamp' (Prodromou et al., 2000). The functional role of this ATP-induced conformational change remains enigmatic although ATP and the anti-tumor drug geldanamycin, which also binds to the nucleotide binding pocket (Stebbins et al., 1997), have been shown to reduce the heat induced oligomerization of Hsp90 (Chadli et al., 1999). Similar, but not identical observations have been made for the ER-resident Gp96. After incubation of Gp96 at 50 °C or induced by the fluorophore bis-ANS (1,1'-bis(4-anilino-5-naphthalenesulfonic acid)), a tertiary conformational change can be observed that goes along with oligomerization, increased peptide binding and chaperone activity (Blachere et al., 1997; Wassenberg et al., 2000). Although Gp96 binds geldanamycin as Hsp90 does, the properties of the nucleotide binding pockets differ. The adenosine derivative 5'-(N-ethylcarboxamido)adenosine (NECA) binds exclusively to Gp96 with a stoichiometry of 1 mol NECA per 1 mol of Gp96 dimer (Rosser and Nicchitta, 2000). Weak binding of other adenosine derivatives including ATP and ADP is also reported in this work, but in contrast to Hsp90 no ATPase activity was observed. Based on this results it has been proposed that Gp96 substrate binding is allosterically regulated with ATP and ADP as negative regulators and possibly another yet unidentified ligand involved (Wassenberg et al., 2000).

## Peptides as HSP binding substrates

By definition, chaperones interact with non-native proteins. They do so by binding to hydrophobic stretches which are normally buried and not exposed on the surface of the protein. For DnaK, the Hsp70 homologue in *E. coli*, Hsc70 and BIP substrate specificities have been analyzed in detail and the crystal structure of a substrate peptide bound to DnaK has been solved (Zhu et al., 1996). The HSP70 family members bind with highest affinity to small hydrophobic segments in extended conformation. Due to negatively charged surfaces at the ends of the hydrophobic binding tunnel basic residues flanking a hydrophobic core of 4 to 5 residues are preferred. In parallel, Gp96 has a binding preference for hydrophobic and aromatic residues (Spee and Neefjes, 1997). As the affinity for the bound peptide segment seems not to be altered by distant parts of the substrate proteins (Zhu et al., 1996), small peptides bind to HSP70s equally strong as peptides in the context of partially denatured proteins. Similarly, the heat shock proteins Gp96, Hsp90, Grp170, Hsp110 and the ER-resident chaperone Calreticulin have been shown to be peptide-binding proteins (reviewed in Srivastava et al., 1998; Schild et al., 1999; Wang et al., 2001). In some cases direct isolation and sequencing of the antigenic peptide from purified heat shock proteins was successful (Nieland et al., 1996; Breloer et al., 1998; Ishii et al., 1999).

Recently, the peptide binding site of Gp96 has been mapped in the C-terminal domain near the dimerization site by cross-linking experiments (Linderoth et al., 2000). This finding is surprising with regard to the two observed binding sites on the highly homologous cytosolic Hsp90 (Scheibel et al., 1998). In the later case, the N-terminal domain seems to have a high affinity for unfolded proteins and peptides longer than 10 amino acids while the C–terminal domain binds preferentially partially folded proteins.

Despite the fact that there are still many open questions concerning the way how peptides meets HSPs, there is no doubt that peptides can associate with heat shock proteins *in vivo*. The main source of peptides inside the cell is the proteasome. They are produced as intermediates of protein turnover and further degraded by cytosolic peptidases. However, a fraction of these peptides in mammals escapes further degradation and is translocated into the ER by the Transporter associated with antigen processing (TAP) in an ATP-dependent matter. In this compartment, a small subset of peptides is loaded on Major Histocompatibility Complex (MHC) class I molecules. These MHC-peptide complexes are finally transported to the cell surface and presented to CD8-positive cytotoxic T cells. Chaperones are involved in

different steps along the way of MHC-peptide complex formation. On the one hand, the specialized chaperone tapasin, which establishes the physical interaction between some MHC class I allelic products and TAP, influences the peptide loading process (Garbi et al., 2000). On the other hand the chaperones Gp96, Grp170 and Calreticulin have been shown to be major peptide binding proteins in the ER that can bind TAP-dependent and TAP-independent peptides of immunological relevance (Arnold et al., 1997; Lammert et al., 1997a; Spee and Neefjes, 1997; Spee et al., 1999). Moreover, it has been proposed that peptides are transferred directly from one HSP to the other without ever occurring as free peptides until they finally reach the MHC class I molecule. However, so far there is little evidence that supports this intriguing 'relay line hypothesis' (Srivastava et al., 1994a). The involvement of HSPs in the MHC class I presentation pathway suggests that heat shock proteins that reside in compartments involved in antigen processing may be associated with peptides representative for the entire cellular protein content in a similar fashion as MHC class I molecules on the cell surface. The pool of available peptides may be even more diverse due to the less stringent binding specificities of heat shock proteins.

### Heat shock proteins require associated peptides to be immunogenic

The immunogenic potential of HSP-peptide complexes was first demonstrated by Srivastava and coworkers (reviewed in Srivastava et al., 1998). Gp96 purified from tumor cells was able to protect mice against a subsequent challenge of the same tumor. Moreover, treatment of mice with HSPs could also be used for therapy against the pre-existing primary tumor and its metastases (Tamura et al., 1997). In the latter case only Gp96 from tumor but not from liver cells was able to elicit an immune response against the cancer cells due to tumor-specific peptides bound to the heat shock protein. The requirement for peptide binding has been validated by findings that HSPs deprived of peptides do not induce immunity (Udono and Srivastava, 1993) and that loading of 'empty' HSPs with peptides results in reconstitution of the HSP immunogenicity (Blachere et al., 1997).

Although Gp96 is the best described HSP concerning its role in the induction of immune responses, other HSPs have been shown since to perform similar functions: the constitutive and inducible form of the HSP70 protein (Udono and Srivastava, 1993), HSP90 (Udono and Srivastava, 1994), Calreticulin (Basu and Srivastava, 1999; Nair et al., 1999), Hsp110 and Grp170 (Wang et al., 2001). All these proteins have the ability in common to bind

intracellular peptides forming potent immunogenic HSP-peptide complexes which can be used as vaccines against the cells from which they are purified. As the immunity induced by these complexes relies on associated peptides and the HSP acts primarily as an antigen carrier molecule, heat shock proteins have also been called adjuvants of mammalian origin (Srivastava et al., 1998).

### The second requirement: The antigen-presenting cell

How does the HSP-peptide complex induce an immune response? To answer this question we will look shortly at basic mechanism of antigen processing and presentation. The adaptive immune system relies on the presentation of immunogenic peptides at the cell surface embedded in the extremely polymorphic structure of molecules of MHC. They were originally described as antigens responsible for rejection of transplants as consequence of non-matched MHC haplotypes. Complexes of MHC molecules and peptides are recognized specifically by the T cell receptor (TCR) leading to activation (or in some cases silencing) of the T cell. There are two occasions when the interaction of the MHC-peptide complex and the TCR are required in the immune response, e.g. against a virus: first, during the inital activation of naive T cells by professional antigen presenting cells (APCs) that display viral antigens on their MHC molecules, a process referred to as T cell *priming*. Second, during the elimination of virus infected cells by now activated cytotoxic T cells (CTLs). Members of the APC family are macrophages, dendritic cells (DCs) and B cells. The dendritic cell has been suggested to be the most sophisticated professional APC being able to prime T cells as well as to induce tolerance by carefully regulating its arsenal of co-stimulatory surface molecules (Banchereau et al., 2000).

The efficient induction of immune responses elicited by HSP-peptide complexes led to the hypothesis that HSP-induced immunity uses existing pathways of antigen presentation. In consequence, this means that HSP-peptide complexes are taken up by specialized antigen-presenting cells and peptide is transferred from HSPs to MHC molecules inside these APCs for recognition by the T cell (Srivastava et al., 1994b). Meanwhile, substantial evidence has been accumulated to underline this hypothesis. Macrophages and dendritic cells that have been pulsed with Gp96-peptide complexes re-present the associated peptides on their MHC class I molecules for activation of CTLs *in vitro* (Suto and Srivastava, 1995; Singh-Jasuja et

al., 2000b; Castellino et al., 2000). The depletion of phagocytic cells leads to the abrogation of the HSP-mediated immune response *in vivo* (Udono and Srivastava, 1994). DCs that have been pulsed with Gp96 are able to induce protective immunity against tumors *in vivo* (Nicchitta, 1998).

Another important feature of HSP-mediated immunity is the independence of the induced protection from the MHC haplotype of the cell from which the HSPs were isolated. Peptide associated to HSPs purified from cells with one MHC haplotype can be re-presented by antigen-presenting cells of another haplotype (Arnold et al., 1995). This phenomenon – the independence of priming from cellular MHC haplotype – has been coined *cross-priming* (Bevan, 1976) and has recently been shown to be required if the antigen is not expressed by the professional APC itself (Sigal et al., 1999). *In vivo*, cross-priming would be essential in inducing immune responses against non-APCs that have been infected by viruses or other pathogens or have turned into tumor cells. The viral or tumor antigens would then be transferred to APCs where they are cross-presented for eliciting a T cell response.

### The third requirement: Receptor-mediated uptake of heat shock proteins

The ER-resident chaperone Protein disulfide-isomerase (PDI) has been shown to bind peptides that have been translocated into the ER by TAP (Lammert et al., 1997b; Spee and Neefjes, 1997). Its peptide-binding abilities even exceed those of Gp96 making PDI the dominant peptide acceptor in the ER. However, all experiments utilizing PDI-peptide complexes to induce immune responses against the associated peptides have failed so far (Lammert et al., unpublished observations; P. Srivastava, personal communication). Indeed, in contrast to Gp96 and other HSPs, PDI is not found to bind to cell surface receptors (Singh-Jasuja et al., 2000a). This shows that other features of HSPs besides their ability to build stable complexes with peptides are required. Therefore and in line with the extraordinary efficiency of immunizations with Gp96 it has been proposed early that APCs possess specific receptors for HSPs on their surfaces enabling these cells to clear Gp96 efficiently from the extracellular fluid (Srivastava et al., 1994a). Lately, evidence for this hypothesis has been provided. The first indication of a receptor-mediated uptake of HSPs by antigen presenting cells was reported in EM binding studies using labeled Hsc70 and Gp96 and macrophages (Arnold-Schild et al., 1999). This observation was confirmed in confocal microscopy studies (Wassenberg et al., 1999). Evidence for binding to specific receptors was finally provided by

flow cytometry studies using fluorescein isothiocyanate (FITC)-labeled Hsc70 (Castellino et al., 2000) or Gp96 (Singh-Jasuja et al., 2000b). In the latter studies saturation and competition by non-labeled Gp96 was shown demonstrating the specificity of the HSP-receptor interaction. It was also revealed that only APCs like macrophages, DCs and B cells but not T cells bind Gp96 in a receptor-mediated fashion. Both studies also show cross-presentation of HSP-associated peptide on MHC class I molecules for activation of CTL lines. Interestingly, for both molecules – Gp96 and Hsc70 – receptor-mediated uptake of the HSP is essential for re-presentation of HSP-associated peptides on MHC class I molecules. Non-specific endocytosis and macropinocytosis were not able to do so. Consequently, HSP receptors do not only facilitate efficient uptake of HSP-peptide complexes but also seem to be responsible for shuttling HSP-associated antigen into the right processing pathway. For Hsc70 it has also been demonstrated that this pathway is dependent on the nature of the antigen and the requirement for further processing. If C-terminal processing of the peptide for transfer onto MHC molecules is required, re-presentation is dependent on the activity of the proteasome and TAP. If C-terminal processing is not required, transfer of peptide from HSP to MHC can occur inside the endosome as confirmed by confocal microscopy (Castellino et al., 2000).

The search for the identity of one or more HSP receptors is still going on. Meanwhile, the first receptor for Gp96 has been identified as CD91 by cross-linking Gp96 chemically to plasma membrane fractions of macrophages and sequencing the bound protein (Binder et al., 2000b). CD91, also known as $\alpha_2$-macroglobulin receptor or low density lipoprotein receptor related protein, consists of a 420 kDa $\alpha$- and a 85 kDa $\beta$-unit. Its functions are believed to be the clearance of extracellular plasma products as well as regulation of lipid metabolism (reviewed by Strickland et al., 1995). The question whether CD91 is the only receptor for Gp96 or whether CD91 is also a receptor for other HSP, still needs to be resolved. Data from our lab hints into the direction that other receptors are likely to be involved in the binding and uptake of extracellular Gp96 (Singh-Jasuja et al., unpublished observation).

### The fourth requirement: HSPs activate dendritic cells

To efficiently prime naive T cells at least two signals have to be provided by the APC: The first is the recognition of MHC-peptide complexes by the TCR and its co-receptor. To fulfill this requirement, APCs and especially DCs possess unique pathways to shuttle exogenous

antigen effectively into the antigen proccessing pathway leading to the formation of MHC-peptide complexes (Rodriguez et al., 1999). The second signal depends on the interaction of co-stimulatory molecules with e.g. CD28 on the T cell side. The absence of co-stimulatory activity rather leads to tolerance and not priming. The ability to carefully regulate its co-stimulatory molecules makes the dendritic cell exceptional among the APCs. Co-stimuli can be provided by several surface markers including CD80, CD86, 4-1BB ligand and others (reviewed by Watts and DeBenedette, 1999) which are upregulated during DC maturation. There are numerous sources responsible for the maturation of DCs: bacterial products like lipopolysaccharid (LPS), pro-inflammatory cytokines like TNF-α or the engagement of CD40 expressed on APCs by CD40 ligand (CD40L) on T helper cells. It has also been shown, that the content of cells undergoing necrosis (but not apoptosis) is able to activate macrophages and DCs (Sauter et al., 2000). However the substances that are released during necrosis and that are responsible for APC-stimulation, still have to be determined. The question whether HSPs – one of the most abundant classes of cellular proteins – can induce the activation of macrophages and DCs has been clarified in the past year. Hsp60 was the first heat shock protein to be identified as an activator of macrophages in a CD14 (Kol et al., 2000) and Toll-like receptor 4-dependent (Ohashi et al., 2000) manner. However, Hsp60 is unlikely to be involved in cross-priming as it does not possess peptide-binding abilities. Gp96 has been demonstrated to activate human and mouse dendritic cells by up-regulating co-stimulatory molecules and subsequently enhance stimulation of T cells. Furthermore, upon activation by Gp96 dendritic cells release pro-inflammatory cytokines resulting in the induction of an inflammatory response by the innate part immune system (Singh-Jasuja et al., 2000c). Heat-denatured Gp96 was not able to activate DCs, while the same heat-treatment did not diminish the activity of LPS, demonstrating that contaminating LPS in the protein preparations was not the cause of the described effects. Maturation of DCs by Gp96 is dependent on a signal transduction pathway involving the transcription factor NF-κB (Basu et al., 2000). Similar results have been obtained for the cytosolic Hsp70 which induces the activation of human monocytes via two different CD14- and NF-κB-dependent pathways (Asea et al., 2000). Whether the surface molecule CD91 identified as Gp96 receptor plays a role in downstream signaling during Gp96-mediated activation of DCs is still an open question, although this seems unlikely, because CD91 does not possess a typically recognized signaling domain. Skepticism about the *in vivo* relevance of HSP-mediated cross-presentation and APC activation has been raised because of the rather high concentrations of HSP used for DC

activation, ranging from 10 to 100 µg/ml *in vitro*. However, the highly immunostimulating potential of HSPs has been demonstrated *in vivo* by showing maturation of dendritic cells in mice by exogenously applied HSP. The injection of 1 µg of Gp96 led to the massive infiltration of CD11c-positive DCs into the draining lymph nodes from the point of injection. These DCs were of the mature phenotype and were highly efficient in the stimulation of T cells (Binder et al., 2000a).
Taken together recent reports show conclusively that HSPs alone can act as highly stimulating agents to antigen presenting cells, especially dendritic cells. The activation of APCs enables DCs to communicate efficiently with T cells resulting in subsequent stimulation of a strong immune response.

### HSPs as necrosis messengers, HSP receptors as sensors for necrosis

Experiments identifying CD91 as a receptor for Gp96 showed that the re-presentation of Gp96-associated peptides after receptor-mediated uptake can be blocked by antibodies against CD91 or by $\alpha_2$-macroglobulin (Binder et al., 2000b). The authors have incorporated the latter observation into an intriguing model for the general role of HSPs in the regulation of APC function. In the blood uptake of Gp96 (and possibly other HSPs) is inhibited by $\alpha_2$-macroglobulin which is present in the serum in large amounts. In tissues, however, extracellular Gp96 can access CD91 and is taken up via receptor-mediated endocytosis leading to the subsequent presentation of its associated peptides on MHC molecules. Another regulator of extracellular Gp96 activity is also be found in the blood. Platelets have been described to possess receptors for Gp96 and might be involved in the clearance or neutralization of Gp96 and other HSPs in the blood and in wounds (Hilf et al., 2001).
As Gp96 is known to be localized to the ER lumen the question regarding the source of extracellular Gp96 and other HSPs arises. Indeed, early experiments identified Gp96 as a cell surface protein on fibroblasts (Pouyssegur and Yamada, 1978). However, there is no basis so far for regulated secretion of HSPs. It seems much more attractive that HSPs are released in large amounts as result of necrosis. Indeed, it has been demonstrated that necrotic cells induced by repeated freeze-thaw cycles release Gp96, Hsp90, Hsp70 and Calreticulin while apoptotic cells generated by UV radiation do not (Basu et al., 2000). Both, necrosis and apoptosis, although finally leading to the death of a cell, have very different starting points. Apoptosis, also known as programmed or 'silent' cell death, occurs all the time, especially

during development of the organism and is a primary 'tool' in the education of the immune system inside the thymus. On the other hand, necrosis is the result of severe stress or trauma, either caused by injury or by infection. Upon necrosis, cellular proteins including HSPs are released into the surrounding extracellular fluid. APCs in the neighborhood might acquire these HSPs and shuttle the associated peptides into the correct processing pathway for presentation on MHC molecules. Simultaneously, HSPs like Gp96 could activate APCs inducing the release of pro-inflammatory cytokines and the up-regulation of co-stimulatory molecules (Singh-Jasuja et al., 2000c; Basu et al., 2000). Presentation of immunogenic peptide on MHC molecules and providing co-stimuli would result in a T cell response against the source of the HSP. This immune response would be directed against infected cells in case of viral infection or would simply elicit a preventive danger response in case of injury. In the case of apoptosis, apoptotic cells or their remnants, so called apoptotic bodies, expose phosphatidylserin on the outer leaflets of their membranes. Phosphatidylserin is sensed by the Phosphatidylserin receptor present on some macrophages leading to an anti-inflammatory cytokine response including IL-10 and TGF-β release (Fadok et al., 2000). Although apoptotic cells are able to transport antigen to APCs leading to the cross-presentation of these antigens (Albert et al., 1998), co-stimulatory activity is not induced (Sauter et al., 2000). Antigen presentation without co-stimulus results in tolerance and not priming. Other receptors such as CD14 or CD36 recognize other apoptotic cell-associated ligands (ACAMPs) and can transmit similar tolerogenic signals to the APC (Devitt et al., 1998). Taken together this model proposes that apoptosis creates a tolerogenic, necrosis on the other hand an immunogenic milieu. HSPs may well play a vital role in these processes: heat shock proteins as necrosis messengers, HSP receptors as necrosis sensors.

To the biochemists heat shock proteins and chaperones are known as multifunctional proteins. They facilitate folding and unfolding of proteins, they participate in vesicular transport processes, they prevent protein aggregation in the densely packed cytosol, they are involved in signaling processes etc. This multifunctionality has now been extended even further by immunologists: HSPs like Gp96 carry peptides that represent cellular proteins, they transfer these peptides to MHC molecules via binding to receptors on DCs and other APCs, they behave as danger signals by activating DCs to express co-stimuli and pro-inflammatory cytokines and they regulate the surface expression of the HSP receptors themselves (Singh-Jasuja et al., 2000c). These combined features make heat shock proteins powerful weapons. They activate both, the innate and the adaptive immune system. Because of having so many

functions, heat shock proteins have been called 'the Swiss Army Knives of the Immune System'.

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## Chapter 3

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# Cross-presentation of Gp96-associated antigens on MHC class I molecules requires receptor-mediated endocytosis

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Heat-shock proteins (HSPs) like gp96 (grp94) are able to induce specific cytotoxic T-cell (CTL) responses against cells from which they originate. Here, we demonstrate that for CTL activation by gp96-chaperoned peptides specific, receptor-mediated uptake of gp96 by antigen presenting cells (APC) is required. Moreover, we show that both in humans and mice only professional APCs like dendritic cells (DCs), macrophages and B cells, but not T cells are able to bind gp96. The binding is saturable and can be inhibited using unlabelled gp96 molecules. Receptor-binding by APCs leads to a rapid internalization of gp96 which co-localizes with endocytosed MHC class I and class II molecules in endosomal compartments. Incubation of gp96 molecules isolated from cells expressing an Adenovirus type 5 (Ad5) E1B epitope with the dendritic cell line D1 results in the activation of E1B-specific CTLs. This CTL activation can be specifically inhibited by the addition of irrelevant gp96 molecules not associated with E1B peptides. Our results demonstrate that only receptor-mediated endocytosis of gp96 molecules leads to MHC class I-restricted re-presentation of gp96-associated peptides and CTL activation; nonreceptor-mediated, nonspecific endocytosis is not able to do so. Thus, we provide evidence on the mechanisms by which gp96 is participating in the cross-presentation of antigens from cellular origin.

## Introduction

Activation of cytotoxic T lymphocytes (CTLs) with exogenous cell-associated antigens requires efficient uptake and presentation of these antigens by bone marrow-derived antigen presenting cells (APCs). This phenomenon was first observed by Bevan (1-3) for the induction of CTLs against minor H antigens. Because the antigens were expressed in foreign donor cells with different MHC molecules, this process was termed ‚cross-priming'. Since then it has been shown that soluble protein antigens (4, 5), antigens expressed in MHC matched cells (6-8) or antigens encoded by naked DNA (9) also require uptake and re-presentation by MHC molecules expressed on the surface of professional APCs. Therefore, the term ‚cross-presentation' was introduced to describe the general re-presentation of exogenous cell-associated antigens by MHC class I (6) and MHC class II molecules (10). In addition to CTL activation, cross-presentation can also lead to the induction of CTL tolerance (11, 12).

The nature of the APCs that are able to take up and re-present cell-associated antigens on MHC class I molecules remains elusive *in vivo*. However, *in vitro* studies suggest that dendritic cells (DCs) (11-13), macrophages (14-16) or B cells (17) might be involved.

Several pathways for antigen uptake have been described, ranging from non-specific mechanisms such as phagocytosis, pinocytosis or macropinocytosis (18-23) to specific, receptor-operated mechanisms that include mannose and scavenger-type receptors (22). Depending on the nature of the antigens, and consequently on the mode of uptake, antigens might be targeted to different processing compartments and be able to gain access to different antigen presentation pathways. CTL activation can be mediated by macropinocytosis or phagocytosis of exogenous, soluble antigens (24-26). However, these pathways require high antigen concentrations and might therefore be of limited relevance in providing a mechanism for cross-presentation *in vivo* (23).

More recently, apoptotic bodies were shown to be phagocytosed by immature dendritic cells resulting in the activation of MHC class I-restricted T cells (13, 27). This uptake involves CD36 and the integrin receptor $\alpha_v\beta_5$ (28), which explains the high efficiency.

An additional pathway with potential relevance for cross-presentation became evident when the induction of tumor immunity and CTL activation through the injection of heat-shock proteins, such as gp96, HSP70 and HSP90, was discovered (reviewed in 29). The specificities of the CTL response were directed against the cells from which the HSPs were isolated. This

can be explained by the association of the HSPs with peptides of cellular origin. Immune responses against several cellular antigens including minor H, tumor and viral antigens were induced (reviewed in 30) by using as little as 1-2 ng HSP/peptide complex in one particular case (31). It was postulated that the extremely efficient MHC presentation of HSP-associated peptides is accomplished by the receptor-mediated uptake of HSPs by professional APCs (32). Recently, binding of Hsp70 and gp96 to a macrophage- and dendritic-like cell line was observed (33). This observation provides a possible explanation for the high immunogenic potential of HSPs in situations in which they are injected into mice or released from dying cells in that they shuttle antigenic peptides to APCs (32). Receptor-mediated endocytosis of HSPs by professional APCs will lead to the accumulation of these peptide chaperones in cells crucially involved in the activation of CTLs.

We therefore decided to characterize the cell populations involved in receptor-mediated endocytosis of HSPs in detail, to follow the fate of endocytosed HSPs and to test whether or not receptor-mediated endocytosis of HSPs indeed results in the re-presentation of HSP-associated peptides and subsequent activation of CTLs. The latter issue in particular is of crucial importance for the understanding of HSP-mediated cross-presentation since antigen uptake by APCs does not necessarily correlate with the ability to cross-present antigens. Despite the fact that macrophages and DCs phagocytose apoptotic cells, only immature DC are able to cross-present antigens and to activate CTLs (28).

We now describe that members of the family of professional APCs, such as macrophages, DC and B cells, are able to bind the ER-resident HSP gp96 specifically. The binding was saturable and could be competed for with unlabelled gp96 molecules. The uptake of gp96 isolated from cells expressing the Adenovirus type 5 (Ad5) E1B epitope by the dendritic cell line D1 resulted in the activation of E1B-specific CTLs. More importantly, activation of Ad5 E1B-specific CTLs could be inhibited by competition with gp96 not associated with E1b peptide. This result clearly demonstrates that CTL activation is the consequence of receptor-mediated endocytosis of gp96 molecules followed by the class I-restricted re-presentation of associated peptides and supports the participation of HSPs in cross-presentation of cell-associated antigens.

## Materials and Methods

### Mice, cells, antibodies and proteins

The DEC205-Knockout mice were kindly provided by Michel Nussenzweig and Ralph Steinman. BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). MHC class II-deficient mice ABBN5 (34) and littermate ABBN6 were obtained from Taconic (Germantown, NY). P388D1, RMA and RMA-S mouse cell lines (ATCC, Manassas, VA) were cultured in Minimal Essential Medium (α-MEM). The cell line D2SC/1, representing an early progenitor of mouse splenic DC, and D1, a non-transformed, growth-factor dependent long-term DC culture (35), were cultured in Iscove's modified Dulbeccos's Medium (IMDM). All tissue culture media were supplemented with 10% FCS, 0.3 mg/ml L-glutamin, 100 U/ml penicillin/streptomycin and 50 μM β-mercaptoethanol. To grow D1 cells, medium was additionally supplemented with 30% conditioned medium from the fibroblast cell line R1. Antibody to gp96 (SPA-850) was obtained from StressGen Biotechnologies (Victoria, BC, Canada). Following labelled antibodies to mouse and human antigens were obtained from Pharmingen (San Diego, CA): H2-$K^b$-Biotin, H2-$A^b$-Biotin, CD8-FITC, Interferon-γ-PE, CD16/CD32 (Fc block), CD45R/B220-PE, CD19-PE, CD14-PE, CD90.2 (Thy1.2)-PE, CD86 (B7.2)-PE, CD11c-PE, Mac3-PE, CD1a-PE, CD83-PE, IgG1-PE and IgG2a-PE isotype controls. Goat-anti-rabbit-Alexa™ 546 and Streptavidin-Alexa™ 546 (Molecular Probes, Leiden, Netherlands) were used as secondary reagents. Bovine Serum Albumin (BSA), biotinylated BSA, Ovalbumin (OVA) and Fluorescinisothiocyanate (FITC) were obtained from Sigma-Aldrich (St. Louis, Missouri). Streptavidin-PE was purchased from Jackson Laboratories, West Grove, PA. BSA and OVA were labeled with FITC or Biotin according to standard protocols. Free FITC molecules were removed by reaction with Tris and gelfiltration through a Sephadex G-25 (Sigma-Aldrich) column. gp96 and gp96-FITC from the mouse cell line IGELa2 were obtained from Immunosome (Tübingen, Germany). All animal studies were performed according to our institutional guidelines and approved by our Institutional Review Board.

### Purification of gp96

The TAP-deficient RMA-S SigE1B cell line has been generated by transfection of RMA-S with the adenovirus early region 1 H2-$D^b$-restricted E1b epitope (VNIRNCCYI) targeted to the endoplasmic reticulum in a TAP-independent fashion (36). Gp96 was purified from RMA,

RMA-S and RMA-S SigE1B cell lines as described (37). The approximate concentrations were determined by measuring the OD at 280 nm using an extinction coefficient of 1.0.

**Cytometry (FACS) binding assay**

100,000 cells were incubated for 30 min on ice in 100 µl IMDM, 10% FCS containing 30 µg/ml gp96-FITC or Ovalbumin-FITC, washed three times and fixed in 1% paraformaldehyde. For competition experiments, a given excess of unlabelled gp96 was added together with 50 µg/ml gp96-FITC simultaneously. For staining of mouse spleen cells (including erythrocytes) and human peripheral blood lymphocytes (PBL), PE-conjugated antibodies were added as markers for different cell types. Immature DCs were prepared from bone-marrow of C57BL/6 mice (38) and human blood monocytes (39) as described. Cytometry measurements were performed on a FACSCalibur® (Becton Dickinson).

**Internalization studies in confocal microscopy**

Bone-marrow derived, immature DCs (BMDCs) were prepared from C57BL/6 mice as described (38). On day 6 of their preparation the BMDCs were tested for CD11c, CD86 and MHC class II expression and seeded on cover slips, pre-cooled and incubated for 30 min on ice with IMDM containing 10% FCS and 50 µg/ml gp96-FITC („pulse"). The coverslips were washed twice and incubated in IMDM medium for 15 min or longer at 37°C („chase"), washed and fixed in 3.7% Paraformaldehyde in PBS. For the co-localization experiments cells were pre-incubated with Fc-Block (α-CD16/CD32) followed by biotinylated antibodies to H2-$K^b$ or H2-$A^b$ and 50 µg/ml gp96-FITC together with Streptavidin-Alexa™ 546. For staining of lysosomes cells were fixed with methanol/aceton (1:1, -20°C) and incubated with α-Lamp-1 (kindly provided by M. Fukuda) and goat-anti-rabbit-Alexa™ 546. For microscopy a Zeiss LSM 510 laser scanning microscope was used. „Bleeding" of emission into other detection channels was excluded usingh the multitracking modus of the LSM 510. Thickness of the optical plane was adjusted by the pinhole to be less than 1 µm.

**Immunization of mice with gp96**

C57BL/6 mice were immunized intraperitoneally with 30 µg gp96 purified from RMA-S SigE1B cells. After 10 days mice were killed and the spleen cells restimulated with E1B-expressing XC3 cells or Ad5 E1B peptide (50 ng/ml). Specific lysis of RMA-S SigE1B cells by CTLs contained in the spleen culture was determined by a standard chromium release

assay 5 days after restimulation and after a second restimulation with XC3 cells or Ad5 E1B peptide (50 ng/ml).

**CTL cross-presentation assay**

The cytotoxic T-lymphocyte (CTL) clones 100B6, 0.1C2 and LN5 were described previously (36, 40). CTL clones were restimulated on a weekly basis by incubation with the Ad5 E1B/E1A-expressing tumor cell line XC3. The E1B peptide was synthesized on a ABI 432 A peptide snythesizer (Applied Biosystems) applying Fmoc strategy.

Activation of CTL clones was assessed by measurement of intracellular Interferon-γ production. 25,000 D1 cells were incubated with 20 µg/ml gp96 purified from RMA-S SigE1B, RMA or RMA-S cells for 2 hours at 37°C, for competition experiments an excess of gp96 from RMA or RMA-S was added, washed four times and incubated with 250,000 CTLs for 12 hours at 37°C. 10 µg/ml Brefeldin A were added for additional 5 hours at 37°C. Cells were washed, fixed and perforated with Saponin. The fixed cells were stained with PE-labeled anti-IFN-γ or isotype control and FITC-labeled anti-CD8 antibodies and measured in flow cytometry.

## Results

### Gp96 binds specifically to antigen presenting cell lines

Recent experiments demonstrated that HSPs are able to interact specifically with a macrophage- and a DC-like cell line (33). We therefore further characterized the cell types able to interact with gp96 in a specific manner. For this purpose, we incubated several cell lines with FITC-labelled gp96, always at 4°C to exclude endocytosis. We only observed a specific interaction of gp96 with APC lines, like P388D1, D2SC/1 and D1, but not with the lymphoma cell lines RMA, EG.7 and T1 (Fig. 1A, B). Increasing the total concentration of gp96-FITC the binding displayed saturation at a total concentration of 30 µg/ml (Fig. 1C) and could only be competed for by unlabelled gp96, but not by ovalbumin (Fig. 1A) or BSA (not shown). A 1-fold excess of unlabelled gp96 resulted in a 50% reduction, a 5-fold excess in a approx. 75% reduction of gp96-FITC binding at saturation point, which could be inhibited completely using an excess of up to a 100-fold (Fig. 1D). These data correspond to the theoretical values of 50% and 83% (1:1 and 1:5 dilution of gp96-FITC with unlabelled gp96), demonstrating that the FITC-labelling of gp96 did not affect the binding characteristics to its putative receptor significantly. No inhibition was observed using an excess of up to 400-fold of ovalbumin or BSA (data not shown). These data demonstrate the presence of a specific gp96 receptor that is expressed on APCs but not on other cell lines (Fig. 1A, B, C, D).

### Gp96 interacts specifically with primary antigen presenting cells in mice and humans

More importantly, gp96 (Fig. 1E) but not BSA (Fig. 1F) bound efficiently to immature bone-marrow-derived primary DCs prepared (as described in 38) from C57BL/6 mice, and could be competed for by increasing amounts of unlabelled gp96 (Fig. 1G) but not by BSA (Fig. 1H). Specific binding was also observed when mouse spleen cells from BALB/c mice were incubated with gp96-FITC (Fig. 2). Gp96 interacted specifically with cells that stained positive for MHC class II and CD45(B220) but not with cells positive for CD90 (Thy-1) molecules. Setting the forward and sideward scatter gate on the bigger cells, including cells of the myeloid lineage, CD11c and Mac-3 positive cells were also positive for gp96-FITC, indicating that the expression of the gp96 receptor is restricted to professional APCs. No staining was observed using OVA-FITC (all panels on the left) or BSA-FITC (data not shown). The identical outcome was observed using spleen cells from C57BL/6 mice (not

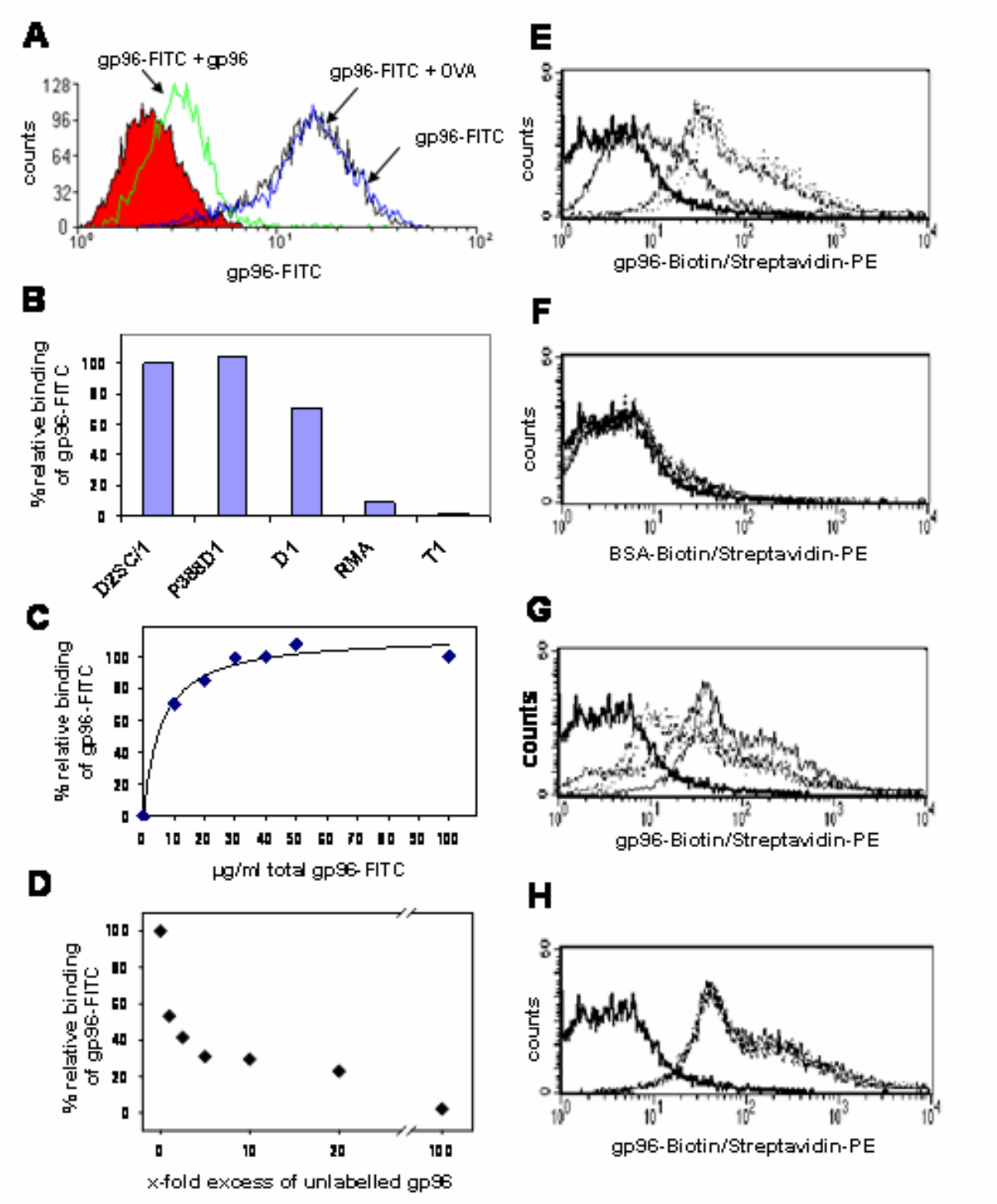


Figure 1: Specific binding of gp96-FITC to APC cell lines and BMDC.
Binding of 3 µg gp96-FITC to 100,000 D2SC/1 cells was performed always at 4°C in 100 µl IMDM containing 10% FCS. This binding could be competed by a 10-fold excess of unlabelled gp96 but not Ovalbumin (**A**). Specific binding of gp96-FITC was observed on D2SC/1 (DC progenitor), P388D1 (macrophage) and D1 (DC) but not on RMA and T1 cells (**B**). Binding could be saturated at approx. 30 µg/ml for 100,000 D2SC/1 cells (**C**) and competed almost completely by an 100-fold excess of unlabelled gp96 (**D**). Binding is given as relative values where 100% represents maximum binding of gp96-FITC. The concentration values shown give total concentration of gp96-FITC added to the cells. (**E**) Binding of 1 µg (—), 5 µg (---) and 10 µg (**…**) gp96-Biotin/Streptavidin-PE to immature bone-marrow derived primary DCs (BMDCs) from C57BL/6 mice, no binding was observed for BSA-Biotin/Streptavidin-PE (**F**); binding of 10 µg gp96-Biotin (—) to BMDC is competed in a similar fashion to **D** by unlabelled gp96 (**G**), but not by unlabelled BSA (**H**).

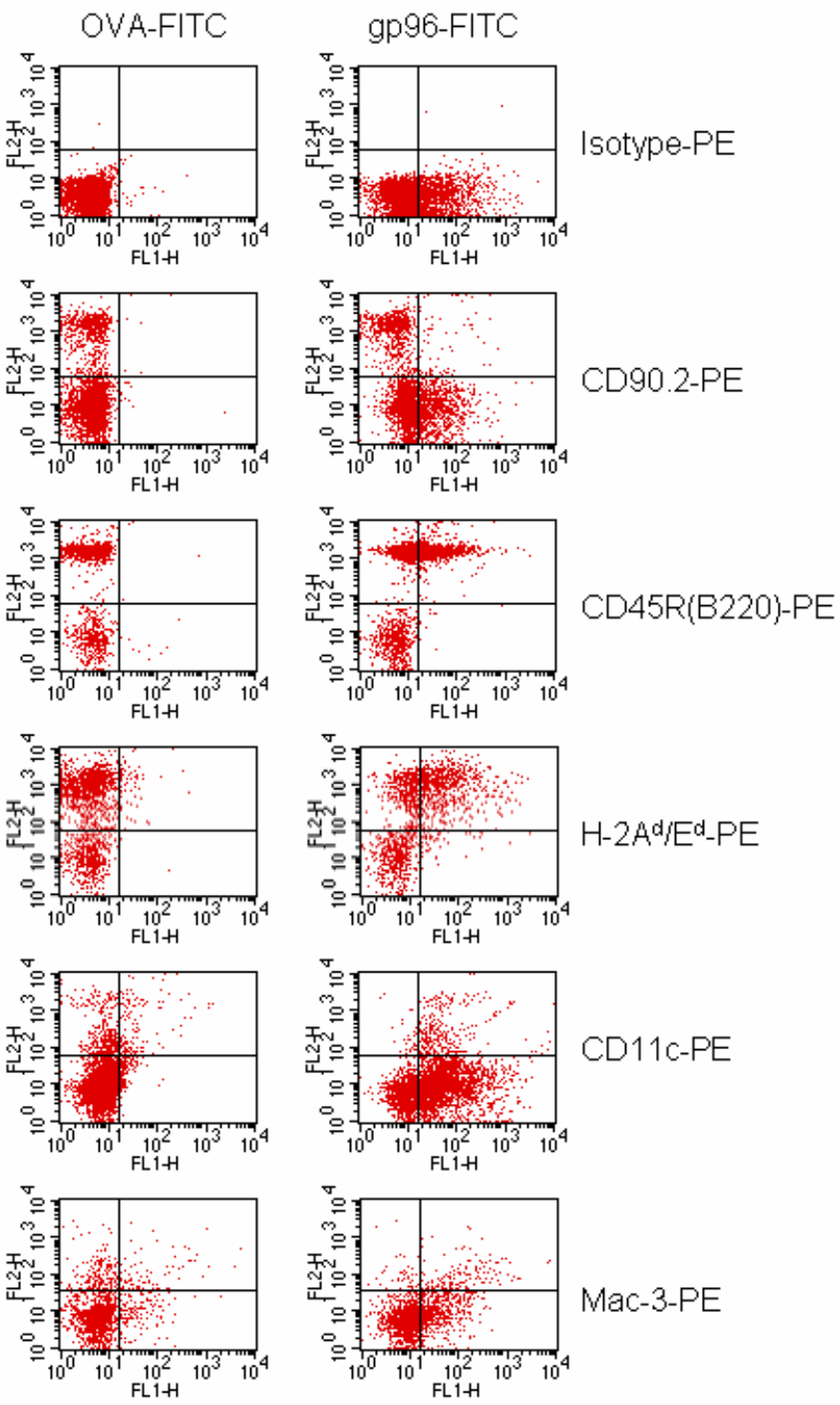


Figure 2: Specific binding of gp96-FITC to B cells, macrophages and dendritic cells, but not to T cells of a spleen cell culture.
100,000 fresh Balb/c spleen cells were stained with 5 µg Ovalbumin-FITC (left panels) or gp96-FITC (right panels) and different PE-labelled cell type marker antibodies to: CD90.2 (Thy-1.2, T cells), CD45R/B220 (B cells), I-A$^d$/E$^d$, CD11c (dendritic cells) and Mac-3 (monocytes and macrophages). Macrophages and DCs were counted in a different gate than lymphocytes with a higher forward scatter value.

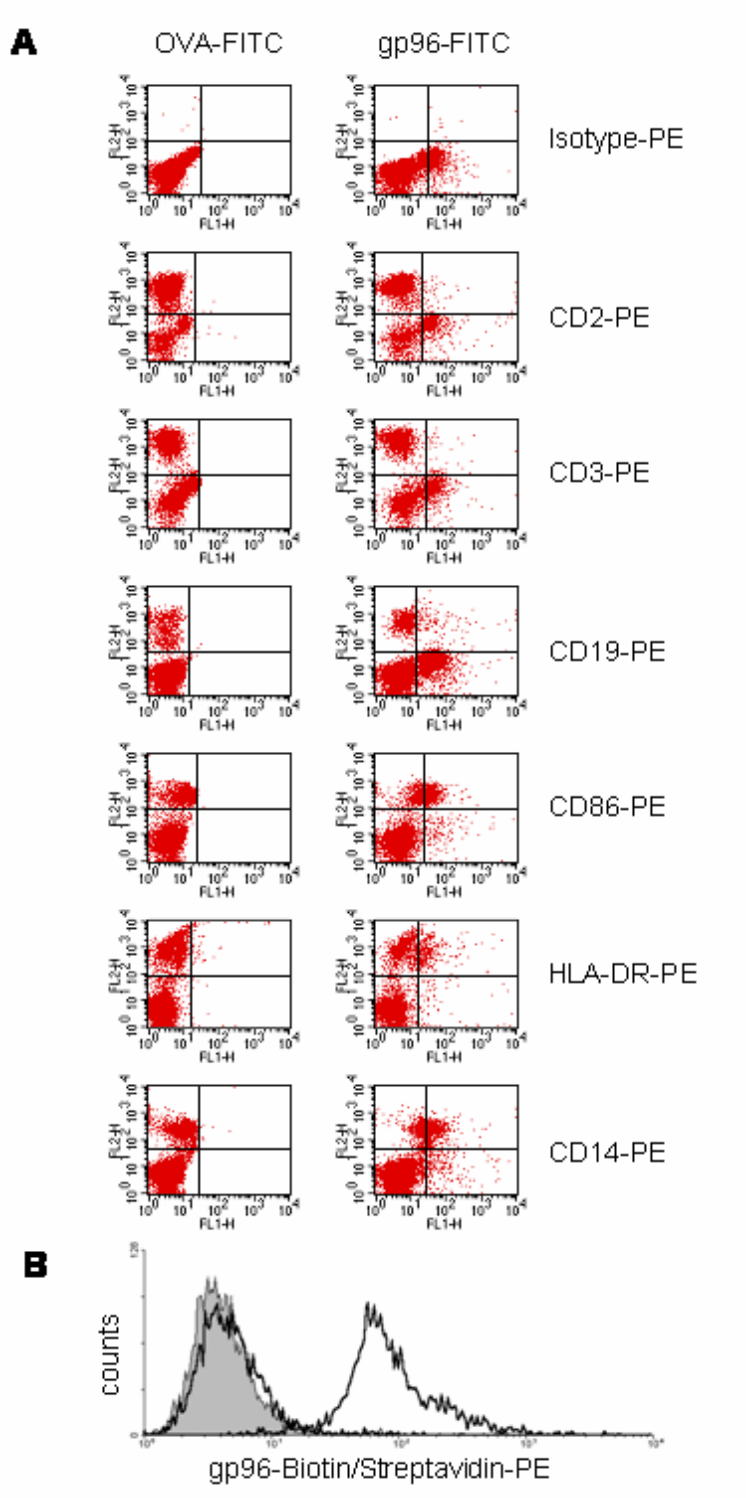


Figure 3: gp96-FITC binds to APCs in human PBL culture but not to T cells.
(**A**) 100,000 fresh human PBL were stained with 5 µg Ovalbumin-FITC (left panels) or gp96-FITC (right panels) together with PE-labelled antibodies to following cell surface antigens: CD2 (T and NK cells), CD3 (T cells), CD19 (B cells), CD86, HLA-DR and CD14 (monocytes). The gate was set on all living cells. Therefore monocytes appear as a population with a slightly higher autofluorescence than lymphocytes in both fluorescence channels. Comparing the shifts of each population monocytes showed slightly better binding of gp96 than B cells. (**B**) Binding of 0 µg (filled grey) and 5 µg (—) gp96-Biotin to immature DCs prepared from human PBL. 10 µg of BSA-Biotin (—) did not display binding.

shown). A similar gp96-FITC staining pattern was obtained for human peripheral blood lymphocytes (PBL). HLA-DR, CD86, CD19 and CD14, but not CD2 or CD3 positive cells interacted specifically with gp96-FITC (Fig. 3A). Again, no staining was observed using OVA-FITC. Gp96 binding to monocytes was slightly better than to B cells in human PBL. As expected, DCs expressing CD1a and CD83 were not detected. To determine gp96-FITC binding to this cell type, we differentiated DCs from human PBL by the application of GM-CSF and IL-4. The whole DC population generated stained positive with gp96 but not BSA (Fig. 3B).

### DEC-205 and MHC class II molecules are not required for gp96 binding

Since gp96 molecules contain a single, high mannose oligosaccharide (41, 42), we addressed the question whether this might allow the uptake by the DEC-205 receptor. DEC-205 is expressed on DCs and thymic epithelial cells and is capable of directing captured soluble, exogenous antigens to a specialized antigen processing compartment (43). DCs were prepared from bone marrow of wild-type and DEC-205$^{-/-}$ mice (kindly provided by Michel Nussenzweig and Ralph Steinman) and incubated with increasing amounts of gp96-FITC. FACS analysis revealed identical staining (Fig. 4A), suggesting that the DEC-205 receptor is not involved in the binding of gp96 molecules by DCs.

We further speculated whether MHC class II might function as a receptor for gp96 because gp96 showed binding to all MHC class II-positive mouse spleen cells and human PBL. Binding together with marker antibodies to spleen cells from MHC class II-Knockout mice and their littermates did not reveal any difference (shown for MHC class II antibody in Fig. 4B, other markers not shown), indicating that MHC class II molecules do not function as gp96 receptors.

### Gp96 is endocytosed efficiently and co-localizes with recycled MHC class I and class II molecules

Recently it has been suggested that gp96-FITC bound to peritoneal macrophages is endocytosed into early endosomes but does not reach the stage of lysosomes (44). We also attempted to determine the faith of receptor-bound gp96 at the cell surface of APCs by confocal microscopy (Fig. 5) using authentic dendritic cells (bone-marrow derived DCs from C57BL/6 mice). Initial binding of FITC-labelled gp96 to the cell surface, at 4 °C to prevent endocytosis, revealed a patched pattern. Further incubation at 37 °C led to efficient

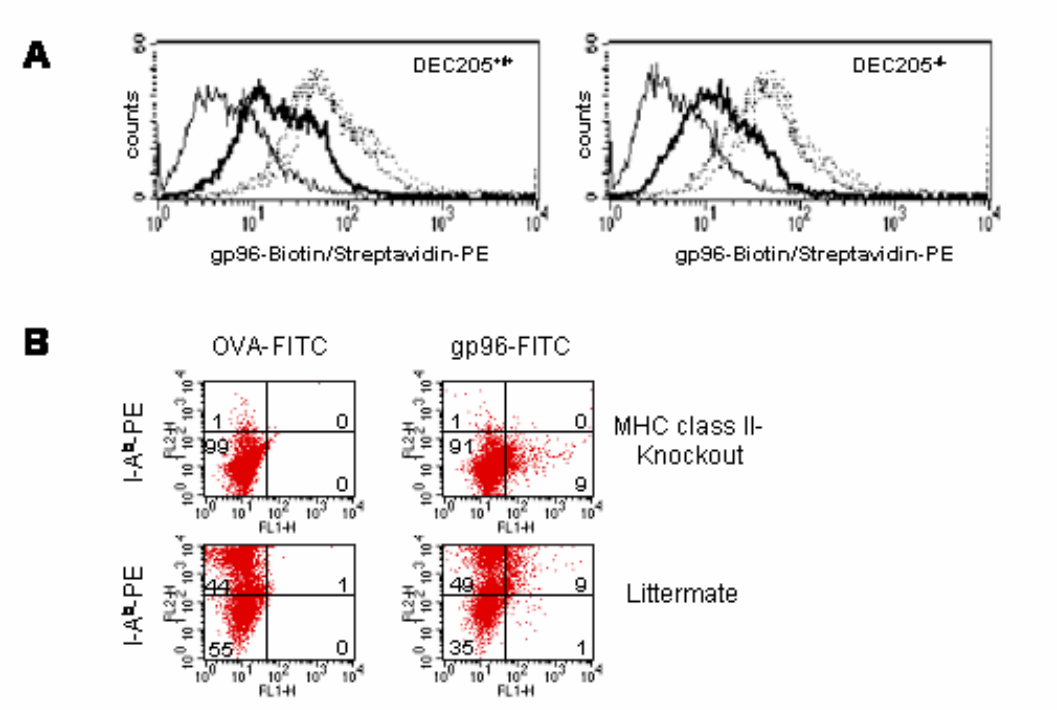


Figure 4: DEC-205 and MHC class II do not function as receptors for gp96.
Binding of gp96-Biotin to bone-marrow derived DCs from wildtype and DEC205$^{-/-}$ mice (A) as well as binding of gp96-FITC to spleen cells from MHC class II$^{-/-}$ mice and their littermate (B) showed identical staining. For staining of spleen cells in B different cell surface markers were used (see Fig. 2), only antibody to MHC class II is shown.

endocytosis of gp96. Co-localization with lysosomes, labelled with Lamp-1 antibody (45), was not observed after 15, 30, 45, 60 and 90 min of endocytosis (shown for 60 min in Fig. 5). Recently, it has been reported that internalized cell surface MHC class I, like class II molecules, are able to bind their antigen in endosomal compartments suggesting these vesicles to be putative MHC class I and class II loading compartments for exogenously derived antigen (46). We therefore attempted to determine whether gp96 taken up by receptor-mediated endocytosis can be found in compartments containing recycled MHC class I and class II molecules. Indeed, after 15 min of endocytosis nearly all of the endocytosed H2-K$^b$ and H2-A$^b$ molecules co-localized with gp96. Similar results were obtained using gp96-FITC bound to the cell surface of the D2SC/1 cell line, where after 15 min of endocytosis gp96 co-localized with Transferrin-Texas Red (as marker for early endosomes) and endocytosed H2-K$^d$ molecules but were excluded from lysosomes after 30 min (data not shown).

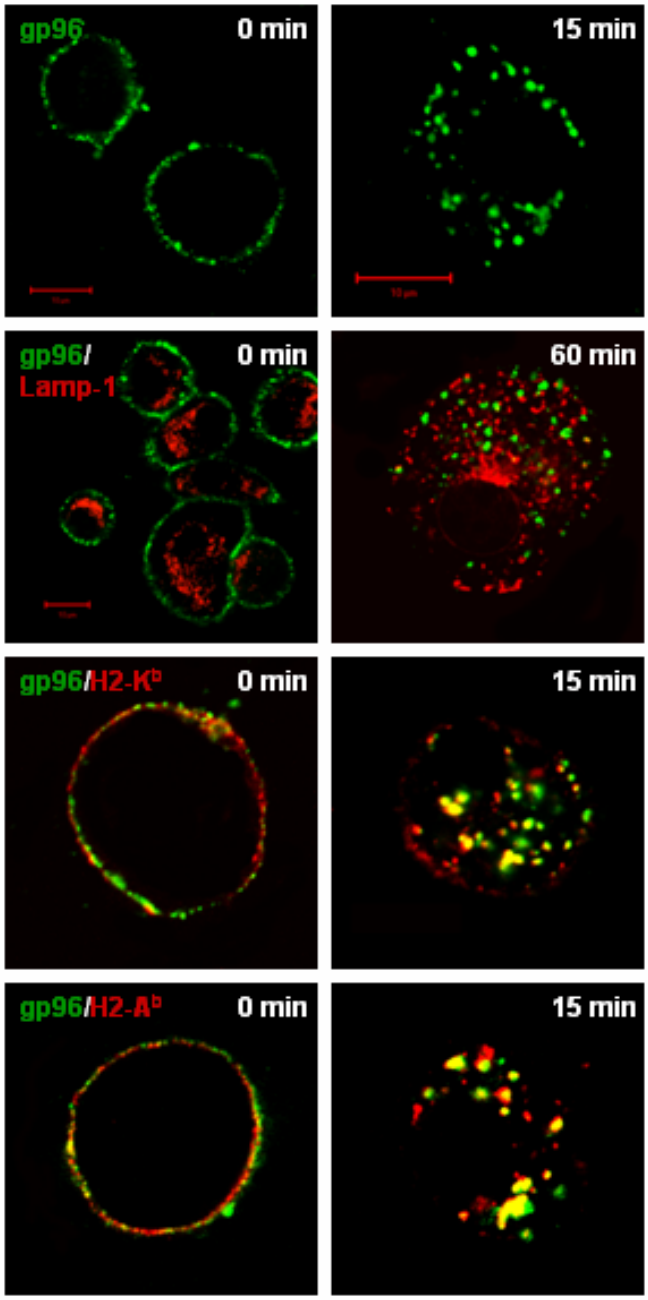


Figure 5: gp96-FITC is endocytosed by bone-marrow derived DCs efficiently and co-localizes with endocytosed MHC class I and class II molecules but does not target to lysosomes.
Internalization of gp96-FITC was followed with confocal microscopy, representative sections are displayed. Coverslip-grown BMDCs were incubated with 50 µg/ml gp96-FITC (shown in false colour green) on ice, washed, chased for 15 min or longer at 37°C and fixed in paraformaldehyde. To follow the fate of gp96-FITC after 15, 30, 45, 60 and 90 min (only 60 min is shown) of endocytosis cells were fixed and permeabilized with Methanol/Aceton and stained with antibody to the Lamp-1 and secondary Alexa™ 546-coupled antibody to visualize lysosomes (shown in false colour red). No co-localization of gp96 and Lamp-1 was observed. Furthermore, cells were stained with biotinylated antibodies to MHC class I (H2-$K^b$) and class II (H2-$A^b$) and secondary Streptavidin-Alexa™ 546 (both shown in red) as well as gp96-FITC (green) on ice, washed and chased at 37°C for 15 min. After 15 min of endocytosis nearly all vesicles containing endocytosed gp96 and MHC class I and class II molecules co-localize (shown in yellow as result of overlapping green and red).

## Gp96-associated peptides are loaded onto MHC class I molecules as a result of receptor-mediated endocytosis

Gp96 molecules have been observed to enter APCs by receptor-mediated endocytosis as well as by non-receptor-mediated, non-specific endocytosis or macropinocytosis (33, 44). The latter non-specific pathways have been described many times before to introduce exogenous proteins into the MHC class I-restricted antigen pathway, but unlike receptor-mediated endocytosis require high concentrations of antigens (reviewed in 22).

To investigate whether receptor-mediated endocytosis can lead to cross-presentation of gp96-associated antigens, we have isolated gp96 from RMA-S SigE1b cells that stably express the H2-$D^b$-restricted E1B epitope of Adenovirus type 5, fused with an ER-targeting signal sequence. C57BL/6 mice immunized with these gp96 molecules generated CTLs that recognized RMA-S SigE1B and RMA cells pulsed with the Ad5-E1B peptide efficiently but not RMA cells, demonstrating the presence of the Ad5-E1B epitope on gp96 molecules. Immunization with control gp96 molecules from RMA-S cells did not induce Ad5-E1B specific CTL responses (Fig. 6).

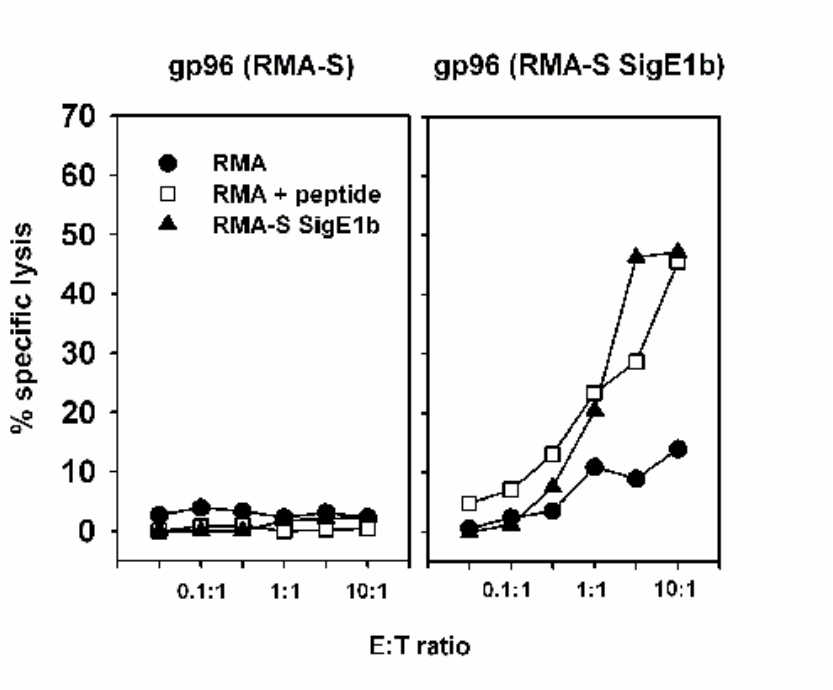


Figure 6: gp96-E1B complexes generate an CTL response *in vivo*.
gp96 was purified from RMA-S SigE1B and RMA-S cells. 30 µg of gp96 from either cell type were injected into C57BL/6 mice intraperitoneally. The specificity of the generated CTLs was assayed by $^{51}$Cr-release of RMA-S SigE1B cells (▲), RMA cells incubated with 100 ng/ml Ad5 E1B peptide ( ) or RMA cells (●). The figure shows one representative of three independent experiments.

To test whether the E1b epitope attached to gp96 was re-presented to CTLs after uptake by APCs, gp96 isolated from RMA-S SigE1B (or control gp96 from RMA-S cells) was incubated with the DC cell line D1 for 2 h at 37°C. The D1 cells were further incubated overnight with the Ad5-E1B-specific CTL clones 100B6, 0.1C2 or control CTL clone LN5, specific for the Ad5-E1A epitope. Intracellular IFN-γ production was measured to determine CTL activation via the re-presentation of the Ad5 E1B peptide. As shown in Fig. 7A, incubation of 0.1C2 CTLs with D1 cells pulsed with RMA-S SigE1B gp96 resulted in the activation of T cells. This activation was not observed if control gp96 isolated from RMA-S cells was used or if gp96 isolated from RMA-S SigE1B cells was incubated with the CTLs in the absence of D1 cells. The latter experiment clearly demonstrates that D1 cells, which efficiently bind gp96 molecules (see Fig. 1B), are required for the re-presentation. The T cells themselves are not able to bind gp96 (see Fig. 2 and 3) and consequently do not stimulate each other.

Most importanly, however, the activation of Ad5 E1B-specific CTLs by gp96 from RMA-S SigE1B cells could be inhibited by the addition of a 2-fold excess of irrelevant gp96 molecules from RMA-S and RMA cells. This excess of gp96 was able to reduce the binding of gp96-FITC by 60% (Fig. 1D) and eliminated the activation of 0.1C2 CTLs by gp96 molecules from RMA-S SigE1B cells almost completely. The identical scenario was observed for a different Ad5-E1B specific CTL clone, 100B6 but not for LN5 CTLs, which are specific for the control Ad5-E1A CTL epitope (Fig. 7B). No competition was observed by using a 2-fold excess of BSA as control (data not shown).

## Discussion

HSPs have been shown previously to induce specific immune responses against tumor, minor H and viral antigens (reviewed in 29, 30). This feature is based on peptides that are associated with HSPs and on the fact that by an unknown mechanism, HSPs can interact very efficiently with APCs to result in the re-presentation of HSP-associated peptides and subsequent activation of T cells (31, 47). We have now shown in this study that indeed specific binding of low amounts of gp96 to a receptor present on mouse and human professional APCs is required for the MHC class I-restricted re-presentation of gp96-associated peptides.

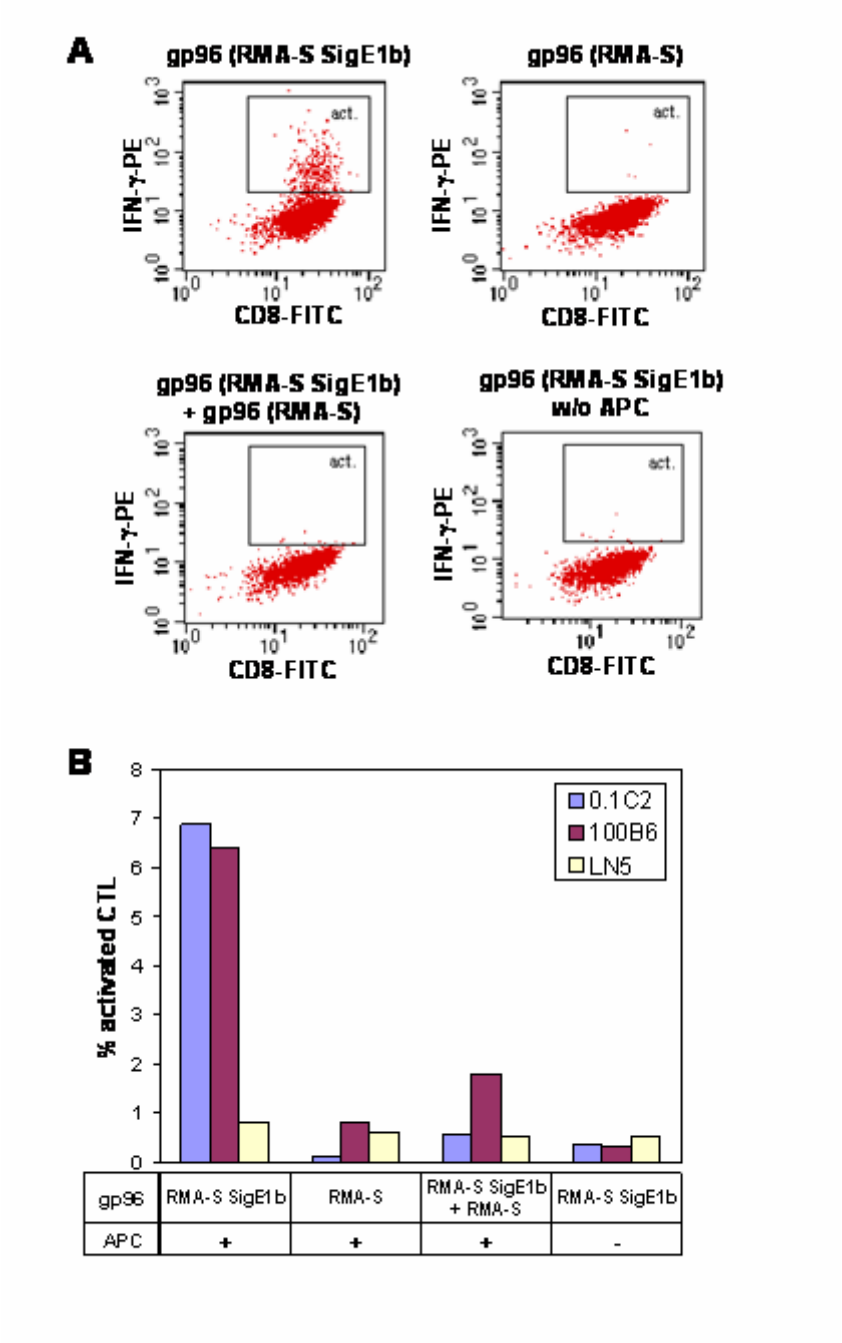


Figure 7: Specific activation of CTLs by dendritic cell-mediated cross-presentation of gp96-associated antigen requires receptor-mediated endocytosis of gp96-antigen complexes.
(**A**) Activation of Ad5 E1B specific CTL clones 0.1C2 was assayed by intracellular Interferon-γ staining in flow cytometry. D1 dendritic cells as APCs could activate the CTLs after prior incubation of D1 with gp96-E1B complexes purified from RMA-S SigE1B (upper left panel), but not with irrelevant gp96 isolated from RMA-S (upper right panel) or RMA cells (data not shown) or in the absence of D1 cells (lower right panel). Moreover, activation by gp96-E1B complexes could be competed with a 2-fold excess of gp96 from RMA-S (lower left panel) or RMA (not shown) but not with the same excess of BSA (not shown) indicating the presence of a receptor-mediated pathway responsible for processing of gp96 by D1 cells. PE-labelled isotype control antibody was always negative (data not shown). Results are representative for at least three experiments. (**B**) Summary of the activation of Ad5 E1B specific CTL clones 0.1C2 and 100B6 as well as control CTL clone LN5 specific for Ad5 E1A. Graph shows the percentage of activated CTLs present in the gate shown in **A**. Addition of Ad5 E1B peptide to D1 cells resulted in the activation of around 25% of CTL clones 0.1C2 and 100B6 (data not shown).

The nature of the gp96 receptor still remains unclear. We reported earlier that gp96 binding to the macrophage line P388D1 cannot be inhibited by mannan, thus arguing against the participation of the mannose receptor. Using DCs from DEC-205$^{-/-}$ mice, we show here that this receptor as well, which displays strong homology to the mannose receptor present on macrophages (43), is unlikely to be involved, because gp96-FITC binding is indistinguishable from that observed for DCs of wild-type mice (Fig. 4A). Because DnaK and HSP73 molecules have been reported to bind to certain allelic products of MHC class II (48, 49), they could represent another potential receptor for HSPs on the surface of APCs. The observation that gp96 binds to all MHC class II positive cells could indicate that gp96, too, uses MHC class II molecules as a receptor. However, anti-MHC class II antibodies were not able to inhibit the binding of gp96-FITC molecules (data not shown) and cells from MHC class II$^{-/-}$ mice showed identical gp96 binding compared to wildtype mice, thus arguing against MHC class II molecules being the receptor for gp96 (Fig. 4B).

We further demonstrate in this study that the specific interaction of gp96 molecules with DCs results in re-presentation of associated peptides and specific activation of CTLs. Gp96 molecules isolated from RMA-S SigE1B cells that carry the Ad5 E1B CTL epitope (Fig. 6) are able to activate Ad5 E1B-specific CTLs after incubation with the DC line D1, as visualized by intracellular IFN-γ staining. The control CTL line LN5 is not activated by any of the gp96 preparations tested (Fig. 7). More importantly, we are able to show here for the first time that receptor-mediated endocytosis of gp96 is indeed required for the re-presentation and subsequent activation of CTLs. By inhibiting the specific binding of RMA-S SigE1B-derived gp96 with a 2-fold excess of unrelated gp96 molecules that have been shown to reduce gp96-FITC binding by 60 % (Fig. 1D), we completely abolish the activation of Ad5 E1B specific CTLs (Fig. 5). This low excess of unrelated gp96 was chosen on purpose to exclude potential toxic effects of a high gp96 concentration. Using synthetic E1b peptide approx. 25 % of CTLs could be activated (data not shown) compared to 6-7% activated CTLs as shown in Fig. 7. Therefore, the amount of RMA-S SigE1b gp96 was not able to activate all possible CTLs, most likely because of limiting amounts of peptide. As the activation of CTLs requires the activating signal to be above a certain threshold the amount of antigen presented by MHC class I molecules in the presence of competitor could easily be below this threshold explaining the lack of a CTL response with a 2-fold excess of irrelevant gp96 not associated with E1b peptide.

Because only receptor-mediated endocytosis of labelled gp96, but not unspecific, nonreceptor-mediated uptake such as pinocytosis or macropinocytosis, can be inhibited by an excess of unlabelled gp96 (33), our results clearly demonstrate that receptor-mediated endocytosis of gp96 molecules is the cellular pathway responsible for re-presentation of gp96-associated peptides by MHC class I molecules. Therefore, our results provide evidence for the hypothesis that professional APCs possess receptors that are able to interact specifically with HSPs (32) and direct HSP-associated peptides into the MHC class I-restricted antigen presentation pathway. This now explains why very small amounts of gp96/peptide complexes can activate T cells.

The exact intracellular pathway for the re-presentation of gp96-associated peptides requires further clarification. Confocal microscopy data point into the direction that gp96 heads for early endosomes but does not enter lysosomes. We could show that gp96 after receptor-mediated uptake enters compartments containing MHC class I and class II molecules. It can be speculated that these compartments function as putative loading compartments where antigen could be transferred to MHC class I and class II molecules (46) but it cannot be excluded that gp96-antigen-complexes enter the cytosol specifically, as recently suggested for immunoglobulin-antigen complexes (ICs) after endocytosis by Fc receptors in dendritic cells (50).

Further identification of the pathway responsible for the re-presentation of gp96-associated peptides will also contribute to the understanding of the phenomenon termed cross-presentation. Until now, cross-presentation of MHC class I-restricted antigens has been shown to be induced by receptor-mediated phagocytosis of apoptotic bodies (27, 28), exosomes (51), bacteria (52) and proteins, either denatured or immobilized (26), by phagocytic or nonphagocytic mechanisms (22). Unlike the latter two pathways, which require in most cases high concentrations of the antigens, receptor-mediated endocytosis of HSPs operates efficiently at antigen concentrations around 1-2 ng per mouse (31) and might be as efficient as receptor-mediated phagocytosis of apoptotic cells or receptor-mediated endocytosis of proteins by surface-immunoglobulins on B cells. One can envisage that HSPs, released from dying cells, bind to HSP receptors of professional APCs and are endocytosed before the associated peptides are re-presented by MHC class I molecules.

The antigen carriers in apoptotic cells or exosomes are unknown but one interesting possibility is that HSPs chaperone the antigenic peptides, thus protecting them from further degradation and directing them to the correct intracellular loading compartment. In line with

this speculation is the observation that HSP70 is one of the proteins found in close association with the transferrin receptor in exosomes derived from reticulocytes (53). Whether or not the induction of apoptosis leads to a general increase of HSPs is still controversial and might depend on factors that are still to be determined. For tumor cells, it was reported that apoptotic death was associated with low HSP expression levels (54), whereas for polymorphonuclear leukocytes, increased apoptosis coincided with induction of Hsp72 (55). Nevertheless, an increase of HSP expression levels generally seems to correlate with increased immunogenicity (54, 56), supporting the above mentioned hypothesis.

The finding that cells deficient in the transporter associated with antigen processing (TAP) are still able to cross-prime as efficiently as wild-type cells (57) does not contradict the involvement of HSPs in cross-presentation. It shows that the endoplasmic reticulum ER-resident HSP gp96 alone is not essential for cross-priming, but it also does not exclude the participation of other HSPs such as HSP70 or HSP90 that might compensate for the absence of immunogenic gp96/peptide complexes. Another argument formulated against the participation of HSPs in cross-presentation of cellular antigens is based on an experiment performed by Bevan and Carbone (58), in which splenocytes were incubated with ovalbumin or β-galactosidase, washed and injected into mice. Because of the non-specific coating of cells with the soluble proteins, an association with HSPs might be difficult to imagine. The incubation conditions (37°C, 10 mg/ml protein, 10 min.), however, do not exclude the pinocytic or phagocytic uptake of the proteins by splenocytes, which process and release antigens, possibly even associated with HSPs. In addition, several different pathways for cross-presentation, including apoptotic cells, exosomes and receptor-mediated endocytosis of HSPs, might exist in parallel, each one able to induce the cross-presentation of different types of antigens.

More detailed knowledge about the gp96 receptor, its intracellular transport and the regulation of expression in different cell types will deepen our understanding of the role of gp96 and possibly HSPs in general in cross-presentation and could greatly improve the application of gp96 for the induction of specific immune responses in vivo.

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## Chapter 4

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# The heat shock protein Gp96 induces maturation of dendritic cells and down-regulation of its receptor

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Peptides associated with the heat shock protein gp96 induce a specific T cell response against cells from which gp96 is isolated. Recently, we have shown that gp96 binds to a yet unknown receptor present on dendritic cells (DCs) and that receptor-mediated uptake is required for cross-presentation of gp96-associated peptides by DCs. We now describe that gp96 mediates maturation of DCs as determined by up-regulation of MHC class II and CD86 molecules, secretion of the cytokines IL-12 and TNF-$\alpha$ and enhanced T-cell stimulatory capacity. Heated, and therefore denatured gp96 is not able to induce DC maturation and cytokine secretion. Furthermore, we show that mature DCs are no longer able to bind gp96 molecules. Hence, the gp96 receptor is down-regulated on mature DCs suggesting that this receptor behaves similar to other receptors involved in antigen uptake like the scavenger receptor CD36, the mannose receptor or the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$.

Alltogether, our findings provide an additional explanation for the remarkable immunogenicity of gp96 as a cross-priming antigen carrier and direct activator of dendritic cells.

## 1 Introduction

Dendritic cells are very effective activators of T cells. Immature DCs are experts in antigen acquisition, whereas mature DC are specialized in T cell activation most likely caused by increased expression of MHC and co-stimulatory molecules, like CD86. DC maturation can be induced by multiple stimuli including LPS, bacteria and viruses, CpG oligonucleotides and signaling molecules, like CD40L [1-5]. Especially the ability to present exogenous antigens through a process called 'cross-presentation' is a key feature of DCs. These exogenous antigens include proteins, bacteria and apoptotic cells [6]. In addition, we have shown recently that also peptides chaperoned by heat shock proteins (HSPs), like gp96 can be represented by APCs in the context of MHC class I molecules to CTLs. The representation requires the uptake of gp96 via a so far unidentified receptor expressed by DCs [7;8].

Heat shock proteins, like HSP60 and HSP70, also induce the activation of monocytes and the secretion of the pro-inflammatory cytokines TNF-α and IL-12 via interaction with CD14 molecules [9-11]. Thus, HSPs may not only serve as a vehicle for antigenic peptides recognized by T cells but also as a danger signal to the innate immune system when released from stressed cells, as postulated [12]. In line with this is the finding that increased HSP70 expression in tumor cells, induced by non-apoptotic cell death or transfection, resulted in enhanced tumor immunogenicity [13] via a T-cell mediated pathway [14].

Among all HSPs analyzed, the ER-resident heat shock protein gp96 has the best documented history with respect to the induction of specific CTL responses and tumor protection [15;16]. These features were attributed to the fact that gp96 is associated with peptides derived from intracellular proteins which are efficiently represented on MHC class I molecules on DCs after receptor-mediated endocytosis of gp96 [8;15]. Nonspecific phagocytosis did not result in CTL activation [8]. However, recently it has been demonstrated that for activation of CTLs from their naive precursors immunogenic peptides need to be presented by DCs that display their full potential of co-stimulatory molecules. Given these insights into the requirements for T-cell priming we tested the effect of gp96 on DC maturation and T cell activation. We found that gp96-treated immature DCs secrete TNF-α and IL-12 and convert to the mature phenotype, expressing increased levels of CD86, MHC class II and CD83 molecules in the case of human DCs. This change in phenotype has functional consequences which are visualized by the increase in activation of allogenic T cells. Interestingly, after maturation

DCs loose their capacity to bind exogenous gp96. This observation is well in line with the reduced abilities of mature DCs to acquire antigen [17].

## 2 Results

### 2.1 Gp96 induces maturation of human DCs

To study the effect of gp96 on phenotypical changes of DCs, immature DCs were generated through incubation of human PBMCs with GM-CSF and IL-4 for 7 days. For additional 24 h gp96 or LPS as a positive control were added to the cultures. As shown in Figure 1, gp96 as well as LPS induced maturation of DCs, now displaying increased levels of CD83 and CD86 on their surface. Denaturation of gp96 by heat destroyed its ability to activate DCs, whereas LPS was not affected by this treatment. These latter observations strongly argue for gp96-mediated DC activation not being a consequence of endotoxin contamination but the result of binding of native gp96 to its receptor [7;8]. This was further supported by the finding that the gp96-flanking fractions from the FPLC purification (lacking gp96) did not induce DC maturation and that normal medium and gp96 did not differ in their endotoxin content as measured by the *Limulus* amebocyte assay kit (data not shown). In addition, gp96 was purified from a cell line not infected with mycoplasma. This is important to note because it has been shown recently that supernatant from mycoplasma-infected cells is able to induce DC maturation [18]. BSA and Concanavalin A added as control proteins in similar amounts as gp96 did not activate DCs (data not shown).

### 2.2 Gp96 induces maturation of mouse DCs

A comparable gp96-mediated activation was also obtained using mouse bone-marrow derived DCs. Incubation of immature DCs with gp96 at different concentrations induced a heat-labile maturation of DCs, as visualized by increased expression of CD86 molecules (Figure 2A) and MHC class II molecules (data not shown). In addition to cell surface expression of maturation markers, gp96 also induces the secretion of pro-inflammatory cytokines IL-12 and TNF-α (Figure 2B). Again, the effect is heat-sensitive and not due to endotoxin contaminations possibly present in our gp96 preparations.

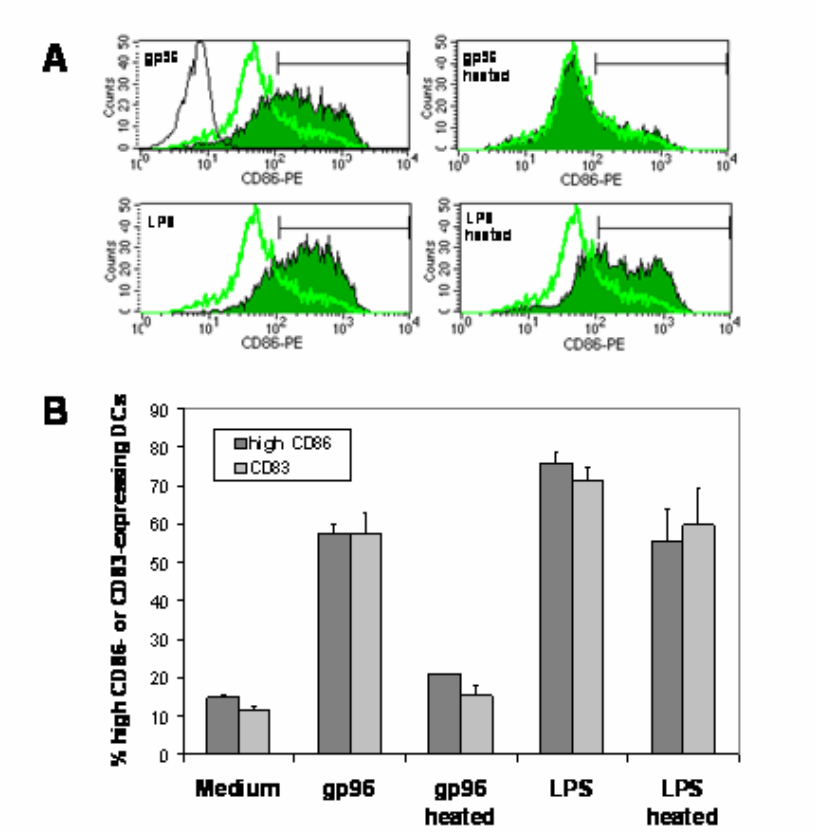


**Fig. 1: gp96 activates human dendritic cells.**
Human dendritic cells were prepared *in vitro* from $CD14^+$ PBMCs with GM-CSF and IL-4 after 7 days and incubated with gp96, heat-treated gp96, LPS or heat-treated LPS for 24 h. **A**: CD86 expression levels of DCs treated with gp96/LPS (filled histogram) or non-treated DCs (in grey; black line represents isotype control antibody which showed same fluorescence intensity for all treatments). **B** shows the percentage of high CD86 (as indicated by marker bar in A) and activation marker CD83 expressing DCs after treatment with the different effector molecules. Error bars give standard deviation. The results are representative of three independent experiments.

## 2.3 Gp96-activated DCs induce strong T cell proliferation

To investigate whether the gp96-mediated DC maturation has functional consequences, DCs matured by gp96 or LPS were incubated with allogenic PBMCs for 4 days and cell proliferation was determined by incubation with $^3$H-thymidin. As shown in Figure 3, DCs displaying a mature phenotype either after LPS or gp96 activation for 24 h, induced 3-fold better T cell proliferation than immature DCs incubated with medium only. As observed before, the gp96-mediated effect is heat-sensitive because DCs incubated with heated gp96 did not display an enhanced T cell stimulatory capacity. Comparable T cell proliferation was observed using mouse DCs and allogenic splenocytes (data not shown).

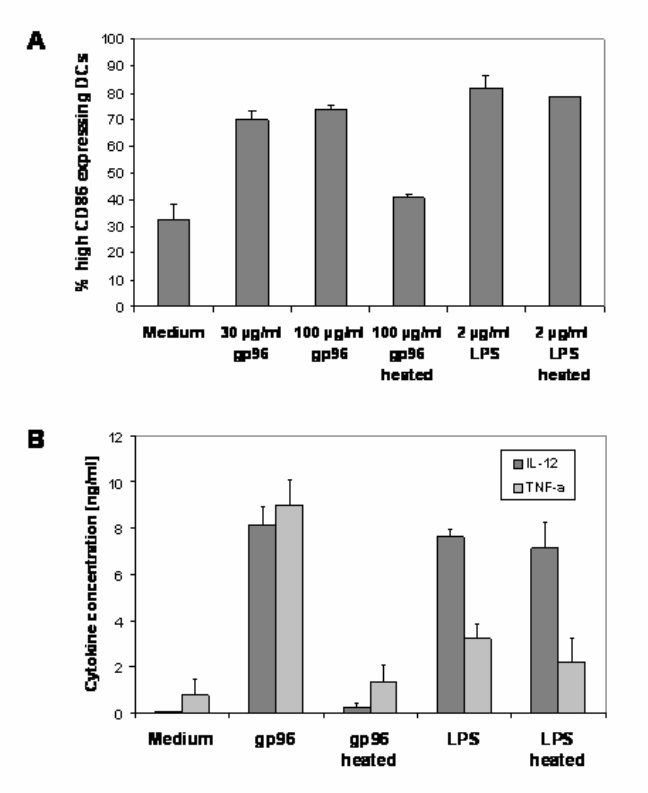


**Fig. 2: gp96 activates mouse dendritic cells.**
Mouse dendritic cells were prepared from bone marrow of C57BL/6 or BALB/c mice with GM-CSF after 7 days. **A**: Treatment with 30 and 100 µg/ml gp96 and 2 µg/ml LPS and heat-treated LPS after 24 h led to an up-regulation of CD86 (as measured by FACS double staining with CD11c and CD86 antibodies) while heat-treated gp96 did not activate DCs. **B**: Supernatants of the experiment above were analysed by ELISA for contents of the cytokines IL-12 and TNF-α showing similar results as in A. Error bars show standard deviation of triplicates. The results are representative of at least three independent experiments.

## 2.4 Mature DCs express reduced levels of the gp96 receptor

Maturation of DCs induces upregulation of MHC class II, CD83 and CD86 molecules, resulting in increased T cell proliferation. In addition, once activated, the DCs are unable to further receive gp96-mediated stimuli. As shown in Figure 4, all CD11c positive mouse DCs bind gp96 but not ovalbumin. However, only immature DCs, expressing low levels of CD86, are able to bind gp96. As gp96 is complexed with peptides from the cell it has been isolated from [15;16] and DCs are able to cross-present these peptides on MHC class I molecules [8], mature DCs can be expected to no longer be able to present gp96-associated peptides to T cells.

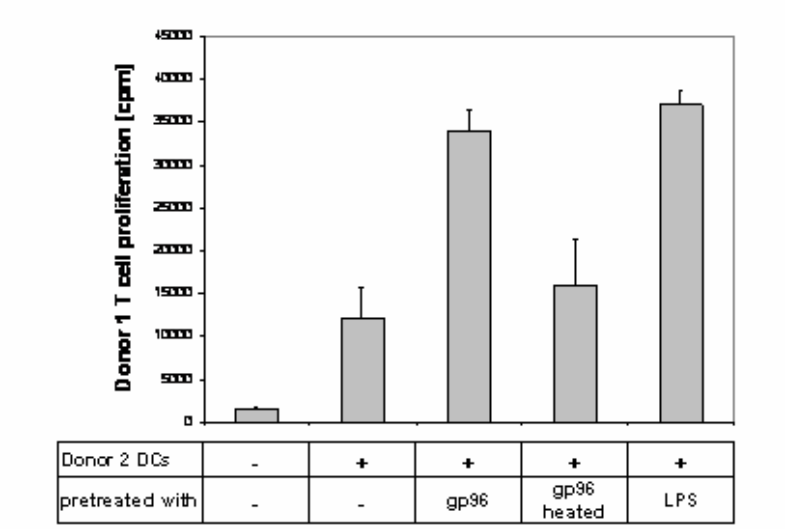


**Fig. 3: Human and mouse dendritic cells activated by gp96 are able to induce strong proliferation of alloreactive T cells.**
Human DCs were prepared and treated with 30 µg/ml gp96, heat-treated gp96 or 2 µg/ml LPS for 24 h as described above. After extensive washing, these pre-treated DCs were incubated with $10^5$ PBMCs of a different donor for 4 days in different stimulator/responder ratios (shown is ratio 1:30). Proliferation of T cells was assayed by addition of 1 µCi $^3$H-Thymidin for 16 h. Error bars give standard deviation of triplicates. Results are representative of two independent experiments. Similar results were obtained for mouse BALB/c DCs inducing proliferation of C57BL/6 T cells (data not shown).

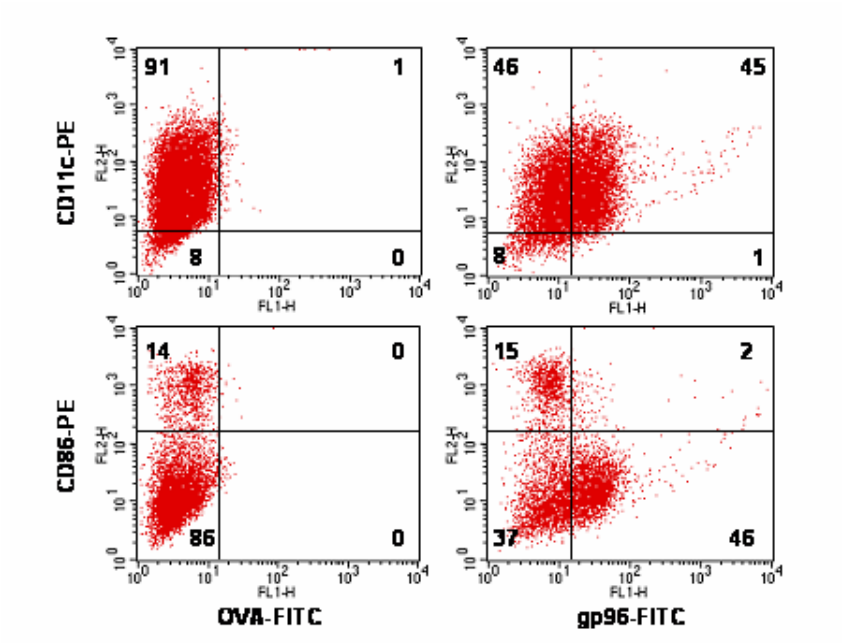


**Fig. 4: Activated DCs down-regulate the gp96 receptor.**
Bone-marrow derived DCs from C57BL/6 mice show a heterogenous population of activated (high CD86 positive on FL-2) and non-activated (low CD86 positive) CD11c-positive DCs. OVA-FITC as control protein did not bind at all, while gp96-FITC bound only to non-activated DCs (lower panels). The upper panels show CD11c expression of the DC preparation and binding of gp96-FITC. The values give percentage of total cells in the specified quadrant. Results are representative of three different experiments.

## 3 Concluding remarks

Our experiments show that the ER-resident heat shock protein gp96 is able to induce maturation of mouse and human DCs. This observation is especially remarkable in the light of previous findings where gp96 has been shown to specifically bind to DCs resulting in MHC class I-restricted cross-presentation of gp96-associated peptides [7;8]. The finding of down-regulation of the gp96 receptor on the surface of mature DCs also allows the first speculations on the nature of the gp96 receptor expressed on DCs. Possible candidates are endocytic receptors like the scavenger receptor CD36 or the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, all of which have been shown to be down-regulated upon DC maturation [19].

We provide here the first evidence that gp96 is not only a peptide carrier directing the associated peptides to professional APCs but also a direct activator of DCs, inducing a conversion to the mature phenotype highly efficient in T cell activation. Gp96 might therefore act as a danger signal when released from necrotic or stressed cells delivering both unspecific and specific stimuli to the immune system.

Once activated, the DCs loose the capacity to acquire new gp96-associated peptides and gain the ability to communicate efficiently with T cells specific for the presented MHC/peptide complexes. This situation resembles closely what has been observed initially for the presentation of soluble antigens on MHC class II molecules [20], where it has been described that DCs are 'locked' in a state of antigen presentation and highly efficient in the activation of T cells.

This new feature of gp96 provides an additional, so far unknown, explanation for its high immunogenicity and will allow to improve its application in the induction of specific immune responses *in vivo*.

## 4 Maerials & Methods

### 4.1 Generation of dendritic cells

The medium used throughout was Iscove's Modified Dulbecco's Medium (IMDM, BioWhittaker, Verviers, Belgium) supplemented with 2 mM L-glutamine (GibcoBRL Life Technologies, Paisley, UK), 100 IU/ml Penicillin/Streptomycin (Gibco), 10% FCS (PAA, Linz, Austria) and cytokines as indicated below. Mouse immature DCs were generated from

bone-marrow of C57BL/6 or BALB/c mice according to [21]. Briefly, bone marrow cells were incubated with 150 U/ml GM-CSF (PeproTech, London, UK) for 6-8 days renewing medium every two days. Approx. 90-100 % of all cells in the FACS® gate used for monocytes were DCs as determined by flow cytometry with antibodies (obtained from Pharmingen, San Diego, CA): they were CD11c-, CD86- and MHC class II-positive and CD14-negative. Human immature dendritic cells were prepared from PBMCs according to [22]. Briefly, $CD14^+$ monocytes were purified by 1 h adherence to culture dishes and extensive washing; monocytes were incubated with 1000 IU/ml IL-4 and 800 IU/ml GM-CSF for 6-8 days renewing cytokines every three days. The cells generated in this way showed a large number of dendrites up to day 12 and were only lightly adherent. They expressed CD1a, low CD14, CD86, HLA-DR and very low CD83 on their surface as determined by antibodies (Pharmingen).

**4.2 Stimulation of dendritic cells**

Mouse and human DCs were stimulated by addition of 30 to 100 µg/ml gp96 or 2 µg/ml LPS (from *Salmonella typhimurium*, Sigma Chemicals, St. Louis, MO) for 24 h. In some cases gp96 or LPS were pre-treated at 95°C for at least 20 min. Gp96 (kindly provided by Immunosome, Tübingen) was purified from mycoplasma-free IGELa2 mouse cell line as described [23]. FPLC fractions preceeding and following fractions not containing gp96 according to Western blot are referred to as 'flanking fractions' (provided by Immunosome, Tübingen). Endotoxin that might be present in gp96 preparations was tested by a Limulus Amebocyte Lysate Kit (QCL-1000, BioWhittaker) according to the guidelines published by the US Food and Drug Administration (FDA). The endotoxin content determined in all cases was at or below 0.05 EU/µg gp96. For detection of possible mycoplasma contaminations of the IGELa2 cell line and gp96 FPLC fractions the Mycoplasma Plus™ Kit by Stratagene, La Jolla, CA was used.

**4.3 Cytokine detection**

Mouse IL-12 (p40) and TNF-α were measured in culture supernatants using standard sandwich ELISA protocols. Antibodies and recombinant standards of both cytokines were obtained from Pharmingen. The capture antibody was bound to the ELISA plate (MaxiSorb™, Nunc, Roskilde, Denmark), the biotinylated detection antibody was recognized

by streptavidin-conjugated horse-radish peroxidase and detected by ABTS substrate (Sigma) emitting at 415 nm.

### 4.4 Stimulation of alloreactive T cells

Human or BALB/c DCs were stimulated in a 96-well plate as described above, washed extensively and incubated with PBL from a different donor or C57BL/6 spleen cells, respectively, for 4 days at different responder/stimulator ratios. On day 4, 1 μCi of $^{3}$H-Thymidin was added per well, cells were harvested after additional 16 h and incorporated $^{3}$H-Thymidin was detected using a Wallac 1450 MicroBetacounter.

### 4.5 FACS® analysis

Cell surface staining was performed using antibodies as mentioned above, Ovalbumin-FITC or gp96-FITC (kindly provided by Immunosome, Tübingen) which were incubated with cells for 30 min on ice in IMDM containing 10% FCS. Dead cells were excluded by PI staining. All FACS® analysis was performed on a FACSCalibur® (Becton Dickinson, Mountain View, CA) using Cell Quest Software.

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# Chapter 5

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## The heat shock protein Gp96 – a receptor-targeted cross-priming carrier and activator of dendritic cells

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Heat shock proteins like gp96 (grp94) are able to induce specific cytotoxic T-cell (CTL) responses against cells from which they originate and are currently studied in clinical trials for use in immunotherapy of tumors. We have recently demonstrated that gp96 binds to at least one yet unidentified receptor restricted to antigen-presenting cells (APCs) like dendritic cells (DCs) but not to T cells. Moreover we have shown, that for CTL activation by gp96-chaperoned peptides receptor-mediated uptake of gp96 by APCs is required.

Lately, we have discovered a second function of gp96 when interacting with professional APCs. gp96 is able to mediate maturation of DCs as determined by up-regulation of MHC class II, CD86 and CD83 molecules, secretion of pro-inflammatory cytokines IL-12 and TNF-α and enhanced T-cell stimulatory capacity. Furthermore, the gp96 receptor(s) are down-regulated on mature DCs, suggesting that the gp96 receptor(s) behave similar to other endocytic receptors like CD36, mannose receptor etc. Our findings now provide additional evidence for the remarkable immunogenicity of gp96: first, the existence of specific gp96 receptors on APCs and second, the capacity to activate dendritic cells which is strictly required to enable these highly sophisticated APCs to prime CTL responses.

In order to activate naive T cells the adaptive immune system has evolved a very specialized and powerful tool, the antigen presenting cell (APC). APCs do not only present peptide ligands bound to their cell surface major histocompatibility complex (MHC) molecules, they are also equipped with a large set of co-stimulatory molecules such as CD80, CD86 and CD40 molecules. Both, the recognition of MHC/peptide complexes and co-stimulators, are required for the activation of naive T cells. The ability of the APC to carefully regulate the cell surface expression levels of co-stimulatory as well as MHC molecules puts these sophisticated cells into the position of most important regulators of the immune system's T cell limb.

## Antigen processing – the dichotomy of MHC class I and II pathways

Pivotal to antigen presentation is the display of peptides in the context of MHC class I and II molecules which allow specific recognition by the T cell receptor (TCR). The antigen processing apparatus of the APC can be grouped into two distinct pathways involving the degradation of protein antigens into short peptide fragments finally embedded in the polymorphic binding domains of the MHC molecules. For MHC class I molecules, cytosolic antigen is degraded by the machinery of 20S and 26S proteasome. Both multi-protein complexes generate sets of peptides (Emmerich et al 2000) which in many cases already have the right C terminus to bind to their correspondent MHC class I molecule (Stoltze et al 2000). These peptides are then transported to the endoplasmic reticulum (ER) via the ER membrane-resident transporter associated with antigen presentation (TAP) 1 and 2 molecules. The lumen of the ER provides a specialized environment allowing these peptides to be finally – presumably N-terminally – processed and associated to MHC class I molecules. MHC class I-peptide complexes are then shuttled towards the cell surface via the secretory pathway for recognition by $CD8^+$ T cells (for review, see Rock and Goldberg 1999). On the other hand, MHC class II molecules associate with longer peptides generated by exopeptidases in the acidic milieu of endosomal compartments. The resulting MHC class II-peptide complexes are exported to the cell surface where they can be recognized by TCRs of $CD4^+$ T cells (for review, see Watts 1997). In most cases peptides associated to MHC class I are derived from intracellular, ligands of MHC class II from extracellular proteins.

## Heat Shock Proteins as intra- and extracellular peptide carriers

Molecular Chaperones such as heat shock proteins (HSPs) are peptide-binding molecules which facilitate folding and refolding of proteins by preventing their aggregation and stabilizing high energy folding intermediates. Recently, it has been shown by several groups that chaperones as well as co-chaperones can be associated with pathways of antigen processing at various points (Lammert et al 1997; Spee and Neefjes 1997; Panjwani et al 1999; Luders et al 2000). It seems to be an attractive idea that HSPs are involved in the shuttling of peptides between the different antigen processing compartments inside the cell (Srivastava et al 1994). Moreover, once the heat shock proteins are released from cells by necrosis or possibly even secretion, they can function as peptide carriers in an even wider sense – from cell to cell. This extraordinary function of HSPs was discovered by purification of HSP-peptide complexes and their use in eliciting $CD8^+$ cytotoxic T cell responses. In pioneering experiments, Srivastava and co-workers demonstrated that the heat shock proteins gp96 (Grp94), Hsc70 (the constitutive form of the Hsp70 protein) and Hsp90 are able to confer immunity against autologous tumor preparations from which these HSPs had been isolated before (reviewed by Srivastava et al 1998; Schild et al 1999).

## Cross-presentation – breaking the classical MHC class I/II dichotomy

It was established, that HSP-mediated immunity requires the presence of APCs (Udono et al 1994; Suto and Srivastava 1995) in line with the idea that HSP-peptide complexes are taken up by APCs which in a subsequent step elicit a CTL response. Hence, peptides which were associated to HSPs are transferred to MHC class I molecules for recognition by $CD8^+$ T cells. Specific activation of CTLs is independent from the MHC haplotype of the cells from which the HSP-peptide complexes originate (Arnold et al 1995). This phenomenon of cross-priming was originally discovered by Bevan (Bevan 1976). Today, the term cross-presentation is used as a more general description for the re-presentation of exogenously derived, cell-associated antigens (Carbone et al 1998).

The phenomenon of cross-presentation requires the existence of processing pathways distinct from the classical dichotomy in antigen processing from endogenous and exogenous origin. Several pathways for presentation of *exogenous* antigen on MHC class I molecules have been described (reviewed by Rock 1996; Jondal et al 1996) which can be put into two fundamentally different processing groups: one involving transport of antigen from endosomes into the cytosol and then further processing similar to the classical MHC class I

processing pathway in a proteasome- and TAP-dependent fashion. In the other pathway recycled MHC class I molecules are directed to endosomes similar to processing of MHC class II molecules.

## The dendritic cell – well equipped for cross-priming

The nature of the APC possessing cross-priming abilities is still not completely unveiled. However, accumulating evidence suggests that bone-marrow derived dendritic cells (DCs) are the only cells that are capable of efficiently stimulating resting, naive T cells and inducing CTL responses *in vivo* (reviewed by Banchereau and Steinman 1998; Banchereau et al 2000). Immature DCs usually reside in non-lymphoid tissue such as skin, where they are specialized in endocytosis and antigen processing. To use these antigens for T cell stimulation the DC must undergo a differentiation process called maturation or activation. Several stimuli such as pathogens, microbial products like lipopolysaccharid (LPS) and tissue damage induce their initial maturation and migration to the T cell areas of the secondary lymphoid organs (Cyster 1999). Mature DCs lose their capability to efficiently capture and process antigen while becoming highly specialized in antigen presentation. MHC class II molecules – now loaded with antigen from the endocytosed material – are forced to move to the cell surface for presentation to $CD4^+$ T helper cells (Cella et al 1997; Pierre et al 1997). Besides MHC molecules, co-stimulatory molecules such as CD40, CD80 and CD86 are up-regulated (Inaba et al 1994; Caux et al 1994) which deliver a second signal to $CD4^+$ T helper cells recognizing antigen on MHC class II molecules. In turn the T cell can further activate the DC via CD40 ligand (CD40L)-CD40 interactions (Ridge et al 1998; Schoenberger et al 1998). Such a fully activated DC is able to secrete cytokines like IL-12 to instruct T helper cells to differentiate along the Th1 or Th2 pathway (Macatonia et al 1995; Cella et al 1996; Koch et al 1996). More importantly, activated DCs are capable of cross-priming naive $CD8^+$ T cells to effector CTLs (Heath and Carbone 1999).

The unique position of dendritic cells among APCs has been confirmed by various *in vitro* antigen re-presentation systems. Bhardwaj and collegues were able to show that apoptotic cells can be endocytosed by macrophages as well a DCs via CD36, but only DCs, which co-express the $\alpha_v\beta_5$ integrins, can stimulate T cells with antigen derived from the apoptotic vesicles (Albert et al 1998a; Albert et al 1998b). Amigorena and co-workers have demonstrated that uptake of ovalbumin (OVA)-immune complexes by Fcγ receptors on the surface of APCs only leads to re-presentation of an OVA epitope on MHC class I molecules

in dendritic cells but not in macrophages or B cells (Rodriguez et al 1999; Amigorena and Bonnerot 1999). Soluble antigen could also be cross-presented but had to be present at significantly higher concentrations in the surrounding medium. Interestingly, the latter group discovered a transport route of the antigen from endosomes to the cytosol specific for dendritic cells. It seems possible that DCs possess either special endosomal proteins facilitating this antigen transfer, or a subset of specialized translocator endosomes. In both cases - apoptotic bodies and immune complexes - DCs use receptors to internalize antigen for cross-presentation. The advantage of receptor-mediated endocytosis does not only lie in a much more efficient and specific uptake in comparison to unspecific means such as macropinocytosis. Receptors might also shuttle the antigen into the right pathway for antigen presentation.

Because immunization and cross-priming in mice with gp96-peptide complexes works very effectively even in the nanomolar range, Srivastava and coworkers have speculated a few years ago, that gp96 and other heat shock proteins might be taken up by receptors found on the surface of professional APCs (Srivastava et al 1994). The existence of such HSP-specific receptors would also account for the cross-priming abilities of heat shock proteins like gp96. For instance, the ER chaperone Proteindisulfideisomerase (PDI) displays much better peptide binding capacities than gp96 as demonstrated by TAP translocation and cross-linking experiments (Lammert et al 1997), but is not able to induce specific CTL responses (unpublished observation) – possibly because APCs do not possess receptors for PDI.

## Evidence for existence of one or more gp96 receptors on APCs

First indications for specific interactions of HSPs with APCs were available from electron microscope studies (Arnold-Schild et al 1999). Gold-labelled gp96 and Hsc70 (the constitutive form of Hsp70) were found to bind to macrophage and monocytic cell lines. Both HSPs were specifically localized in clathrin-coated pits on the cell surface, indicating receptor-mediated binding. This binding was not observed on fibroblasts. Using a large excess of unlabeled gp96 or Hsc70, binding of gold-labeled HSPs to clathrin-coated plasma membrane regions could be blocked. Later, Nicchitta and co-workers were also able to demonstrate binding of FITC-labeled gp96 molecules to peritoneal macrophages but not to COS or CHO cells (Wassenberg et al 1999). However, in this latter work saturation and competition experiments were not shown.

There are several criteria for specific, receptor-mediated binding of molecules to cells. The binding must be saturable, as the number of receptors per cell is finite. Furthermore, the binding of the labeled molecule should be blocked by the same unlabeled molecule and this competition should be titratable showing a function corresponding to saturation. Blocking of binding of the labeled molecules should already work at low concentrations of unlabeled competitor. Theoretically, if the labeled molecule is added to the cells in saturating concentration, a 1:1 ratio of labeled/unlabeled protein should reduce the binding of the labeled protein by 50%. In many cases such optimal blocking is rarely seen because of additional unspecific binding of the labeled molecule. The fraction of unspecific binding is largely dependent on the binding buffer and the quality of the protein and its labeling.

In our case FITC-labeled gp96 molecules (kindly provided by Immatics Biotechnologies) were bound to bone-marrow derived mouse dendritic cells. Saturation of binding was reached at 30 to 50 μg/ml gp96. At the latter concentration 45% inhibition of binding was reached with a 1:1 ratio of labeled versus unlabeled gp96 molecules. Binding of gp96-FITC was observed for mouse splenic and human blood monocytes and macrophages, B cells showed less but still significant binding. In no case was binding to T cells and NK cells observed, hence the expression of gp96 receptor(s) is restricted to antigen presenting cells (Singh-Jasuja et al 2000a).

Furthermore, we were interested to know whether or not PDI, which has very strong peptide binding abilities, would also bind to cells. Fig. 1 shows binding of gp96-FITC compared to PDI-FITC to D2SC/1 cells, a splenic DC precursor cell line. In no case binding of PDI-FITC was observed, indicating that a cell surface receptor does not exist for PDI. This might explain why PDI molecules cannot be used for immunization in mice (Lammert et al, unpublished observation; Nair et al 1999).

## Endocytosed gp96 colocalizes with recycled MHC class I molecules in dendritic cells

Confocal microscopy allows a high-resolution visualization of the binding of gp96-FITC to cells. Gp96-FITC molecules bound to bone-marrow derived mouse dendritic cells with a characteristic patched staining on the cell surface. We could demonstrate that receptor-bound gp96 – when chased at 37°C – is endocytosed into compartments containing MHC class I and II molecules (Singh-Jasuja et al 2000a). Fig. 2 shows binding on and endocytosis into D2SC/1 cells. After 15 min of endocytosis gp96 is found in early endosomes – visualized by

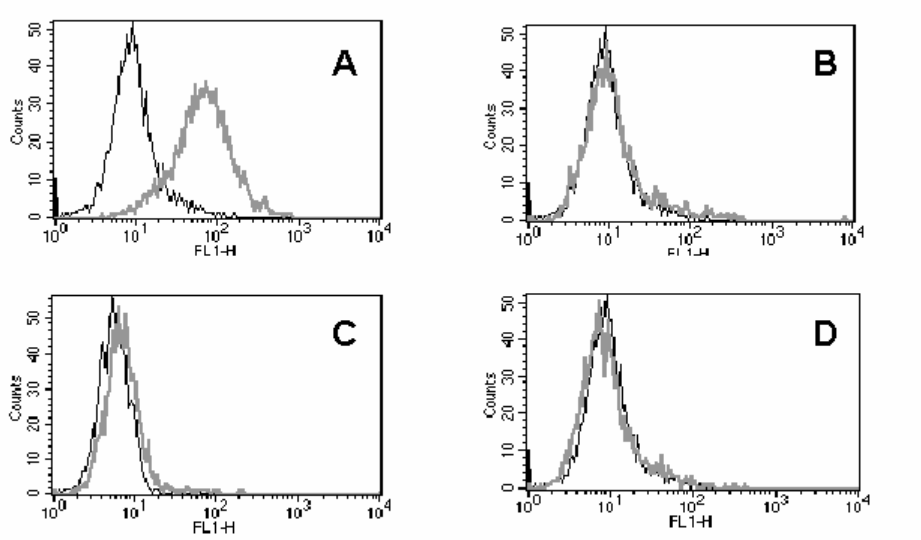


**Figure 1: The heat shock protein gp96 but not Proteindisulfideisomerase (PDI) binds to APC cell lines.**
Binding of 30 µg/ml FITC-labeled gp96 (grey line) compared to control protein OVA-FITC (black line) on the DC precursor cell line D2SC/1 at 4°C (**A**) and competition of this binding with an excess of 100fold unlabeled gp96 (**B**). gp96-FITC does not bind to the T cell line RMA (**C**). Up to 100 µg/ml PDI-FITC do not bind either to D2SC/1 (**D**) or RMA (not shown).

colocalization with endocytosed Transferrin – together with MHC class I molecules. However, no co-localization between gp96 and MHC class I molecules is observed on the surface. This suggests that peptide transfer from gp96 to MHC class I does not happen on the cell surface but requires endocytosis. Interestingly, it has been reported recently that internalized cell surface MHC class I molecules, like class II molecules, are able to bind their antigen in endosomal compartments, suggesting that these vesicles are indeed putative MHC class I and class II loading compartments for exogenous antigen.

Recently Germain and co-workers were able to demonstrate that Hsp70 is taken up by peritoneal macrophages in a receptor-dependent manner (Castellino et al 2000). Subsequently, antigen associated to Hsp70 is introduced into the MHC class I processing pathway via a endosomal *or* cytosolic route, depending on the sequence context of the antigenic peptide bound to the HSP. If the C-terminal anchor residue fits already for association to MHC class I molecules, an endosomal route can be taken. In the other case, the antigen has to be released into the cytosol and requires processing by the proteasome and translocation by TAP. Whether or not gp96 as well might transport its cargo to MHC molecules via distinct processing pathways, depending on further processing requirement of the associated peptides, is currently under investigation

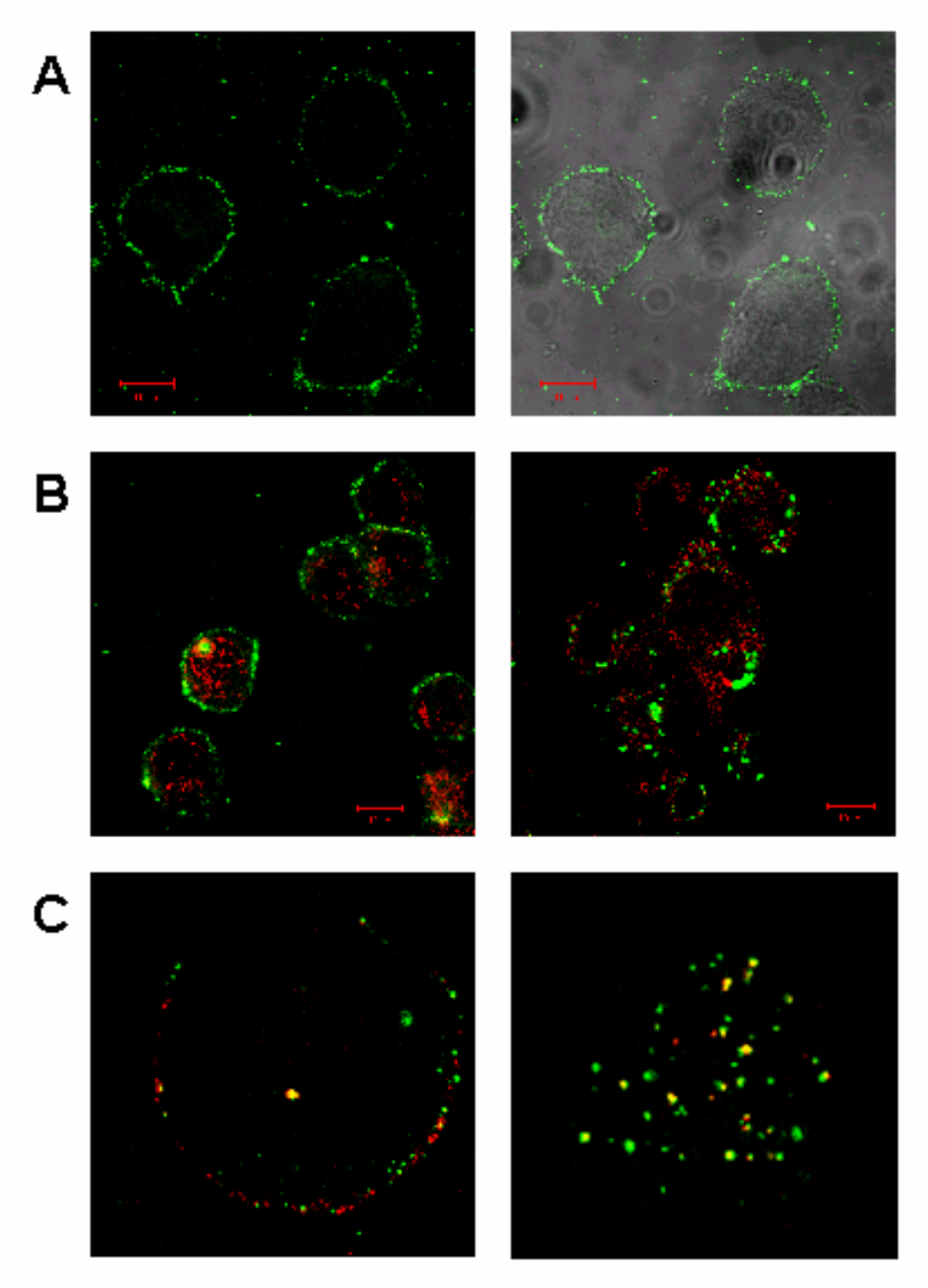


**Figure 2: Confocal micrographs of D2SC/1 cells.**
Binding of gp96-FITC to the surface of D2SC/1 cells at 4°C shown as single-channel picture (left panel of A) and overlap with transmission picture (right panel). B shows co-staining with lysosomes (left panel) and no co-localization with endocytosed gp96-FITC after 15 min treatment at 37°C. gp96-FITC does not co-localize with MHC class I molecules on the surface of D2SC/1 cells (left panel of C) but after 15 min of endocytosis (right panel) in early endosomes identified with Transferrin (not shown). gp96-FITC is diplayed in green, lysosomes (stained by α-Lamp1) and MHC class I molecules (stained by α-H2-$K^d$) in red, co-localization is shown in yellow.

### Cross-presentation of gp96-associated antigen on MHC class I molecules requires receptor-mediated endocytosis

To investigate whether unspecific uptake or receptor-mediated endocytosis of gp96 molecules leads to presentation of the associated peptides on MHC class I molecules, we purified gp96 from a RMA-S SigE1b cells, a cell line expressing the Adenovirus-5 E1b epitope (Toes et al 1995) exclusively in the ER. Such *in vivo* generated gp96-E1B complexes were able to elicit a CTL response in mice immunized with these complexes, demonstrating the association of E1B to gp96 molecules. Incubation of the mouse dendritic cell line D1 or bone-marrow derived DCs (unpublished data) together with gp96-E1b resulted in activation of E1B-specific CTL clones as measured by intracellular IFN-γ staining of CTLs. Gp96 associated with no or irrelevant peptides or a CTL clone recognizing the different E1A epitope were used as controls and showed no CTL activation in any case. Furthermore, the addition of excess irrelevant gp96 to gp96-E1B complexes was able to completely block activation (Singh-Jasuja et al 2000a). These results demonstrate that the gp96 receptor(s) on APCs are strictly required for cross-presentation of gp96-associated peptides on MHC class I molecules. Unspecific endocytosis, which cannot be blocked by low amounts of competitor gp96 molecules, is not able to do so. Similar results were also obtained by Germain and collegues (Castellino et al 2000). Antigenic precursor peptides associated to HSP70 *in vitro* were taken up by macrophages in a saturable fashion. They were re-presented on MHC class I molecules assayed by IL-2 secretion of CTLs specific for the epitope sequence embedded in the precursor peptide. As shown for gp96, these experiments, too, demonstrated blocking of cross-presentation by unloaded Hsp70 molecules as competitors.

### gp96 is able to induce maturation of dendritic cells

It has been shown recently that other HSPs like Hsp60 (Chen et al 1999; Kol et al 2000) and Hsp70 (Asea et al 2000; Moroi et al 2000) can induce the activation of monocytes via interaction with CD14. Thus, HSPs may not only serve as a vehicle for antigenic peptides but also as a danger signal to the innate and specific immune system as suggested for other endogenous activators of DCs (Matzinger 1998). Activated, mature dendritic cells display their full co-stimulatory potential and are able to communicate very effectively with naive T cells. Only activated DCs have the ability to prime CTL responses. Thus, we investigated whether gp96 was able to deliver a maturation signal to DCs.

Dendritic cells were derived *in vitro* from mouse bone marrow stem cells or $CD14^+$ monocytes from human blood by incubation with GM-CSF or GM-CSF and IL-4, respectively, according to existing protocols (Inaba et al 1992; Bender et al 1996).

After 12 h up to 10 ng/ml IL-12 and TNF-α had accumulated in the medium supernatant of the DCs activated with gp96 or LPS. Controls including boiled gp96 did not exceed 0.1 ng/ml IL-12 and 1 ng/ml TNF-α, while the activity of LPS could not be diminished by boiling. Thus, we were able to demonstrate that dendritic cells treated with gp96 were activated as shown by secretion of pro-inflammatory cytokines. This effect was not due to contaminations of the protein preparations by endotoxins such as lipopolysaccharid (LPS). Furthermore, 48 h after initial treatment of immature DCs, we observed up-regulation of co-stimulatory molecules like CD86 (B7.2), MHC class II molecules and, in the case of human DCs, CD83.

To test whether these mature DCs were also able to activate T cells better than immature DCs we incubated mouse and human DCs treated with gp96 for 48 h (as well as all controls mentioned above) with mouse or human T cells of different haplotype or donor, respectively, to observe the activation of allogeneic T cells. Proliferation of T cells was assayed after 5 days by incorporation of $^3H$ thymidine. Indeed, proliferation of T cells incubated with gp96- or LPS-activated DCs was two to three times higher than of T cells incubated with immature DCs (Singh-Jasuja et al 2000b). At the same time proliferation of activated DCs was substantially lower compared to non-activated DCs, confirming that activated DCs grow slower than immature DCs.

### Activated dendritic cells downregulate their gp96 receptor(s)

As shown previously, human and mouse dendritic cells are able to bind gp96 in a specific, receptor-mediated manner. Furthermore, receptor-mediated endocytosis of gp96 is required for activation of CTLs specific for gp96-associated antigen (Singh-Jasuja et al 2000a). We investigated whether the gp96 receptor(s), like other endocytic receptors, are down-regulated on mature DCs, which are specialized rather in presentation than uptake and processing of antigen (Albert et al 1998b). We found that activated mouse and human DCs expressing high levels of CD86 and in the case of human DCs expressing CD83 did no longer bind gp96. Hence, the gp96 receptor behaves similar to other endocytic receptors like the scavenger receptor CD36, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ and Fc receptors (Singh-Jasuja et al 2000b).

## Heat Shock Proteins as ideal cross-priming vehicles

These latest results provide some important information on the nature of gp96-mediated as well as inflammatory immunity in a more general way. Indeed, gp96 and other HSPs could provide one missing link to the question what triggers inflammation induced by necrosis. Necrotic cells resulting from tissue damage or viral infection release their contents, including gp96, into the extracellular fluid. Surrounding immature dendritic cells could take up gp96 and other HSPs in a receptor-mediated fashion for presentation of gp96-bound antigen on MHC class I molecules. At the same time gp96 would deliver a maturation signal enabling the DCs to up-regulate their co-stimulatory molecules like CD86. Presentation of antigen in the context of MHC class I molecules and co-stimulators together would lead to priming of $CD8^+$ T cells to cytotoxic effector cells and the induction of a pro-inflammatory cytokine response. In this model, recognition of antigen on MHC class II molecules by $CD4^+$ T cells is not required, as the activation signal is already delivered by the antigen carrier gp96 itself. The question regarding the requirement of $CD4^+$ T cell help in HSP immunization is still not clear; it has been shown that depletion of $CD4^+$ T cells in the effector but not in the priming phase abrogates gp96-mediated immunity (Udono et al 1994).

Until now, debates about the nature of the cross-priming antigen carrier have included apoptotic vesicles (Albert et al 1998a), heat shock proteins, immune complexes (Amigorena and Bonnerot 1998; Regnault et al 1999) and exosomes (Zitvogel et al 1998). Apoptotic cells, that occur rather frequently not only in embryonal development, were originally distinguished from necrotic cells on the basis of morphological differences and the ability of necrotic but not apoptotic cells to induce a pro-inflammatory cytokine response. While necrosis is associated with release of pro-inflammatory cytokines like TNF-α and IL-12 by APCs, apoptotic cells induce anti-inflammatory cytokines like TGF-β and IL-10 (Fadok et al 1998). Apoptotic bodies can be engulfed by dendritic cells; containing antigens can be effectively processed and cross-presented on MHC class I molecules to activate T cells. Uptake of apoptotic cells, however, does not result in activation of dendritic cells while necrotic cells do so (Sauter et al 2000). Hence, apoptotic cells – although shown to be effective antigen carriers for cross-*presentation* – do not trigger co-stimulator expression and would be, on their own, ineffective for cross-*priming*. On the contrary, presentation of antigen to T cells lacking co-stimulators rather inactivates T cells by inducing periferal tolerance (Steinman et al 2000).

From the current viewpoint cross-priming mediated by heat shock protein-antigen complexes seems to be more attractive than by apoptotic bodies. It looks like that heat shock

proteins and apoptotic cells, rather than being competitors in cross-priming, fulfill not only different but opposing tasks. Interestingly, in this model, both processes – cross-priming by gp96 and cross-tolerance by apoptotic cells – require receptors for their initiation. While the gp96 receptor is essential for cross-presentation of associated antigen (Singh-Jasuja et al 2000a), Fadok and colleagues have recently identified the phosphatidylserine (PS) receptor on bone-marrow derived macrophages to mediate the uptake of apoptotic cells (Fadok et al 2000). A few years ago, the same group was already able to show that late-stage apoptotic cells expose the lipid phosphatidylserin – which is normally localized to the inner leaflet of the plasma membrane – on the outer membrane-leaflet (Fadok et al 1992).

The heat shock protein gp96 has been described years ago by Srivastava and colleagues as an effective antigen carrier which has some potential in immunotherapy of tumors. Today, we are able to understand the nature of the immune response elicited by gp96 much better, as a number of attributes meanwhile have been associated with this multi-functional molecule: firstly, the existence of one or more gp96 receptors enabling the very efficient uptake of gp96 from the extracellular fluid. Secondly, the ability of gp96 to induce up-regulation of co-stimulatory activity and down-regulation of endocytic activity (such as the gp96 receptor) in dendritic cells make these cells ideally suited to prime T cell responses. Thirdly, the ability of gp96 to elicit the release of pro-inflammatory cytokines, hereby providing an effective danger signal also to the innate immune system, further supports the efficient induction of immune responses. And fourthly, recent observations (Hilf et al., unpublished data) point into the direction that gp96 can, by specifically binding to platelets, even activate these and might play an additional role in wound healing.

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## Chapter 6

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# The ER-resident heat shock protein Gp96 activates dendritic cells via the TLR2/4 pathway

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In this study, we have analysed the consequences of Gp96 interaction with cells expressing different toll-like receptors (TLR)[1] and with bone marrow-derived dendritic cells (BMDC) from mice lacking functional TLR2 and/or TLR4 molecules. We find that the Gp96-TLR2/4 interaction results in the activation of NF-κB-driven reporter genes, mitogen- and stress-activated protein kinases and induces IκBα degradation. BMDCs of C3H/HeJ and more pronounced C3H/HeJ / $TLR2^{-/-}$ mice fail to respond to Gp96. Interestingly, activation of bone marrow-derived dendritic cells depends on endocytosis of Gp96 molecules. Our results provide, for the first time, the molecular basis for understanding the Gp96-mediated activation of antigen presenting cells by describing the simultaneously stimulation of the innate and adaptive immune system. This feature explains the remarkable ability of Gp96 to induce specific immune responses against tumors and pathogens.

## Introduction

The immunogenic potential of heat shock proteins (HSPs) is a well established phenomenon, first observed by Srivastava and co-workers during the immunotherapy of mouse tumors (reviewed in [1]). Specificity of the immune response is based on peptides that associate with HSPs as a consequence of their function as molecular chaperones [2,3]. Tumor specific protection is mediated by $CD8^+$ T cells as shown by *in vivo* cell depletion studies [4] and by the ability to generate CTL lines specific for a variety of antigens from mice immunized with HSP molecules [5 6]. The HSPs that mediated this effect include the cytosolic Hsp70 and Hsp90 and the ER-resident chaperones calreticulin and Gp96 [7,8]. Furthermore, Gp96 molecules have been shown to induce CTL cross-priming against viral and minor histocompatibility antigens supporting the hypothesis that Gp96 molecules are associated with a large repertoire of peptides not influenced by the cellular MHC expression [6,9]. HSP molecules can also provide an immunogenic context to synthetic peptides complexed to Hsp70 or Gp96 molecules in vitro [10,11]. Because of this, HSP molecules have been called adjuvants of mammalian origin [12]. Recently, progress has been made to understand the mechanisms contributing to the efficient induction of immune responses against the HSP-associated peptides.

A receptor responsible for the uptake of HSP-peptide complexes has been identified as the $\alpha_2$-macroglobulin ($\alpha_2$m)-receptor CD91, expressed on professional antigen presenting cells (APCs) [8,13]. Only receptor-mediated endocytosis has been shown to result in the representation of HSP-associated peptides, thus explaning the high efficiency of this process [14]. However, the ideal adjuvant should not only target the antigen to professional APCs, it also should induce APC activation to provide the proper costimuli required for efficient induction of the immune response. For HSPs, especially Hsp70, Hsp90 and Gp96, this ability has been demonstrated recently. The exposure of macrophages or DCs to HSPs resulted in the upregulation of MHC class II and costimulatory molecules as well as in TNF-$\alpha$ and IL-12 secretion [15,16]. The contribution of this mechanism in situtations of physiological relevance is supported by the observation that necrotic but not apoptotic cell death leads to the release of HSPs [16,17], thus activating the innate arm of the immune system to attract cells equipped with antigen specific receptors.

The molecular basis for this process, however, has not been understood so far. Studies investigating the stimulatory effect of Hsp60 on epithelial cells and Hsp60 and Hsp70 on macrophages demonstrated the involvement of CD14 molecules, suggesting the participation

of toll-like receptors (TLR) [18]. In drosophila, toll participates, in addition to the induction (coordination) of dorsal-ventral patterning during embryogenesis, in the defence against fungi by the induction of drosomycin secretion as an early form of innate immune responses against infection [19]. In mammals, TLRs are involved in the response to pathogens by the recognition of so-called pathogen-associated molecular patterns (PAMPs). These include lipopolysaccharide (LPS), peptidoglycans, lipoproteins and bacterial CpG-DNA which are recognized by TLR4, TLR2 and TLR9, respectively [20,21]. The TLR signaling pathway shares most components with the IL-1 receptor (IL-1R) signaling pathway responsible for the activation of the innate immune system [20].

Recently, HSPs have been linked to TLRs by the observation that Hsp60 failed to activate TLR4 defective macrophages from C3H7HeJ mice [22]. Subsequently, it was shown that genetic complementation of non responder cells with TLR4 or TLR2 restores responsiveness (gain of function) to HSP60 while $TLR2^{-/-}$ or TLR4 deficient cells exhibit a "loss of function". Surprisingly, macrophage activation was equally well induced by bacterial as well as endogenous, mammalian Hsp60. Thus, the presence of molecular patterns that interact with members of the TLR family is not limited to pathogen-derived molecules. Because of this, Hsp60 has been proposed to serve as a danger signal for the innate immune system [18]. Hsp60 is not associated with antigenic peptides (Arnold-Schild and Schild, unpublished observation) and found much earlier in phylogeny, as is the TLR and IL-1R family. Therefore, it remains unclear if the HSP/TLR-pathway is solely used by the innate immune system to fight pathogens by unspecific mechanisms or if HSPs with peptide-binding ability, like Gp96, also use this mechanism to link nonspecific immunostimulatory capacities with their specific, peptide-based features with the activation of the adaptive immune system.

In this study, we analyzed the functional consequences of the interaction of Gp96 with members of the TLR family.

## Results

### Gp96 interacts with TLR2 and TLR4

Among the members of the TLR family, the ligands for TLR2, TLR4 and TLR9 are studied in considerable detail. Interestingly, Hsp60 has been shown to activate macrophages via TLR4[22]. Inspired by these observations, we investigated the potential of these TLRs to trigger Gp96-mediated APC activation [14,16]. For this purpose we incubated the human embryonic kidney

fibroblast cell line 293T transiently transfected with different TLRs and the luciferase reporter driven by synthetic enhancer containing NF-κB binding consensus sites with Gp96 or LPS as a control. As shown in Fig. 1, the expression of TLR2 and also TLR4/MD-2 conferred responsiveness to the Gp96 stimulus in a dose-dependent manner. Boiling of Gp96 abolished the induction of luciferase activity, thus demonstrating that possible endotoxin contaminations in the Gp96 preparation were not responsible for the observed effect. Likewise, the presence of polymyxin B (an LPS inhibitor) did not interfere with Gp96-mediated activation (data not shown).

TLR4-mediated Gp96 activation is dependent on the presence of MD2 as transfection with TLR4 or MD2 alone did not result in the induction of luciferase activity (Fig. 1). LPS mediates NF-κB driven luciferase induction via TLR2 and TLR4. This finding is in line with previous reports showing that in addition to TLR4-mediated activation by LPS, other endotoxin contaminations present in commercially available LPS also mediate APC activation via TLR2 [23]. Transfection of TLR3, TLR7, TRL8 and TLR9 did not conferr responsiveness to the Gp96 stimulus (stimulation indices between 1.2 and 1.5; data not shown).

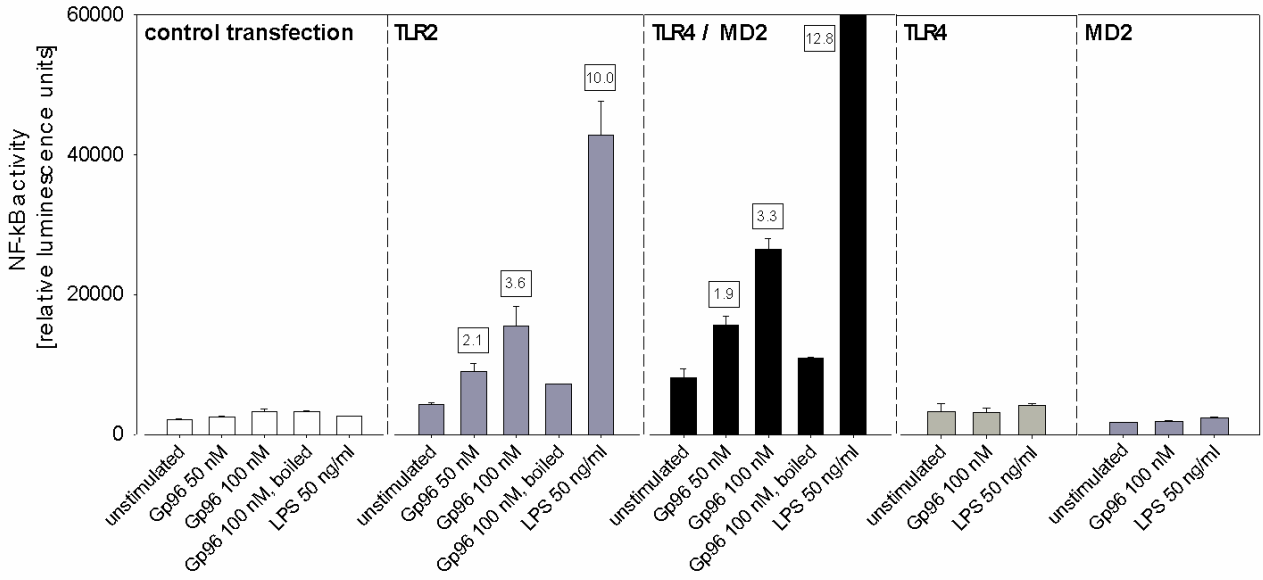


**Figure 1.** Gp96 activate cells via TRL2 and TLR4. Human embryonic kidney fibroblasts 293T were transiently transfected with the indicated Toll-like receptors. At the same time they were cotransfected with luciferase reporter driven by a synthetic enhancer harboring NF-κB binding consensus sites. Luciferase activity was measured after stimulation by Gp96 , boiled Gp96 and LPS. Only cells transfected with TLR2 or TLR4 plus MD2 respond to Gp96 activation but cells without Toll-like receptor transfection did not. Each error bar represents the deviation of duplicates. The results are representative of three independent experiments. In some cases, the x-fold induction of luciferase activity relative to the unstimulated cells is indicated.

## Gp96 mediates DC activation via TLR4 and TLR2

To analyze the contribution of TLR2 and TLR4 under more physiological situations, we studied the Gp96-mediated activation of bone marrow-derived DCs (BMDCs) from mice lacking functional TLR2 or TLR4 molecules, or both. In the first set of experiments we investigated the secretion of the proinflammatory cytokine IL-12 in response to Gp96, LPS and CpG-DNA. As shown in Fig. 2, BMDCs from C3H/HeN, TLR2$^{-/-}$ and TLR2 wild-type but not C3H/HeJ (a TLR4 deficient mouse) and C3H/HeJ / TLR2$^{-/-}$ (TLR2$^{-/-}$/4deficient) mice responded to the Gp96 stimulus by secretion of IL-12. CpG-DNA induced IL-12 secretion in all cultures and LPS-mediated activation was impaired in BMDCs from C3H/HeJ and C3H/HeJ / TLR2$^{-/-}$ mice, in line with previous reports. Again, boiled Gp96 did not induce any stimulation. The lack of TLR2 did not affect Gp96-mediated IL-12 secretion from BMDCs.

During Gp96-mediated DC activation, secretion of IL-12 is accompanied by the upregulation of the costimulatory molecule CD86 [14,16]. Therefore, CD86 expression in mice lacking functional TLR2 and/or TLR4 molecules was investigated. As observed for the secretion of IL-12, BMDCs from C3H/HeN, TLR2$^{-/-}$ and TLR2 wild-type but not C3H/HeJ and C3H/HeJ / TLR2$^{-/-}$ mice upregulated CD86 molecules after 48 h coculture with Gp96. Fig. 3a shows CD86 upregulation for one C3H/HeN and one C3H/HeJ mouse, Fig. 3b represents mean values of CD86 upregulation of three individual mice. This effect was again heat-sensitive. CpG-DNA induced CD86 upregulation on all BMDCs, whereas LPS was impaired in

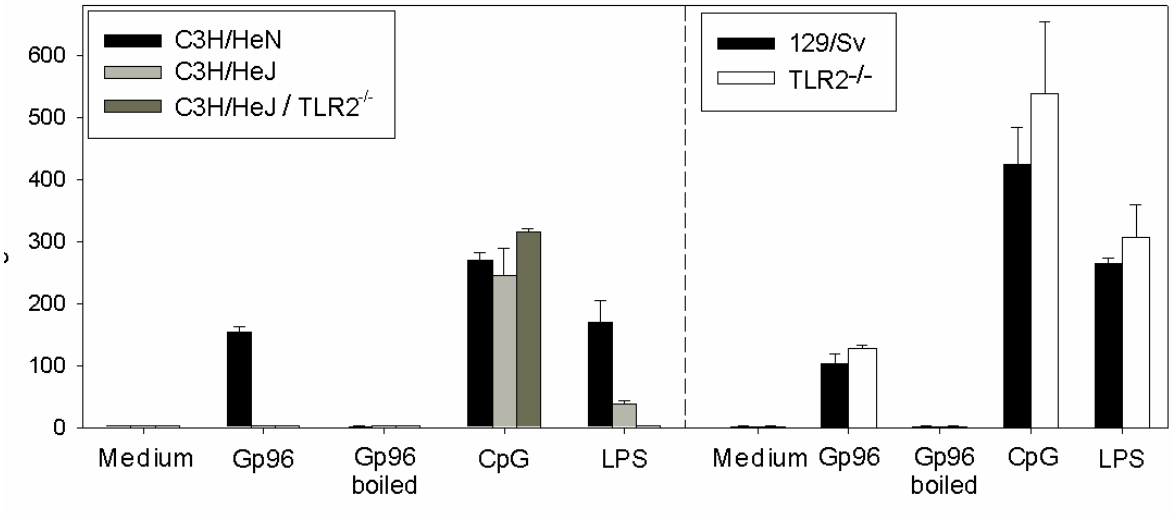


**Figure 2. Gp96 -induced IL-12 production by dendritic cells is TLR4 dependent.** Bone marrow-derived dendritic cells from the indicated mice strains were cultured in the presence of Gp96 (1 µM), boiled Gp96 , CpG (oligonucleotide 1668, 2 µM) or LPS (2 µg/ml).After 20 h IL-12 concentration in cell culture supernatant was measured by sandwich ELISA. The mean value of triplicates is shown and error bars represent standard deviation.

BMDCs from C3H/HeJ and TLR2/4$^{-/-}$ mice. A minimal upregulation of CD86 molecules in C3H/HeJ compared to TLR2/4$^{-/-}$ mice was observed in three independent experiments and might be caused by the interaction of Gp96 with TLR2 as observed for the NF-κB driven luciferase induction reported in Fig. 1. In TLR2$^{-/-}$ mice this effect can obviously be compensated by TLR4.

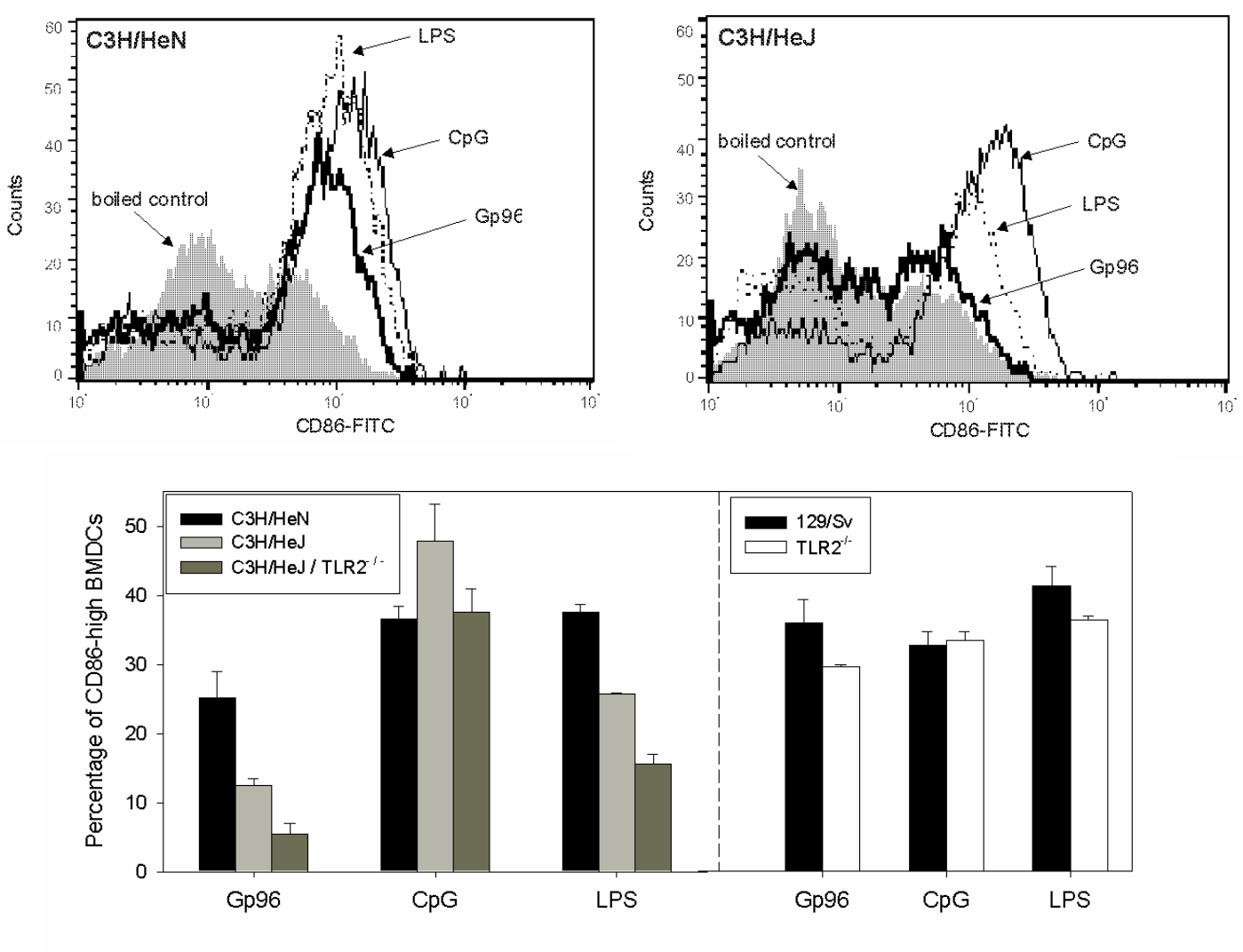


**Figure 3. Gp96 mediates DC activation via TLR4 and TLR2.** Bone marrow-derived dendritic cells were stimulated with Gp96, CpG or LPS (same concentrations as described in Fig. 2). After two days surface expression of CD86 was evaluated. Appropriate isotype control stainings were included. **(a)** CD86 surface expression by BMDCs from C3H/HeJ and C3H/HeN control mice is shown. C3H/HeJ derived dendritic cells do not upregulate CD86 in response to Gp96, while CpG activation is not and LPS activation only partially affected. **(b)** The percentage of CD86-high cells of triplicates was evaluated. Mean values were corrected by the percentage of CD86 positive cells in control samples (20%, as shown in part a). Error bars represent standard deviation of mean of mean. Gp96-induced DC maturation is impaired in C3H/HeJ mice and even more in C3H/HeJ / TLR2$^{-/-}$ mice. In contrast, mice missing only TLR2 are not affected, suggesting that the function of TLR2 can be compensated by TLR4 in these cells.

## Gp96 activates classical signaling cascades

The interaction of Gp96 with TLR2 and TLR4 suggests that the activation of APCs involves the classical signaling cascades described for other TLR2 and TLR4 ligands [21,24].To analyse this issue, the macrophage cell line RAW264.7 was incubated with different concentrations of Gp96 and probed for the phosphorylation of the kinases c-Jun N-terminal kinase 1/2 (JNK1/2), p38, extracellular signal-regulated kinase 1/2 (ERK1/2) and the degradation of IκB-α as an indication for the activation of the NF-κB pathway. Fig. 4 shows that all kinases tested were activated. The Gp96 effect was again heat sensitive. CpG-DNA was used as a positive control.

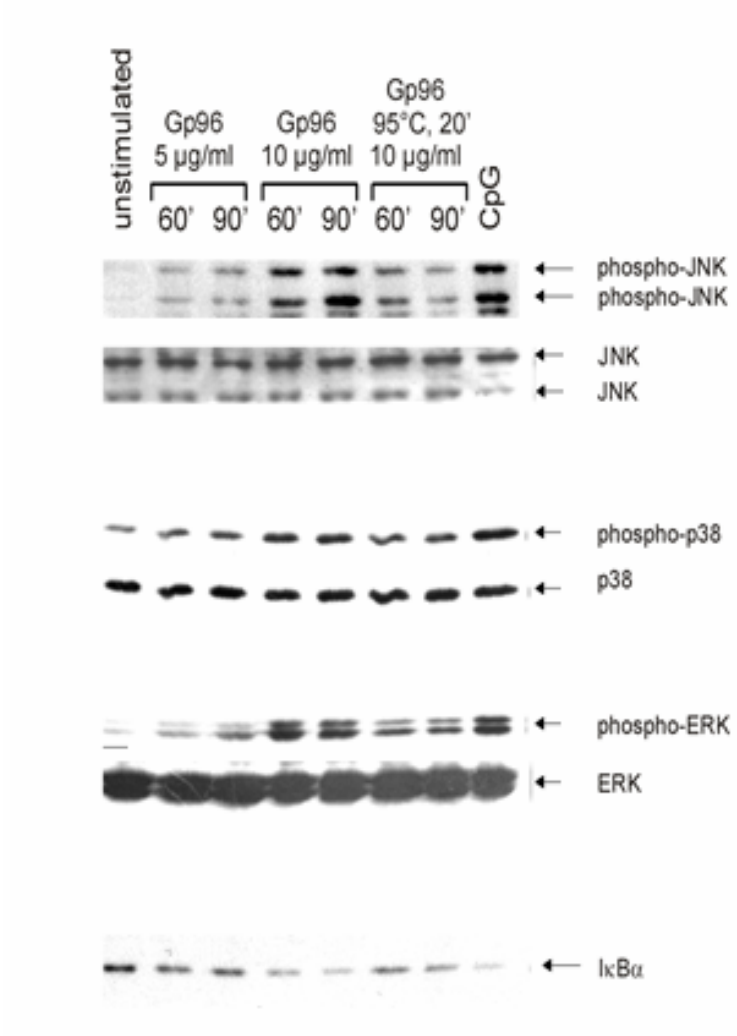


**Figure 4. Gp96 triggers classical signaling cascades.** RAW267.4 macrophages were incubated with different concentrations of Gp96 for 60 or 90 min and with 2 µg/ml CpG-DNA as a positive control for 30 min. Cells were lysed and the amount of phospho-JNK, phospho-p38, phospho-ERK and was determined by western blot. In addition, the total amount of JNK, p38 and ERK in lysates was determined. JNK1/2, p38 and ERK1/2 were activated upon stimulation with Gp96 in a time and concentration dependent manner. At the same time, the NF-κB pathway was triggered as indicated by the degradation of IκB-α. The stimulatory effect of Gp96 was heat sensitive.

## DC activation requires endocytosis of Gp96

Because MHC class I-restricted representation of Hsp70- as well as Gp96-associated peptides depends on receptor-mediated, clathrin-dependent endocytosis of these molecules and subsequent transport to multivesicular compartments [14,25,26], we investigated whether endocytosis of Gp96 might be a prerequisite for DC activation. Monodansylcadaverine (MDC) is an inhibitor of the membrane-bound transglutaminase and interferes with clathrin-mediated receptor trafficking as demonstrated for the $\alpha_2$-macroglobulin receptor, CD91 [27]. Interestingly, CD91 has been shown to be responsible for Gp96 uptake and representation of the associated peptides [8,13] after receptor-mediated endocytosis [14].

Indeed, MDC was found to inhibit the endocytosis of FITC-labeled Gp96 molecules by DCs (Fig.5). The presence of 0.5% DMSO alone used to dissolve MDC did not influence Gp96 uptake compared to medium control lacking DMSO (data not shown). Therefore, the accumulation of Gp96-FITC at the cell membrane is due to the effect of MDC.

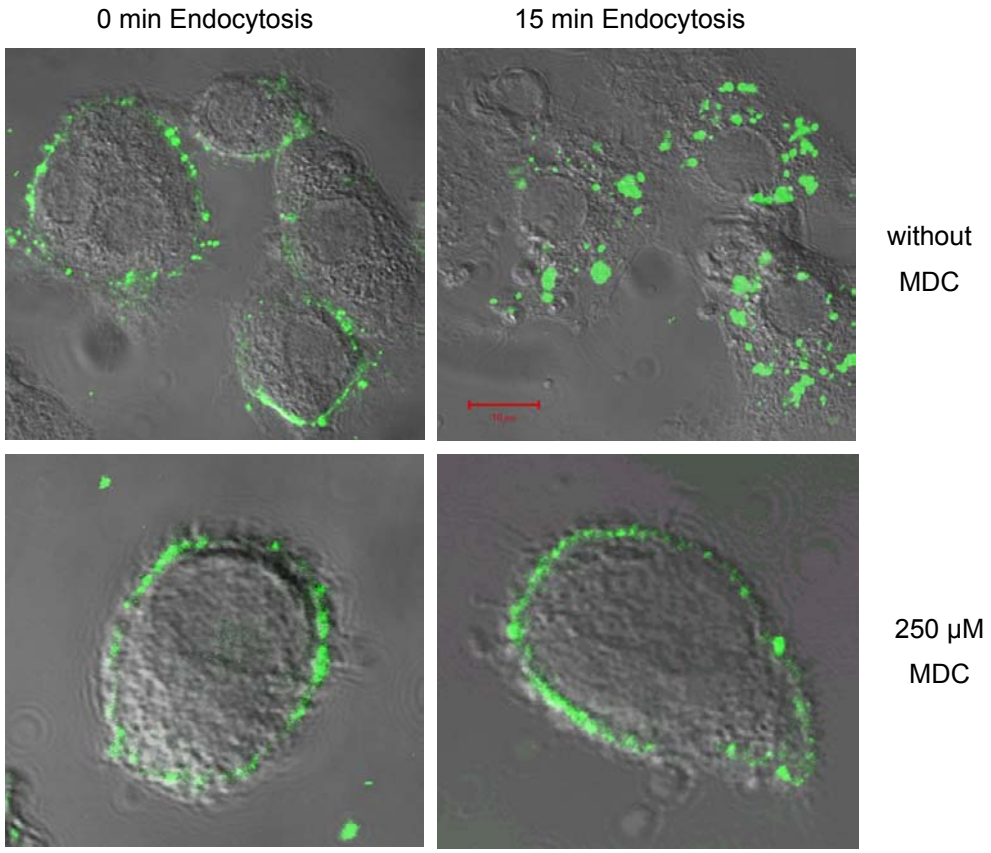


**Figure 5. Monodansylcadaverine inhibits receptor-mediated endocytosis of Gp96.**
Human monocyte-derived, immature dendritic cells were incubated with Gp96-FITC at 4°C, washed and fixed (no endocytosis) or treated at 37°C for further 15 min (endocytosis) and fixed with methanol/acetone. The confocal micrographs show an overlay of the transmission and fluorescence channels. In the presence of MDC uptake of Gp96-FITC was substantially inhibited. MDC is an inhibitor of transglutaminase and has been shown to abrogate clathrin-dependent receptor-mediated endocytosis. Viability of cells (tested by trypan blue staining) in 0.5% DMSO and 250 µM MDC was approximately 95% and 90%, respectively. Representative sections are shown. Identical results were obtained in at least four independent experiments.

The lack of Gp96 endocytosis in the presence of MDC is accompanied by the lack of TNF-α secretion (Fig. 6). As a control, LPS-mediated DC activation is not inhibited by MDC. These results also argue against an endotoxin contamination in the Gp96 preparation being responsible for the observed effect. Similar results were obtained for the Gp96-induced secretion of IL-12 (data not shown).

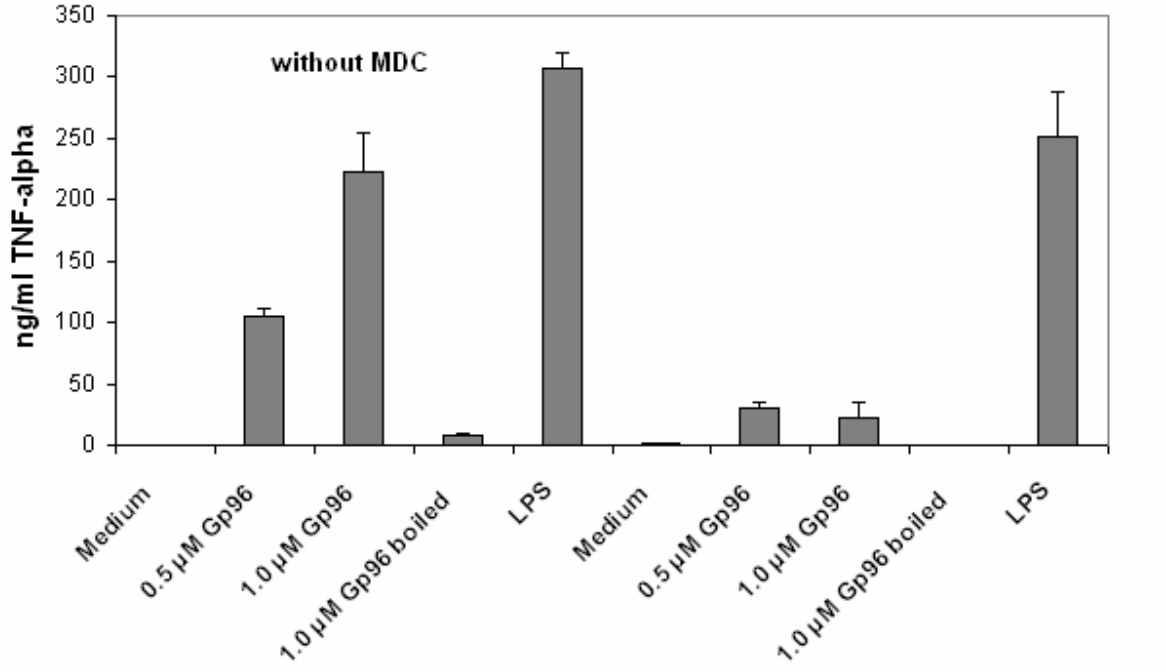


**Figure 6. Receptor-mediated endocytosis is required for Gp96-mediated TNF-□ secretion by DC.** Human monocyte-derived, immature dendritic cells were activated by Gp96 and LPS in the presence and absence of 250 µM MDC. Boiled Gp96 was used as a control to exclude the possibility of endotoxin contaminations in Gp96 preparation. MDC specifically inhibited Gp96-mediated DC activation while the activity of LPS was unchanged in the presence of MDC. This demonstrates that receptor-mediated endocytosis is required for Gp96-mediated activation of dendritic cells. Error bars represent SEM.

We next analyzed the effect of MDC on the signal transduction pathways and found that they were also affected as examplified by the strongly reduced phosphorylation of JNK1/2 (Fig. 7). The inhibitory effect of MDC was dose dependent (data not shown) and did not influence the anisomycin-mediated phosphorylation of JNK1/2 (used as control). Thus, Gp96 mediates activation of DCs via TLR2 and TLR4 using the SAPK, MAPK and NF-kB pathways in a process that requires endocytosis.

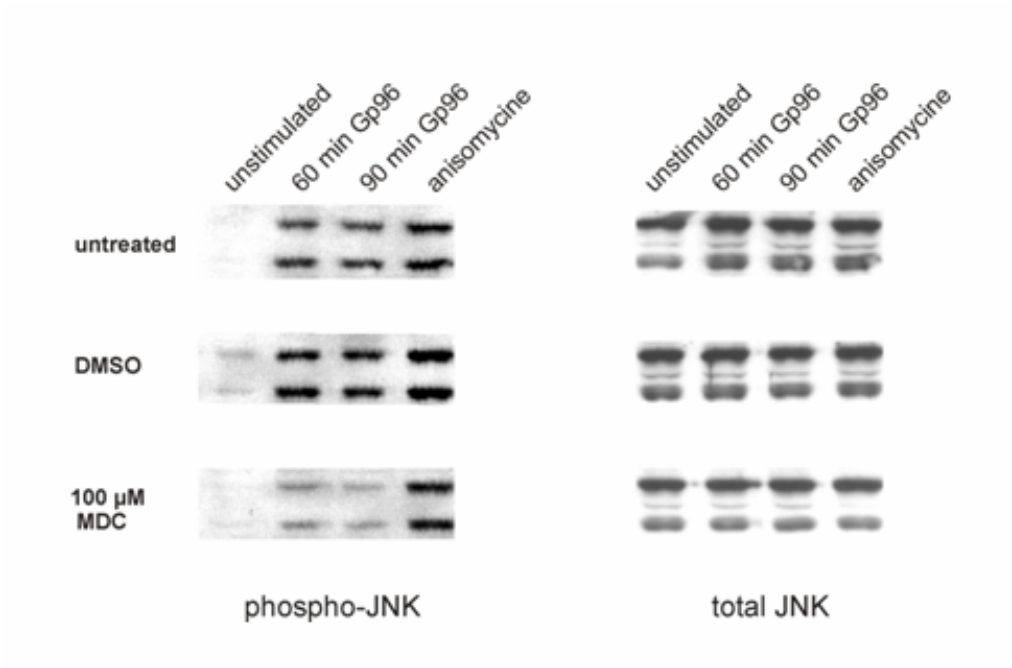


**Figure 7. Endocytosis of Gp96 is required for activation of JNK.** RAW267.4 macrophages were stimulated with different concentrations of Gp96 for 60 or 90 min or with 0.2 µg anisomycine for 10 min. Cells were incubated either in standard medium, in medium containing 100 µM MDC or in medium containing DMSO matching the amount used for solvation of MDC. The assay of JNK was performed as described for Fig. 4. The stimulatory capacity of Gp96 is abrogated upon inhibition of endocytosis by MDC. Stimulation by anisomycine was uneffected by MDC treatment. The overall amount of JNK remained the same in all samples.

## Discussion

We identified the molecular mechansim of APC activation by the ER-resident chaperone Gp96. We find that TLR2 and TLR4 mediate NF-κB driven luciferase induction (Fig. 1) and mainly TLR4, but to small extent also TLR2, are responsible for the upregulation of CD86 and the secretion of IL-12 and TNF-α (Fig. 2 and Fig. 3). IL-10 was not detectable (<1.5 ng/ml, data not shown) which is in line with the previousely reported secretion of only proinflammatory cytokines [14,16]. TLR3, TLR7, TLR8 and TLR9 molecules appear not to be involved in Gp96-mediated signaling (data not shown). Expression of CD86 and cytokine secretion is proceded by the activation of the NF-κB pathway and activation of the stress-activated protein kinases (SAPK) JNK1/2 and p38 as well as the mitogen-activated protein kinase ERK1/2 (Fig. 4). DC activation is inhibited by MDC which interferes with receptor-mediated endocytosis (Fig. 5 – 7).

Despite the fact that Gp96 and LPS are very different molecules, they both mediate DC activation via TLR4 and TLR2. The nature of the PAMP enharboured by HSPs that allows their specific interaction with TLR2 and TLR4 is unknown. We are currently investigating the possibility if Gp96 and microbial components interfere with each others in binding to both TLRs. However, regardless of their shared interaction with TLR2 and TLR4 molecules, several differences in their mode of action are apparent. The most striking observation is that the Gp96 -mediated DC activation strictly depends on the endocytosis of Gp96 whereas LPS-mediated DC activation does not require endocytosis and thus functions in the presence of the endocytosis inhibitor MDC. As a consequence, Gp96 - but not LPS-mediated secretion of cytokines is impaired in the presence of MDC (Fig. 6). This, together with the observed sensitivity of Gp96 -mediated DC-activation to heat denaturation, also demonstrates that an endotoxin contamination in the Gp96 preparation does not account for the observed effects. This is further supported by the observation that cells expressing Gp96 molecules targeted to their surface induce efficient DC maturation upon cell-to-cell contact [34].

Internalization of TLR2 during the activation of macrophages has been observed previously [28]. In this series of experiments, TLR2 was found to accumulate in phagosomes of macrophages activated with the yeast cell-wall particle zymosan. Whether or not endocytosis was a prerequisite for activation was not investigated. Our results obtained with Gp96 as an agonist for TLR2 and TLR4 suggest this to be the case.

The importance of HSP endocytosis for macrophage activation has very recently been reported for the Hsp60/TLR2/4 mediated activation of macrophages [29]. We observe here the same effect for the new TLR2 and TLR4 ligand, Gp96. Our finding parallels the need for Gp96 endocytosis during receptor-mediated uptake and representation of Gp96-associated peptides [14,25] which is mediated through the interaction of Gp96 with CD91, the receptor for $\alpha_2$-macroglobulin [8,13]. Interestingly, MDC has been first described as an inhibitor of endocytosis of the $\alpha_2$-macroglobulin receptor [27].

An interesting scenario can be postulated from the above findings: similar to the proposed requirement of LPS-mediated activation for CD14 and LBP on the cell surface (reviewed in [30]), Gp96 -mediated DC activation might depend on the presence of CD91 molecules which endocytose bound Gp96 molecules and subsequently mediate their transport to endocytic vesicles, as described [25]. This process will increase the local concentration of Gp96 , now able to trigger signaling through TLR2 and TLR4 present in these vesicles [31] by the recruitement of cytosolic MyD88 to the outer membrane of endocytic vesicles.

The contribution of TLR2 to Gp96 -mediated DC activation is not clear. While transfection of TLR2 induces NF-κB-driven luciferase activity (Fig. 1), TLR2$^{-/-}$ mice show normal Gp96 -mediated DC activation profiles (Fig. 2 and Fig. 3b). On the other hand, Gp96 -mediated DC activation in C3H/HeJ mice is always stronger than in TLR2$^{-/-}$/4 deficient mice which are not able to respond to a Gp96 stimulus at all. This observation suggests a minor contribution of TLR2. In TLR2$^{-/-}$ mice the lack of TLR2 signaling can apparantly be completely compensated by TLR4, but TLR2 can only induce minimal activation when TLR4 is not functional, as observed for BMDCs from C3H/HeJ mice. One explanation could be an imbalance in the expression of TLR2 and TLR4 on BMDCs, favouring effects mediated by TLR4. However, the interaction of both TLR molecules with Gp96 is strongly supported by the recent finding that the interaction of TLR2 and TLR4 with Gp96 inside the ER is crucial for the expression of these receptors on the cell surface [32].

So far TLRs have been described as sensors for PAMPs crucial for the initiation of an innate immune response. These mechanisms were developed long before the adaptive immune system. One of the newest addition to the list of TLR ligands identified is Hsp60. Interestingly, not only bacterial but also human Hsp60 molecules crossreact with TLRs [29]. Our results now demonstrate that the exclusive association of TLRs with PAMPs is obsolete. Gp96 is not expressed in bacteria or fungi and provides the first example of a non-pathogen derived ligand of TLRs. More importantly, our results show for the first example of how the innate and adaptive immune system can be stimulated simultaneously by the same molecule which is released under physiological situations from necrotic cells [16,17]. The importance of these TLR-mediated stimuli for the induction of T helper type 1-dominated immune responses has been observed recently using MyD88$^{-/-}$ mice [33].

Gp96 has kept the, probably Hsp60-derived, ability to stimulate APCs unspecifically via TLRs but added a new function: to act as a carrier for antigenic peptides and to promote receptor-mediated uptake by professional APCs [14,25]. The unique combination of both features now allows the MHC-restricted presentation of antigenic peptides to cells of the adaptive immune system in an immunostimulatory context and enables DCs to act as coordinators of innate and adaptive immune responses. Being able to understand these mechanisms will make it possible to interfere with the HSP-mediated activation of APCs and to rationally modulate immune responses, either towards immunity or tolerance.

## Methods

**Reagents, antibodies and plasmids.** Gp96 and FITC-labeled Gp96 was provided by Immatics Biotechnologies, Tübingen, Germany. LPS from *Salmonella minnesota* RE 595, monodansylcadaverine (MDC) and anisomycin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Phosphothioate stabilized CpG oligonucleotide 1668 (TCC-ATC-ACG-TTC-CTG-ATG-C) was purchased from TIB MOLBIOL (Berlin, Germany).
Antibodies to ERK1/2 were obtained from Upstate Biotechnology (Lake Placid, NY), other antibodies used in cell signaling studies were from New Engalnd Biolobs (Frankfurt a. M., Germany) including: anti-phospho-JNK1/2 (Thr183/Tyr185), anti JNK1/2, anti-phospho-p38 (Thr180/Tyr182), anti-p38, anti-IκB-α, anti-phospho-ERK1/2 (THR202/Tyr204), anti-ERK1/2, anti-phospho-STAT1 (Tyr701) and anti-STAT1. Antibodies for FACS analysis were purchased from BD Biosciences (Heidelberg, Germany).
The expression vectors for the N-terminus of human flag-tagged TLR2 and TLR4 were gifts from Tularik, Inc. (South San Francisco, USA). The human MD2 expression vector was kindly provided by K. Miyake (Sage Medical School, Nabeshima, Japan). The luciferase reporter driven by a synthetic enhancer harboring 6 NF-κB binding consensus sites was a gift from P. Baeuerle (München, Germany).

**Analysis of signalling pathways in RAW264.7.** The mouse macrophage cell line RAW264.7 (purchased from ATCC, Manassas, VA) was grown in VLE-RPMI medium (Biochrom KG, Berlin, Germany) supplemented with 100 IU/ml penicillin/streptomycin (Biochrom KG) and 10% FCS (Biochrom KG) . Prior to stimulation, cells were incubated with serum-free medium for 2-4 h. Stimulation was performed for the indicated time period by addition of 10 nM or 100 nM Gp96 , 100 nM Gp96 pre-treated at 95°C for 20 min or 2 µM CpG oligonucleotide 1668.
Cells were then lysed in lysis buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DGTA, 10% glycerol, 1% Trition X-100, 10 mM pyrophosphate, 20 mM β-glycerophosphate, 1 mM orthovanadate, 10 mM sodium fluoride, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF). Lysates were boiled in SDS-sample buffer, sonicated and centrifuged at 10 000 g for 10 min.

Electrophoresis of the lysates was carried out on a 10% SDS-PAGE and Western blotting was performed using Protran nitrocellulose membranes (Schleicher&Schuell, Dassel, Germany). The membranes were blocked in 5% skim milk solution, probed with the indicated antibodies and visualized using Renaissance Chemiluminescence Reagent (NEN, Köln, Germany). When indicated 100 µM MDC was added 20 minutes before stimulation. All following steps were performed as described above.

**Luciferase-reporter assay in human embryonic kidney fibroblasts 293T.** Human embryonic kidney fibroblasts 293T were cultured in DMEM (Biochrom KG) supplemented with 100 IU/ml penicillin/streptomycin (Biochrom KG) and 10% FCS (Biochrom KG). For luciferase reporter assays 5-10 x $10^6$ cells were transfected with 1 ng 6x NF-κB luciferase reporter and 10 ng TLR2 or 10 ng TLR4 plus 10 ng MD2 plasmid DNA. The overall amount of plasmid DNA was held constant at 20 µg per electrophoration by addition of empty expression vector. Cells were electroporated in a final volume of 400 µl (RPMI-25% FCS) at 200 V and 960 µF (Gene Pulser, Bio-Rad-Laboratories, München, Germany). Following electroporation, cells were washed and cultured in 6-well plates. Cells were subsequently stimulated with 100 nM Gp96 , 500 nM Gp96 , 500 nM Gp96 pre-treated at 95°C for 20 min or 100 ng/ml LPS overnight in serum-free medium. Cell lysis and measurement of luciferase activity in extracts was performed with the Luciferase Assay System Kit from Promega (Mannheim, Germany) according to manufacturer's instruction.

**Generation of mouse dendritic cells.** Mouse immature DCs were generated from bone marrow of C3H/HeN, C3H/HeJ, C3H/HeJ / $TLR2^{-/-}$, 129Sv/C57BL/6 and $TLR2^{-/-}$ mice. $TLR2^{-/-}$ mice were obtained from Tularic Inc. (South San Fransisco, CA), C3H/HeJ / $TLR2^{-/-}$. All other mice were obtained from Charles River (Sulzfeld, Germany). For the generation of mouse bone-marrow derived DCs Iscove's Modified Dulbecco's Medium (IMDM; BioWhittaker, Verviers, Belgium) was used supplemented with 2 mM L-glutamine (GibcoBRL Life Technologies, Paisley, GB), 100 IU/ml penicillin/streptomycin (GibcoBRL), 10% FS (PAA, Linz, Austria) and 200 U/ml GM-CSF. Bone marrow cells were incubated in GM-CSF containing medium for 6-8 days and fresh medium with GM-CSF was replaced every 2 days. The obtained DCs were CD11c positive and CD14 negative.

**Generation of human dendritic cells.** Human DCs were prepared from freshly drawn blood from healthy donors. Peripheral mononuclear blood cells (PBMCs) were isolated using a Ficoll density gradient (Lymphoprep; Nycomed, Oslo, Norway). The obtained cells were washed twice with phosphate-buffered saline (PBS) and resuspended in X-Vivo 15 medium (Walkersville, USA) supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. PBMCs were plated at a density of $20x10^6$ cells / well. After 2 h at 37 °C non-adherent cells were removed by washing with PBS. Adherent monocytes were cultured for 6 days in medium supplemented with 1% (v/v) human serum (Peel-Freez, Brown Deer, WI, USA), 1000 U/ml interleukin-4 (IL-4, R&D Systems, Minneapolis, USA) and 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Leukomax, Novartis Pharma GmbH, Nürnberg, Germany). The differentiation state of DCs was examined by flow cytometry. Only immature DCs being $CD1a^+$, $CD14^-$, $CD83^-$, $CD86^{low}$ were used for activation experiments. The fraction of activated DCs analyzed by CD83 expression was always <5 %.

**Stimulation of DCs.** Mouse DCs were stimulated by addtion of 1 µM Gp96 , 1 µM heat pre-treated Gp96 or 2 µg/ml LPS. After 24 h, IL-12 (p40) IL-10 concentrations in the supernatants were measured using standard sandwich ELISA protocols. Antibodies and recombinant standards of both cytokines were obtained from Becton Dickinson Biosciences (Heidelberg, Germany). The capture antibody was bound to the ELISA plate (MaxiSorb™, Nunc, Roskilde, Denmark), the biotinylated detection antibody was revealed by streptavidin-conjugated horseradish peroxidase and ABTS substrate (Sigma) and the assay read at 405 nm. Furthermore, on day 2 after activation, expression of the costimulatory molecule CD86 was measured by flow cytometry (FACSCalibur™, Becton Dickinson). Isotype controls of antibodies were used in all experiments to determine the appropriate background fluorescence.

For experiments in the presence of MDC human monocyte-derived immature dendritic cells (day 7) were incubated with 0.5 µM or 1.0 µM Gp96 , 1.0 µM Gp96 heat-inactivated at 95°C for 20 min or 2 µg/ml LPS in the presence or absence of 250 µM MDC and 0.5% DMSO in both cases for 16 h. Supernatants were assayed for TNF-α and IL-12 by sandwich ELISA as described above.

**Confocal microcopy.** Human monocyte-derived dendritic cells (day 7) were seeded on cover slips. The DCs were pre-cooled and incubated for 30 min on ice with IMDM containing 10% FCS and 100 µg/ml Gp96 -FITC („pulse") in the presence and absence of 250 µM monodansylcadaverine (MDC) and in the presence of 0.5% DMSO in both cases. The coverslips were washed twice and were fixed immediately or incubated in IMDM medium for 15 min at 37°C („chase"). Fixation was done in methanol/acetone (ratio 1:1) at –20°C. For confocal microscopy a Zeiss LSM 510 laser scanning microscope was used. Thickness of the optical plane was adjusted by the pinhole to be less than 1 µm.

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## Chapter 7

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# Human Platelets Express Heat Shock Protein Receptors and Regulate Dendritic Cell Maturation

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Immunizations using the ER-resident heat shock protein Gp96 induce specific immune responses. Specificity is based on the MHC class I restricted cross-presentation of Gp96-associated peptides derived from endogenous proteins. Initiation of the immune response depends on the ability of Gp96 to induce the production of pro-inflammatory cytokines by macrophages and dendritic cells (DCs) and their maturation in a fashion presumably independent of associated peptide. Both events are mediated by Gp96 receptors on antigen presenting cells. It is known that Gp96 is released from cells upon necrosis induced e.g. by virus infection. While this event will support the efficient induction of immune responses on one hand, it might on the other hand interfere with processes that are susceptible to chronic inflammation, like wound healing after tissue damage. Therefore, Gp96-mediated stimulation of the immune system requires tight regulation. Here we describe that human thrombocytes specifically interact with Gp96 and that binding of Gp96 to platelets is 10-fold enhanced upon activation by thrombin. Gp96 does neither interfere with thrombin-induced platelet activation nor with platelet aggregation. However, the presence of platelets during Gp96-mediated DC activation reduces secretion of pro-inflammatory cytokines and activation of DCs. This effect is independent of soluble platelet factors and cell-to-cell contact between DCs and thrombocytes. Thus, we provide evidence for a regulatory mechanism that neutralizes Gp96 molecules systemically, especially in the blood, and thus limits the immunostimulatory capacity of Gp96. This effect might be of significance in wounds where chronic inflammation and immune responses against autoantigens have to be prevented.

## Introduction

The endoplasmic reticulum (ER)-resident heat shock protein (HSP) Gp96 plays multiple roles in mammalian organisms. As a chaperone it assists protein folding and prevents aggregation of partially unfolded proteins in the ER.[1] In this key compartment of the major histocompatibility (MHC) class I presentation pathway, Gp96 is also one of the major peptide binding proteins and associates with a peptide pool representative for the protein content of the cell.[2,3] Gp96 is released from cells after tissue damage caused by severe injury and as a consequence of necrotic cell death induced by freeze-thaw cycles[4] or virus infection.[5] Its presence in the extracellular space reveals surprising immunostimulatory properties: immunization with Gp96 preparations from tumor cells has been shown to elicit protective and therapeutic immune responses against the tumor the HSP had been purified from.[6,7] The specificity of this immune response is due to tumor-derived peptides associated with Gp96.[6] After receptor-mediated endocytosis of Gp96 by professional antigen presenting cells (APCs) [8] these peptides are presented on MHC class I molecules.[9] This process is usually referred to as 'cross-presentation' [10,11] and is one of the key events during priming of naive T cells.[12] In addition, Gp96-mediated APC activation results in the upregulation of co-stimulatory molecules and in the release of the pro-inflammatory and $T_H1$ promoting cytokines tumor necrosis factor alpha (TNF$\alpha$) and interleukin-12 (IL-12).[4,13] The release of the anti-inflammatory and $T_H2$ promoting cytokine IL-10 is only slightly stimulated. The resulting cytokine milieu is an important prerequisite for the triggering of a cytotoxic T cell response.

So far, receptor-mediated interactions for Gp96 have only been described for professional APCs comprising DCs, macrophages and B cells. The alpha-2 macroglobulin receptor (CD91) has been identified as one receptor on a macrophage cell line. Binding of Gp96 to the scavenger receptor CD36 has been shown by Panjwani et al.[14] More recently, physical interaction between Gp96 and Toll-like receptor (TLR) 1,2 and 4 in the ER has been described,[15] and we were able to show that Gp96 signals in bone-marrow derived murine DCs via the TLR4 pathway.[16] However, the potent ability of Gp96 to induce a pro-inflammatory milieu might be a mixed blessing for the organism in some situations. After injuries, transient inflammation during the first phase of wound healing is helpful, but chronic inflammation prevents proper tissue formation.[17] In this scenario the pro-inflammatory potential of Gp96 has to be regulated, otherwise wound healing might be impaired and the uncontrolled maturation of DCs would favor the development of autoimmunity.

A major difference between for example virus-induced cell lysis and cell death caused by injury is the presence of platelets. These are small, non-nucleated cells which play a key role in hemostasis by forming a plug which physically stops blood loss. In addition, activated platelets release several mediators from their granules that contribute to proper wound healing. Platelet aggregation and activation is triggered by many different stimuli, of which the most prominent are thrombin, collagen and ADP. The latter two substances are normally not visible to platelets unless blood vessels are disrupted. From this point of view, collagen and ADP might be considered as messengers for cell death and injury with platelets as appropriate sensors. Although platelets carry MHC class I molecules on their surface, they do not themselves stimulate primary T cell responses.[18] However, thrombocytes possess several immunmodulary properties: Activated platelets have been shown to induce an inflammatory reaction on vascular endothelial cells via CD40 ligand (CD40L) which was originally identified on activated $CD4^+$ T cells.[19] In addition, the maturation of DCs by fixed, activated platelet preparations was demonstrated.[20] On the other hand, upon activation thrombocytes secrete pro-inflammatory cytokines and chemokines (e.g. platelet factor 4, RANTES) as well as anti-inflammatory mediators (e.g. TGF-β).[21-25]

In this work, we show for the first time that human platelets express receptors for the ER-resident heat shock protein Gp96. We investigated in detail the binding of Gp96 to the surface of human platelets and consequences therefrom concerning platelet function. Furthermore, we analyzed the influence of human platelets on the Gp96-mediated activation of DCs.

## Materials and methods

### Materials

Purified mouse Gp96 and fluorescein isothiocyanate (FITC)-labeled Gp96 were provided by Immatics Biotechnologies (Tübingen, Germany). Recombinant ovalbumin and FITC-labeled bovine serum albumin (BSA) was purchased from Sigma (Taufkirchen, Germany). Ovalbumin was labeled with FITC according to standard protocols and purified by gel filtration on a Sephadex G-25 column (Amersham Pharmacia Biotech, Freiburg, Germany)

### Platelet isolation

9 volumes of freshly taken blood from healthy donors was mixed with 1 volume of 110 mM sodium citrate as anticoagulant. The citrated blood was centrifuged at 100 g for 15 min at

room temperature to obtain platelet rich plasma (PRP) in the upper phase. For activation, flow cytometry and co-culture experiments with DCs, platelets were separated from plasma according to the following procedure. The PRP was overlaid on top of a 2 ml 34 % (w/v) BSA cushion and centrifuged at 550 g for 10 min. Platelets were collected from the interphase and washed twice with serum-free cell culture medium. For activation, platelet suspensions were incubated for 3 min with 0.2 U/ml thrombin at 37 °C if not stated otherwise. Thereafter, cells were fixed for 2 min on ice by the addition of paraformaldehyde (PFA) to a final concentration of 1 % (w/v). Residual PFA was removed by two additional washing steps.
Non-fixed platelets were used within three hours after preparation. Contamination by other blood cells (mainly erythrocytes) in thrombocyte preparations used for DC co-cultures was always lower than 0.5 %.

**Dendritic cell preparation**

Human DCs were prepared from freshly drawn blood from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll density gradient (Lymphoprep; Nycomed, Oslo, Norway). The obtained cells were washed twice with phosphate-buffered saline (PBS) and resuspended in X-Vivo 15 medium (Walkersville, USA) supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. PBMCs were plated at a density of $6x10^6$ cells / ml. After 2 h at 37 °C non-adherent cells were removed by washing with PBS. Adherent monocytes were cultured for 6 days in medium supplemented with 1% (v/v) human serum (Peel-Freez, Brown Deer, WI, USA), 1000 U/ml interleukin-4 (IL-4, R&D Systems, Minneapolis, USA) and 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Leukomax, Novartis Pharma GmbH, Nürnberg, Germany). The differentiation state of DCs was examined by flow cytometry. Only immature DCs being $CD14^-$, $CD83^-$, $CD86^{low}$ were used for activation experiments. The amount of CD1a on DCs varied between different DC preparations between low expression and complete absence which is in accordance with earlier findings.[26] The fraction of activated DCs analyzed by CD83 expression was always <5 %.

**Antibodies and staining for flow cytometry**

The following antibodies were used for fluorescence-activated cell sorter (FACS) analysis: fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (moAb) against CD41 (Coulter Immunotech, Marseille, France) exclusively expressed on platelets and

megakaryocytes, to ensure the purity of platelet preparations, phycoerythrine (PE)-CD40L-antibody (Coulter Immunotech, Marseille, France), PE-labeled moAb specific for the α subunit of CD91 (Research Diagnostics Inc., Flanders, USA), PE-CD1a-moAb, PE-CD14-moAb, PE-CD36-moAb, PE-CD83-Ab, PE-CD86-moAb (all BD Biosciences, Heidelberg, Germany). For FACS analysis, aliquots of $1x10^7$ platelets were incubated with labeled antibodies or proteins in FACS buffer (PBS, 1 % (w/v) BSA, 0.02 % (w/v) sodium azide) for 30 min on ice. Staining with Gp96-FITC was performed in cell culture medium supplemented with 10 % (v/v) FCS. Platelets were washed three times with FACS buffer and fixed in 1 % (w/v) PFA prior to analysis on a FACScalibur cytometer (BD Biosciences, Heidelberg, Germany). Appropriate mouse isotype control stainings were included to evaluate background staining. If indicated, platelets were preincubated with competitors for 30 min on ice. For competition experiments the anti-CD36 antibody clones CB38 (BD Biosciences, Heidelberg, Germany) and SM0 (Sigma-Aldrich, Taufkirchen, Germany) and a moAb against the 85 kDa subunit of CD91 (clone 5A6, Research Diagnostics Inc., Flanders, USA) were used.

**Analysis of platelet function**

Freshly isolated platelets from different donors were pre-incubated for 15 min at 37 °C with different effectors. 2.5 µM ADP, being a weak inducer of platelet activation at this concentration, was used as a positive control. Thereafter, thrombin was added in different concentrations varying from non-saturating amounts (1 mU/ml) to saturated activation (100 mU/ml). After 5 min of incubation at 37 °C, platelets were fixed by addition of PFA to a final concentration of 1 % (w/v). Residual PFA was removed by two washing steps with FACS buffer. Platelets were stained with a PE-labeled antibody specific for the platelet activation marker CD40L. CD40L expression was normalized to the value after activation with 500 mU/ml. For aggregation assays freshly prepared PRP from healthy donors who had not taken Aspirin™ for 10 days was used. Platelet concentration was adjusted to $2,5*10^5$ /µl with platelet poor plasma which had been obtained by centrifugation (2500 g, 15 min, RT) of the remaining blood after PRP preparation. Aggregation was analyzed using a APACT 4 aggregometer (Labor GmbH, Ahrensburg, Germany). 300 µl of stirred PRP was incubated at 37 °C with 50 µg/ml Gp96 or with ovalbumin or buffer alone for 3 min. Thereafter, ADP, collagen or adrenaline was added and aggregation was measured for additional 5 min.

**Platelet-DC co-culture**

$2x10^4$ platelets/µl in 200 µl cytokine-free medium were pre-incubated for 45 min with 20 µg/ml Gp96 or 20 ng/ml LPS in a 96-well plate. Thereafter, $2x10^5$ immature DCs were added. After 24 and 48 hours 100 µl of the cell culture supernatant were assayed for IL-10, TNF-alpha and IL-12 by sandwich ELISA with antibodies obtained from BD Biosciences, Heidelberg, Germany. In addition, the maturation state of DCs was measured by determining the amount of $CD83^+$ and $CD86^{high}$ cells by flow cytometry after 48 h. To exclude endotoxin contaminations as the reason for DC activation in the used Gp96 lots, boiled Gp96 was included due to the observation that Gp96-induced DC activation is heat-sensitive while LPS-induced is not.[13] In some experiments direct cell-to-cell contact was prevented with transwell inserts for 96-well plates (NUNC, Roskilde, Denmark). In these experiments platelets filled into the chamber of the insert were separated from DCs by a membrane (0.2 µm pore diameter) allowing only the exchange of soluble factors.

## Results

### Gp96-FITC binds specifically to human platelets

We first investigated whether Gp96 molecules interact with human platelets. Freshly purified platelets were fixed directly or stimulated by 0.2 U/ml thrombin for 3 min at 37 °C before fixation. To control the homogeneity of the cells used, thrombocytes were stained with an antibody against the platelet-specific marker CD41 (Figure 1 A). This marker identified a homogenous platelet preparation with little variations in fluorescence intensity between activated (filled gray) and non-activated (black line) cells. In contrast, only activated thrombocytes show staining with an antibody against CD40L. This is in line with previous findings reporting CD40L expression exclusively on activated platelets.[19] Gp96-FITC binds to non-activated and to thrombin-activated platelets (Figure 1 A, third panel). Comparing fluorescence intensities, binding of Gp96 is approximately tenfold enhanced on activated platelets. FITC-labeled ovalbumin (OVA-FITC) as a control showed no binding on thrombocytes (data not shown). Figure 1 A (fourth panel) shows that CD91, which has been already identified as a Gp96 receptor, is also expressed on human platelets and strongly up-regulated after thrombin-induced activation correlating with the binding characteristics of Gp96-FITC. The scavenger receptor CD36, which has been suggested as an additional

receptor for Gp96 recently,[14] is already present at high levels on non-stimulated platelets and is only slightly up-regulated after activation (Figure 1 A, last panel). To analyze the specificity of the observed binding of Gp96 to platelets we tested whether saturation could be achieved. Non-stimulated and thrombin-activated thrombocytes showed typical saturation curves when incubated with different concentrations of Gp96-FITC (Figure 1 B,C).

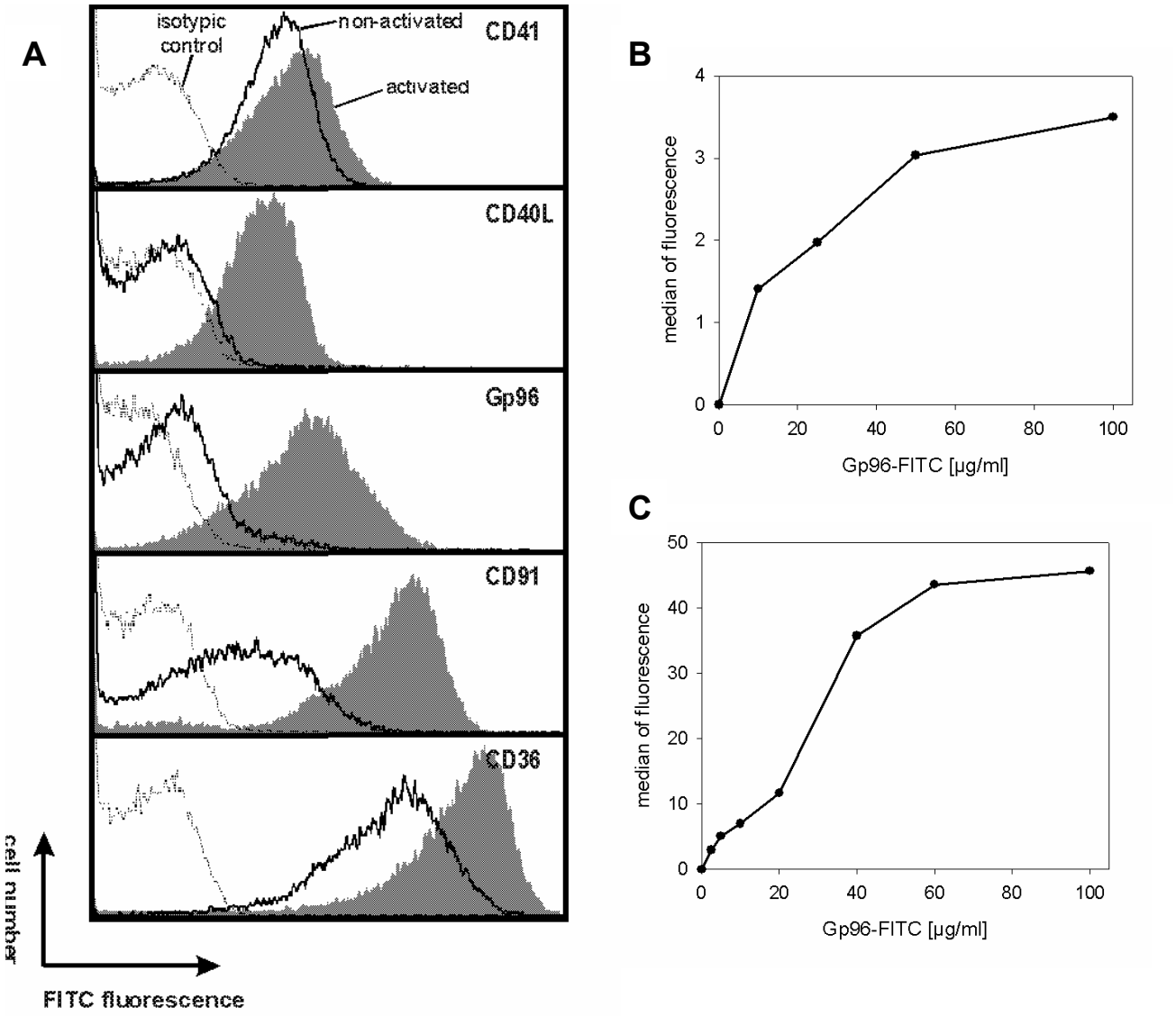


**Figure 1. Gp96 binds to non-activated and thrombin-activated human platelets.**
Freshly prepared platelets from human blood were incubated for 3 min at 37 °C with 0.2 U/ml thrombin or without effector and fixed with paraformaldehyde. (A) After extensive washing platelets were stained with antibodies specific for CD41, CD40L, CD91 and CD36 or with 50 µg/ml FITC-labeled Gp96 and analyzed by flow cytometry. Isotypic control staining is shown as dotted lines, staining of non-activated platelets as solid lines and of thrombin-activated platelets in filled gray. Non-activated (B) or thrombin-activated (C) platelets were stained with different concentrations of Gp96-FITC for 30 min at 4 °C and analyzed by flow cytometry. The data shown are representative for at least four independent experiments.

Maximal binding is again tenfold higher on activated than on non-activated platelets. In both cases, half-maximal binding is achieved between 20 and 30 µg/ml Gp96-FITC which is comparable to the binding characteristics on monocytes and DCs.[9] Another feature of specific binding of a labeled ligand to its receptor at saturating concentrations is that it can be specifically competed by the same unlabeled ligand. Figure 2 A shows the binding of 50 µg/ml Gp96-FITC to activated platelets in the presence of different amounts of unlabeled Gp96. Increasing amounts of competitor reduce the Gp96-FITC binding as expected for specific ligand/receptor interaction. Ovalbumin and BSA as control proteins were not able to compete Gp96-FITC binding (Figure 2 A,B). The same competition pattern was observed for non-stimulated platelets (Figure 2 B). We tried to compete Gp96-FITC binding with monoclonal antibodies against CD36 and CD91, to investigate whether these two receptors are also involved in Gp96 binding to platelets. While competition with appropriate isotypic controls did not result in reduced Gp96-FITC staining, two anti-CD36 clones and an antibody against the 85 kDa subunit of CD91 competed with Gp96 binding (Figure 2 C). An antibody against the platelet marker CD41 did not alter Gp96-FITC staining (data not shown), supporting the specificity of the observed competition. This suggests that both, CD36 and CD91, are involved in Gp96 binding to platelets.

### Gp96 does not influence platelet activation and aggregation

We tested whether Gp96 was able to interfere with thrombin induced platelet activation. Freshly isolated platelets were pre-incubated with 100 µg/ml Gp96 or ovalbumin as control for 15 min at 37 °C or were left untreated. 2.5 µM ADP, a weak inducer of platelet activation at this concentration, was included as positive control. Thereafter, thrombin was added in different concentrations varying from non-saturating amounts to fully activating concentrations. After 5 min incubation with thrombin, cells were fixed and expression of the platelet activation marker CD40L was measured by flow cytometry. Due to variations between different donors in their response to thrombin, data for individual donors are shown (Figure 3). Without thrombin only ADP-treated platelets show a slight increase in CD40L expression. Therefore, Gp96 does not trigger thrombocyte activation. Moreover, compared to control samples, Gp96 had no influence on platelet activation induced by saturating or lower concentrations of thrombin. It cannot be excluded that a weak activating effect of Gp96 was not visible in our experiments due to pre-activation during cell preparation. However, this

seems very unlikely because no significant staining with anti-CD40L antibody compared to the isotypic control was observed. Thus, the influence of platelet pre-activation was negligible.

The second important component of platelet function is their aggregation after injury in order to stop bleeding physically. To analyze the effect of Gp96 on thrombocyte aggregation freshly prepared PRP was incubated at 37 °C with 50 µg/ml Gp96 under continuous stirring in an aggregometer. No formation of aggregates could be observed (Figure 3B, horizontal parts of the curves before addition of other stimuli). Even after 15 min of incubation there was no induction of aggregation by Gp96 alone (data not shown). After 3 min platelet aggregation was induced either by 2.5 or 10 µM ADP, 10 µg/ml collagen or 50 µM adrenaline. Independent of the effector used no differences in the kinetics or final degree of aggregation could be observed between Gp96-pretreated and control samples.

### Platelets inhibit Gp96 induced DC maturation

While no interference of Gp96 with platelet function was apparent in our experiments, we now addressed the question whether the binding of Gp96 to platelets might interfere with the immunostimulatory effect of Gp96. We concentrated on dendritic cells, as the key cell type in the initiation of an immune response. Immature DCs were cultured with Gp96 for 2 days in the presence or absence of $2x10^4$ thrombin-activated autologous platelets per µl (concentration in blood: 1.5 – $4x10^5$ per µl). After 24 h a reduced concentration of the pro-inflammatory cytokines TNF-α and IL-12 in the supernatant could be observed when platelets were present in the cell culture (Figure 4 A). No difference was visible in the secretion of the anti-inflammatory and $T_H2$ promoting cytokine IL-10, which is only slightly induced in DCs by Gp96. Without Gp96-induced activation non of the three cytokines could be detected in the culture supernatant (data not shown). A similar cytokine pattern was observed after 48 h culture, and to a lower extent when non-stimulated platelets were used (data not shown). Additionally, DCs showed reduced upregulation of the activation markers CD83 and CD86 after 48 h activation with Gp96 in the presence of platelets (Figure 4 B). While CD83 expression was only reduced by approximately 20 % in all experiments performed, the reduction in CD86 expression was greater than 50 %.

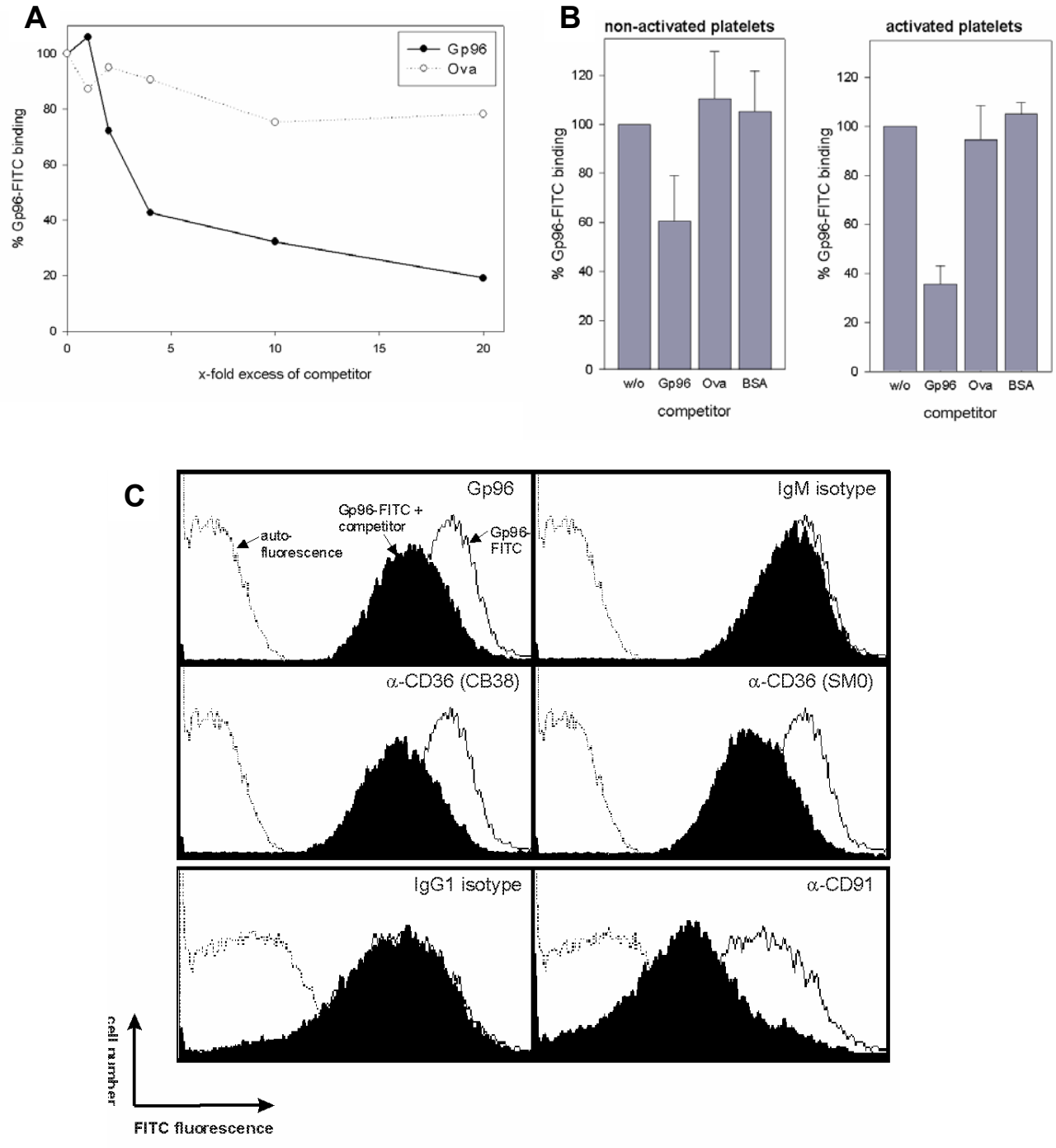


**Figure 2. Gp96-FITC binding to human platelets can be specifically competed by unlabeled Gp96 and by antibodies against CD36 and CD91.**
Platelets were fixed and incubated with 50 µg/ml Gp96-FITC after pre-incubation with competitor for 30 min on ice. Fluorescence of stained cells was analyzed by flow cytometry. (A) Different amounts of unlabeled Gp96 or ovalbumin were used for competition on thrombin-activated cells. Data shown are representative for three independent experiments. (B) Non-stimulated and thrombin-activated platelets were stained using a 10-fold excess of the indicated competitors. Fluorescence intensity without competitor was set as 100 %. Means of triplicates are shown and error bars represent SEM. (C) 500 µg/ml unlabeled Gp96, isotypic controls, monoclonal antibodies against CD36 (CB38, SM0; both IgM isotype) or against the 85 kDa subunit of CD91 (IgG1 isotype) were used for competition prior to staining of thrombin-activated platelets with 50 µg/ml Gp96-FITC (concentration of competing antibodies: 50 µg/ml). Autofluorescence is shown as dotted line, uncompeted and competed staining as solid line and filled histogram, respectively. The experiments were performed in triplicates.

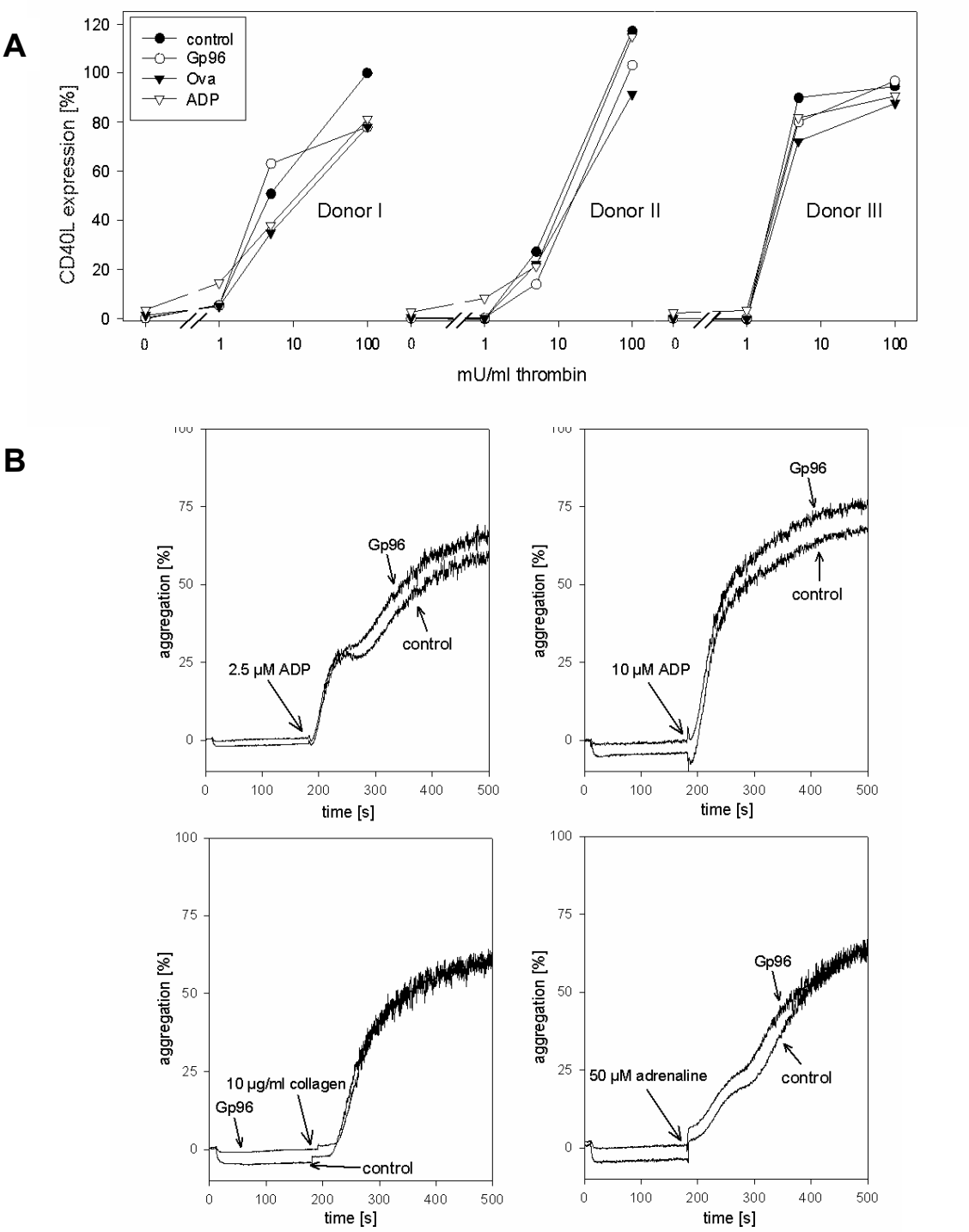


**Figure 3. Gp96 does not interfere with platelet function.**
(A) Freshly isolated platelets from three different healthy donors were pre-incubated with 2.5 µM ADP, 100 µg/ml Gp96 or ovalbumin at 37 °C for 15 min. Thereafter, thrombin at different concentrations was added. Following an additional 5 min incubation at 37 °C platelets were fixed and activation was analyzed by flow cytometry after staining with PE-labeled anti-CD40L antibody. Due to varying levels of CD40L expression between different donors, the obtained fluorescence intensities were converted into relative activation values. The median of fluorescence after maximal stimulation with 500 mU/ml thrombin was set as 100 % activation (donor 1: 23.71; donor 2: 28.13; donor 3: 18.11). The value for non-stimulated platelets was set as 0 % (2.94±0.24). (B) The influence of Gp96 on the aggregation of platelet rich plasma ($2.5x10^5$ cells/µl) was measured in an aggregometer. 10 s after start of measurement Gp96 was added to a final concentration of 50 µg/ml. The control samples were treated with buffer only. After 3 min 2.5µM or 10 µM ADP, 10 µg/ml collagen or 50 µM adrenaline were added and aggregation was followed for additional 320 s. Experiments were done in triplicates and repeated for three different donors.

The DC activating capacities of Gp96 were not caused by low amounts of endotoxin in the Gp96 preparations, since boiled Gp96 showed no stimulating effect on DCs (data not shown). In contrast, LPS-induced activation is heat-resistant as we have shown previously.[13] When PBMCs from the same donor were used instead of platelets, Gp96-mediated DC activation was not influenced (data not shown). Thus, the anti-inflammatory effect of platelet preparations was not due to contamination by other blood cells. Therefore, we conclude that platelets themselves reduce Gp96-induced DC activation resulting in lower secretion of pro-inflammatory cytokines and in a less pronounced differentiation to mature DCs. To analyze further the mechanism of this effect we performed transwell experiments to avoid cell-to-cell contacts between DCs and platelets. As another feature of this method more platelets could be used without affecting DC viability ($1x10^5/\mu l$, which is close to the lower edge of the physiological thrombocyte concentration in blood). As

shown in Figure 5, freshly prepared platelets were able to reduce Gp96-induced TNF-α production and up-regulation of CD86 and CD83 without direct cell-to-cell contact. In contrast to this, LPS-induced DC maturation was not affected by the presence of platelets (Figure 5 B). Moreover, even with PFA-fixed platelets, which had been activated with thrombin prior to fixation, TNF-α production was completely abrogated (Figure 5 A). This strongly suggests that no platelet derived soluble factor can be involved in the observed immunosuppressing effect of platelets, favoring a more passive mechanism where platelets simply compete with DCs for Gp96. In line with this, the inhibitory effect of platelets was more pronounced when thrombin-activated, fixed platelets were used (Figure 5 A), while fixed non-stimulated platelets caused only a lower reduction of DC activation (data not shown).

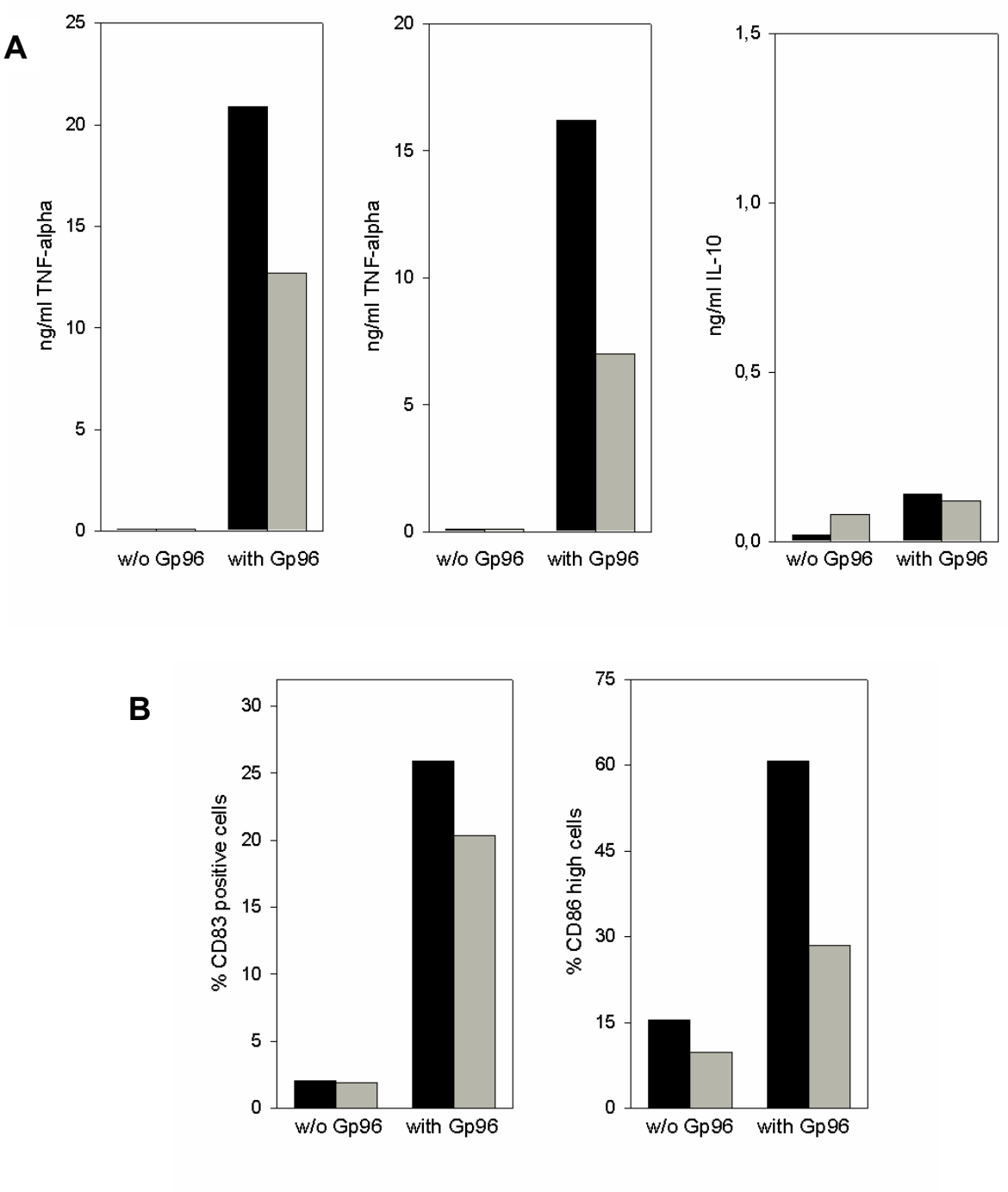


**Figure 4. Autologous platelets interfere with Gp96 induced DC maturation**
Human monocytes were cultured with GM-CSF and IL-4 to obtain immature DCs. After 6 days $4x10^6$ thrombin-activated platelets from the same donor were pre-incubated with 20 µg/ml Gp96 in a 96-well plate for 45 min followed by addition of $2x10^5$ immature DCs per well. (A) Cytokine concentrations of IL-12, TNF-α and IL-10 in the cell culture supernatant were determined after 24 h. (B) The maturation of DCs was measured by determining the number of $CD83^+$ and $CD86^{high}$ cells by flow cytometry. Values for DCs without platelets are shown as black bars, DC-platelet co-cultures as white bars. Data shown are representative for three independent experiments with cells from different donors.

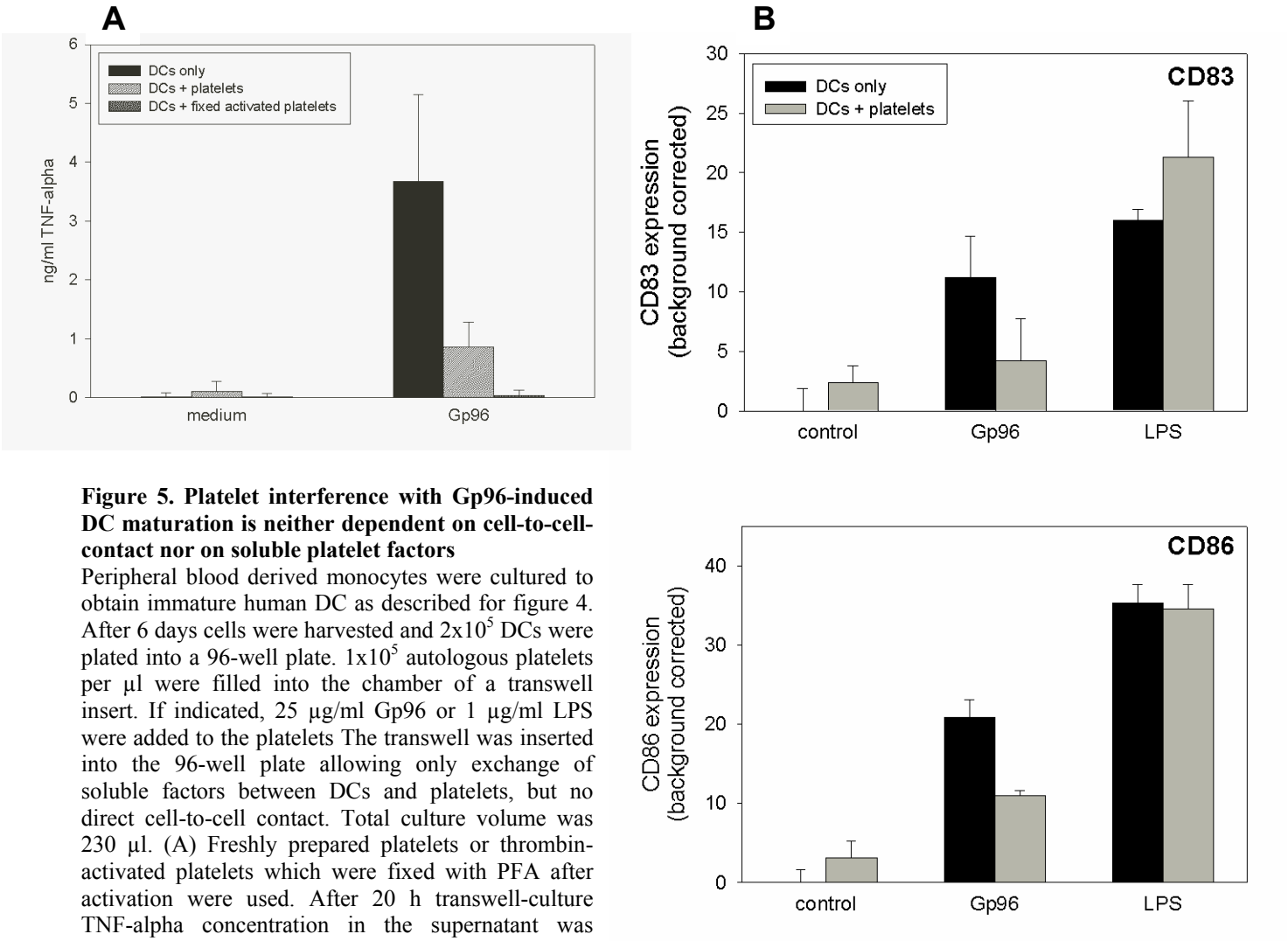


**Figure 5. Platelet interference with Gp96-induced DC maturation is neither dependent on cell-to-cell-contact nor on soluble platelet factors**
Peripheral blood derived monocytes were cultured to obtain immature human DC as described for figure 4. After 6 days cells were harvested and $2x10^5$ DCs were plated into a 96-well plate. $1x10^5$ autologous platelets per µl were filled into the chamber of a transwell insert. If indicated, 25 µg/ml Gp96 or 1 µg/ml LPS were added to the platelets The transwell was inserted into the 96-well plate allowing only exchange of soluble factors between DCs and platelets, but no direct cell-to-cell contact. Total culture volume was 230 µl. (A) Freshly prepared platelets or thrombin-activated platelets which were fixed with PFA after activation were used. After 20 h transwell-culture TNF-alpha concentration in the supernatant was determined. Mean values of duplicates are shown. The experiment was repeated twice. (B) In a different transwell experiment only freshly prepared, non-fixed platelets were used. After 48 h DCs were stained for CD86 and CD83 expression and analyzed by flow cytometry. Mean values of triplicates are shown with error bars representing SEM. The background activation (% activated cells in control samples without platelets) was subtracted from all values.

## Discussion

The ER-resident HSP Gp96 is released during necrotic cell death and activates dendritic cells. This feature, in combination with its ability to transfer intracellular peptides for their MHC class I restricted presentation allows Gp96, together with other HSPs like HSP70 and HSP90, to be a very efficient messaging system alerting the organism of bacterial or viral infections and possibly injury. Since HSPs are also released during mechanical tissue damage, control mechanisms have to exist that limit the HSP-mediated DC activation locally and prevent the release of pro-inflammatory cytokines in healing wounds. One mechanism has been

postulated when CD91 was identified as one of the receptor molecules for Gp96. Interaction of Gp96 with CD91 in the blood stream is inhibited by the presence of $\alpha_2$-macroglobulin which binds to CD91 as well.[27] Our data reported here indicate a second, more general mechanism: The immunostimulatory capacity of Gp96 is neutralized in the blood stream by the binding of Gp96 to thrombocytes. This interaction is specific as the binding of Gp96-FITC can be inhibited by unlabeled Gp96 molecules (Figures 1 and 2). By competition experiments with monoclonal antibodies the two known receptor candidates CD91 and CD36 could be identified as Gp96 receptors on platelets. Further investigations are needed to reveal whether they act together in a receptor complex or whether they form two independent receptors. More interestingly, Gp96-FITC binding is tenfold enhanced after the activation of thrombocytes (Figure 1). This implies an even greater neutralizing effect of platelets on the immunostimulatory capacity of Gp96, when activated during tissue damage accompanied by blood vessel disruption. The presence of physiological amounts of platelets reduces the Gp96-induced secretion of the pro-inflammatory cytokines TNF-$\alpha$ and IL-12 (Figure 4 A) and maturation of DCs as analyzed by the diminished upregulation of the activation markers CD83 and CD86 (Figure 4 B). Since the activation status of DCs correlates with their ability to stimulate T cells,[13] the induction of immune responses will be impaired. Moreover, the immature DC phenotype and the reduced levels of pro-inflammatory cytokines in the presence of platelets might even favor the induction of tolerance towards self antigens released during mechanical tissue damage and possibly presented by immature DCs.[28-31]

It is important to note that the binding of Gp96 to platelets does not interfere with their function. This is demonstrated by unchanged platelet activation and aggregation in the presence of Gp96 (Figure 3 A and B). The functional insensitivity of platelets towards Gp96 binding makes perfect sense because it ensures that while Gp96 is neutralized wound healing still can take place properly.

The binding of Gp96 on platelets has an impact on activation of DCs by HSPs when both are present in culture. This inhibition might be the result of competition: the high number of Gp96 binding sites on platelets is likely to reduce the concentration of free heat shock protein available for binding to APCs. Due to 80 % of the surface of platelets being invaginated building an open canalicular system, a great portion of bound Gp96 is not at all accessible for other cells but hidden in these invaginations. It cannot be excluded, however, that other mechanisms enhance or diminish the observed immunosuppressive effects of platelets. Thrombocytes themselves may respond to Gp96 binding in a yet unidentified way leading to

an altered DC activation. But at least in our system secreted platelet factors are not involved in immunosuppression, otherwise fixed platelets would not have been able to reduce DC activation (Figure 5 A).Another possible mechanism would be that activated platelets adhere to DCs and modify their response to external stimuli through direct interaction which has been shown for other cell types: Isolated monocytes produce various chemokines in the presence of activated platelets.[32-34] Autologous platelets enhance the IL-1 and TNF-α response of PBMCs after LPS stimulation.[35] For this latter effect a direct platelet adhesion to monocytic cells via P-selectin on the platelet surface has proven to be essential, although the activation signal itself is given by other, probably soluble, mediators.[32] P-selectin also mediates the specific interaction between neutrophils and activated thrombocytes,[36] but the implications on inflammatory events are controversially discussed.[37,38] Our results suggest that DCs unlike monocytes are not activated by platelets themselves, at least when low concentrations of platelets are used (Figure 4 B). The inflammatory response to Gp96 is not elevated but diminished. Moreover, this effect is not dependent on cell-to-cell contact as shown by transwell experiments (Figure 5). Nevertheless, a direct contact between platelets and DCs might contribute to the observed immunosuppressing effect of thrombocytes or might alter the activation state of DCs. Recently, it has been reported that Langerhans cells, a special DC subtype, are activated if co-cultured with fixed activated platelets from a heterologous source.[39] The authors attribute the observed maturation of Langerhans cells to the stimulating capacities of CD40L molecules on activated platelets.[19] In our experiments using only low concentration of activated living thrombocytes and DCs, no DC activation or pro-inflammatory cytokine production could be observed without addition of Gp96 (Figure 4).

Taken together, platelets might influence DC activation on at least three different levels: First, neutralization of DC activating substances (e.g. Gp96). Second, release of cytokines. Third, direct interaction via membrane associated molecules (e.g. CD40L/CD40). Another fact supports the relevance of the immunosuppressing effect of platelets *in vivo*. The platelet:DC ratio used in our experiments is far lower than that in peripheral blood. With 0.6 % DCs among all PBMCs and up to $2x10^6$ PBMCs per µl of blood a maximal DC concentration of 12 per µl can be calculated, while the platelet concentration varies between 1.5x and $4x10^5$ per µl. Thus, the platelet:DC ratio in human blood is around 10.000:1. In our experiments we used only ratios of 20:1 (co-culture ) or 115:1 (transwell experiment) with significant influence on Gp96-induced DC maturation. Regarding wounds, there are no reliable data on

the number of DCs and platelets in a scenario of tissue injury with blood vessel disruption, but it seems very unlikely that the platelet:DC ratio might be lower than 20:1.

Recently, other HSPs have been shown to interact with the Gp96 receptor CD91,[40,41] which is also expressed on thrombocytes. It has to be evaluated whether these proteins bind to platelets in a similar way as Gp96 does.

Nevertheless, the interaction of Gp96 and possibly other HSPs with thrombocytes can be expected to have important implications in the prevention of systemic inflammation and in reducing the secretion of pro-inflammatory cytokines in healing wounds.

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# Zusammenfassung

Das Hitzeschockprotein Gp96 ist der Zellbiologie schon lange als molekulares "Chaperone" (engl. für Anstandsdame) bekannt, da es an der Faltung von neu synthetisierten und fehlerhaft gefalteten Proteinen im Endoplasmatischen Retikulum (ER) beteiligt ist (eine Einführung zu Hitzeschockproteinen bietet der erste Teil des **Kapitels 2**). Auf der Suche nach neuen Tumorantigenen wurden von Pramod Srivastava und seinen Kollegen chemisch induzierbare Tumore aus Mäusen fraktioniert und die Fraktionen auf immunogene Komponenten untersucht. Schließlich wurde eine besonders immunogene Fraktion gefunden, die in der Lage war, eine Maus gegen den Tumor effektiv zu immunisieren. Die Analyse dieser Fraktion führte zur Entdeckung von Gp96 als Tumorantigen und seiner Einführung in die Immunologie. Die immunogene Komponente konnte schließlich identifiziert werden: Nicht Gp96 selbst, sondern Peptide, die nicht-kovalent im ER an Gp96 binden, sind für die Immunantwort verantwortlich, die zur Aktivierung zytotoxischer T-Zellen führt. Die Feststellung, daß antigenpräsentierende Zellen aus dem Knochenmark für die Gp96-vermittelte Immunantwort notwendig sind, führte zu folgender Hypothese: Gp96-Peptidkomplexe werden von antigenpräsentierenden Zellen (später wurde vermutet, es handele sich um dendritische Zellen) aufgenommen und die gebundenen Peptide werden auf MHC Klasse I-Moleküle zur Aktivierung von T-Zellen übertragen, ein Mechanismus, der im allgemeinen als "Cross-Presentation" (Kreuzpräsentierung) von Antigenen bezeichnet wird.

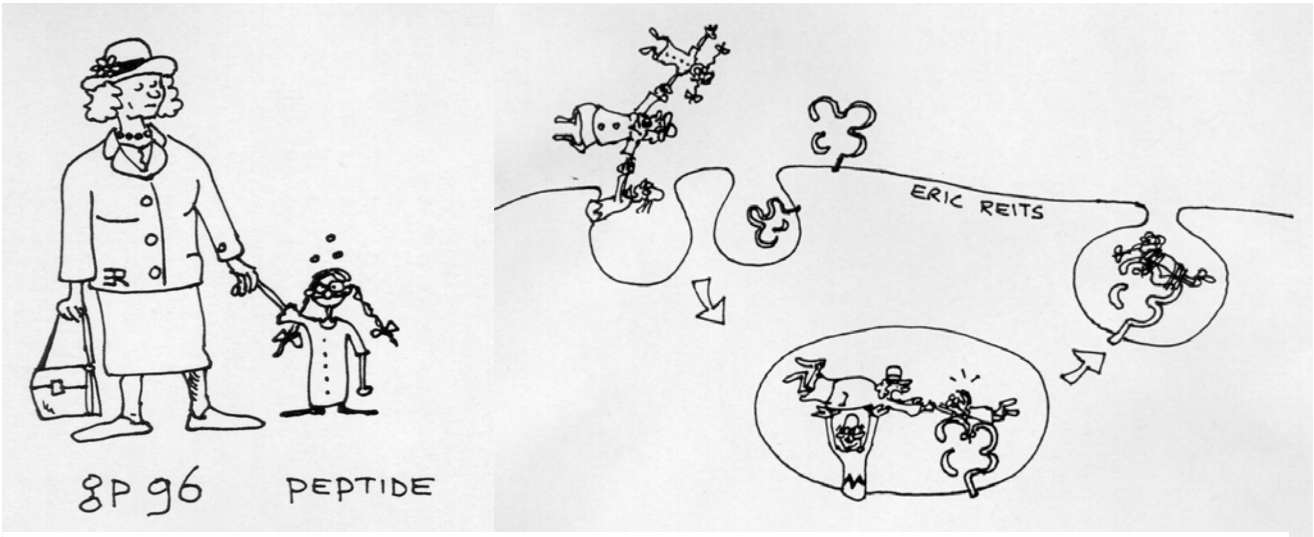


Abb. 1: Die Zeichnungen (angefertigt von Eric Reits während eines Forschungsaufenthalts des Autors dieser Dissertation im Netherlands Cancer Institute in Amsterdam), illustrieren eine Möglichkeit, wie Gp96-gebundene Peptide auf MHC Klasse I-Moleküle übertragen werden können. Die Hypothese, daß hierzu rezeptor-vermittelte Endozytose essentiell ist, wurde in dieser Arbeit nachgewiesen.

In nachfolgenden Arbeiten wurde andere Proteine identifiziert, die ebenfalls Peptide im ER oder Zytosol binden konnten. Eines dieser Proteine, die Proteindisulfidisomerase (PDI), bindet Peptide sogar besser als Gp96. Trotzdem ist nie gelungen, mit PDI-Peptid-Komplexen eine Immunantwort zu induzieren. Es wurde vermutet, daß Gp96 außer der Fähigkeit zur Peptidbindung noch andere Voraussetzungen erfüllen muß, um immunogen zu wirken; Voraussetzungen, die offensichtlich von PDI nicht erfüllt werden. Dabei wurden zwei (unabhängige, sich ergänzende) Hypothesen aufgestellt:

(1) Gp96-Peptid-Komplexe müssen von der antigenpräsentierenden Zelle besonders effektiv aus dem Extrazellulärraum aufgenommen werden. Diese Effektivität ist nur mit spezifischer, rezeptor-vermittelter Endozytose möglich.

(2) Gp96 kann die antigenpräsentierende Zelle (APC) so zu aktivieren, daß die Fähigkeit zur Antigenpräsentation erhöht ist.

Gegenstand dieser Dissertation ist die Verifizierung beider Hypothesen.

Gleichzeitig stellte sich die Frage, welche Relevanz Gp96 und andere Hitzeschockproteine mit ähnlichen Eigenschaften *in vivo* haben könnten. Die Vermutung liegt nahe, daß Gp96 nicht nur als Peptidtransporter bei der Immunisierung funktioniert, sondern auch *in vivo* als Vehikel für Antigene dienen könnte, nämlich dann, wenn eine Zelle – z. B. durch virale Infektionen – stirbt und ihre Proteine (inklusive Gp96) in den Extrazellulärraum entlassen werden. Die hierfür notwendigen Schritte sind in der Abbildung auf der folgenden Seite dargestellt.

(1) Eine Virusinfektion hat zur Folge, daß virales Antigen im Zuge der Antigenprozessierung (siehe hierzu **Kapitel 1** als allgemeine Einführung) im ER sowohl von MHC Klasse I-Molekülen als auch von Gp96 gebunden werden kann. Die Lyse der Zelle führt zum Entlassen der zellulären Proteine, u. a. auch Gp96. Gp96-Peptid-Komplexe werden spezifisch und rezeptorvermittelt von der APC aufgenommen, was ein besonders effektives Einschleusen von Antigen in die Prozessierungswege der APC ermöglicht. Der Nachweis der Bindung von Fluoreszein-markiertem Gp96 an einen oder mehrere spezifische Rezeptoren ist in **Kapitel 3** dargestellt. Der Nachweis der Spezifität gelang durch Kompetitionsexperimente mit unmarkiertem Gp96. Es kann gezeigt werden, daß nur antigenpräsentierende Zellen (untersucht wurden dendritische Zellen, Monozyten, Makrophagen und B-Zellen), nicht jedoch T-Zellen Rezeptoren für Gp96 besitzen.

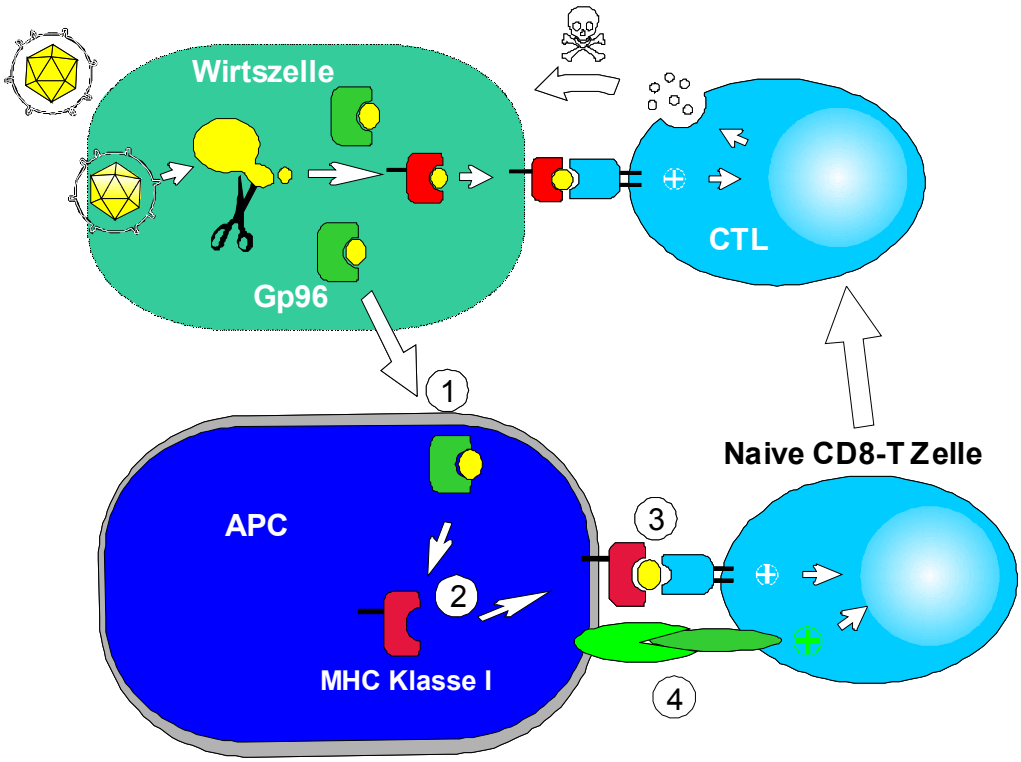


Abb. 2: Schematische Darstellung des 'Cross-Primings'. Die Punkte (1) bis (4) sind im Text erläutert.

Eine solche spezifische, rezeptor-vermittelte Bindung kann bei Gp96, nicht jedoch bei PDI nachgewiesen werden (**Kapitel 5**). Insofern wird bestätigt, daß die Fähigkeit zur Bindung an einen Rezeptor vermutlich eine wichtige Voraussetzung für ein peptidbindendes Protein ist, immunogen zu wirken.

(2) Damit Gp96-gebundene Peptide kreuzpräsentiert werden können, müssen sie nach der Endozytose auf MHC-Klasse I-Moleküle übertragen werden. Es ist immer noch nicht klar, an welcher Stelle der Antigenprozessierung diese "Übergabe" erfolgt. In dieser Arbeit kann jedoch gezeigt werden, daß nach rezeptorvermittelter Endozytose von Gp96 in dendritischen Zellen (**Kapitel 3**) und Monozyten (**Kapitel 5**) Gp96 tatsächlich mit MHC Klasse I-Molekülen in Vesikeln kolokalisiert. Somit wird vermutet, daß in diesen Beladungsvesikeln, die kürzlich charakterisiert worden sind, der Transfer des Peptides stattfindet.

(3) Wesentlich ist der Nachweis, ob solche durch Gp96 kreuzpräsentierte Peptide auch tatsächlich zur Aktivierung von antigenspezifischen T-Zellen führen. Hierzu wurde (wie in **Kapitel 3** dargestellt) Gp96 mit einem viralen Epitop beladen. Tatsächlich werden T-

Zellen nach Aufnahme der Gp96-Peptid-Komplexe durch dendritische Zellen aktiviert. Interessanterweise kann diese Kreuzpräsentation mit irrelvantem, unbeladenem Gp96 verhindert werden, so daß darauf geschlossen wird, daß nur die rezeptor-vermittelte Endozytose und nicht die unspezifische Makropinozytose zur Kreuzpräsentation von Gp96-gebundenem Antigen führt.

(4) Um naive T-Zellen zu aktivieren ("primen"), sind zwei Signale notwendig. Erstens die Präsentation des T-zell-spezifischen Peptids auf dem MHC-Molekül (siehe (3)). Zweitens ein oder mehrere kostimulatorische Signale (z. B. durch die B7-Moleküle), die von der APC ausgelöst werden und stimulatorisch auf die T-Zelle wirken. Im **Kapitel 4** kann gezeigt werden, daß Gp96 nicht nur in der Lage ist, Peptide zu präsentieren und in den Antigenprozessierungsweg von APCs einzuschleusen, sondern auch die Fähigkeit besitzt, dendritische Zellen (DCs) so zu aktivieren, daß diese ihre kostimulatorischen Signale hochregulieren. Solche Gp96-aktivierten DCs sind darüber hinaus in der Lage, T-Zellen effektiver zu aktivieren und können pro-inflammatorische Zytokine ausschütten, die ein Zustandekommen der Immunantwort begünstigen.

Eine solche "geprimte" T-Zelle kann schließlich ihre Effektorfunktion entfalten und die Zellen, die ihr spezifisches Epitop präsentieren (z.B. ein virales Antigen, das auf virusinfizierten Wirtszellen präsentiert wird), eliminieren (Abb. 2).

Somit kann in dieser Dissertation gezeigt werden, daß das Hitzeschockprotein drei Funktionen in sich vereint, die es zum idealen "Cross-Priming"-vermittelnden Antigentransporter machen. Erstens die Möglichkeit, Peptides zu binden; zweitens die Fähigkeit, rezeptorvermittelt APCs anzusteuern und drittens die Befähigung, diese APCs gleichzeitig zu aktivieren und sie so zum Priming von T-Zellen zu prädestinieren.

Es hat sich gezeigt, daß zwei verschiedene Rezeptoren für die Endozytose und für die Aktivierung von dendritischen Zellen notwendig sind. Während die Gruppe von Pramod Srivastava den $\alpha_2$-Makroglobulinrezeptor (CD91) und den "Scavenger"-Rezeptor CD36 als Endozytose-Rezeptoren für Gp96 identifizieren konnte (letzterer wurde in Kapitel 4 schon vor Veröffentlichung durch Srivastava als potentieller Kandidat genannt), kann unsere Gruppe feststellen, daß für die Aktivierung der Toll-like Rezeptor 4 und in geringerem Ausmaß der

Toll-like Rezeptor 2 notwendig ist (siehe **Kapitel 6**). Beide Rezeptoren spielen in der Abwehr von Mikroorganismen durch das angeborene Immunsystem eine große Rolle. Somit konnte gezeigt werden, daß das Hitzeschockprotein Gp96 in der Lage ist, sowohl das angeborene als auch das adaptive (oder spezifische) Immunsystem zu aktivieren.

Es liegt nahe, daß die Fuktion eines Moleküls mit solchen multiplen Eigenschaften vom Organismus kontrolliert werden muß. Insbesondere eine unkontrollierte, systemische Wirkung ist zu vermeiden, die möglicherweise zu einer Überreaktion führen könnte, die einem septischen Schock ähnelt. In **Kapitel 7** ist ein solcher Kontrollmechanismus skizziert. Unsere Gruppe konnte zeigen, daß Blutplättchen, insbesondere aktivierte Bluttplättchen wie sie zum Beispiel nach einer Verwundung vermehrt auftreten, sehr gut und rezeptorspezifisch Gp96 binden können. Dieses gebundene Gp96 steht anschließend nicht mehr für die Aktivierung dendritischer Zellen bereit. Somit wird im Blutkreislauf eine systemische Ausbreitung von Gp96 verhindert.

Basierend auf den Daten dieser Dissertation und der Arbeit von anderen Gruppen kann folgendes Modell aufgestellt werden:
Nekrose ist der Zelltod, der zum Beispiel durch zellulären Streß, Verletzungen und viraler Lyse ausgelöst wird. Apoptose ist der progammierte Zelltod, wie er zum Beispiel bei der

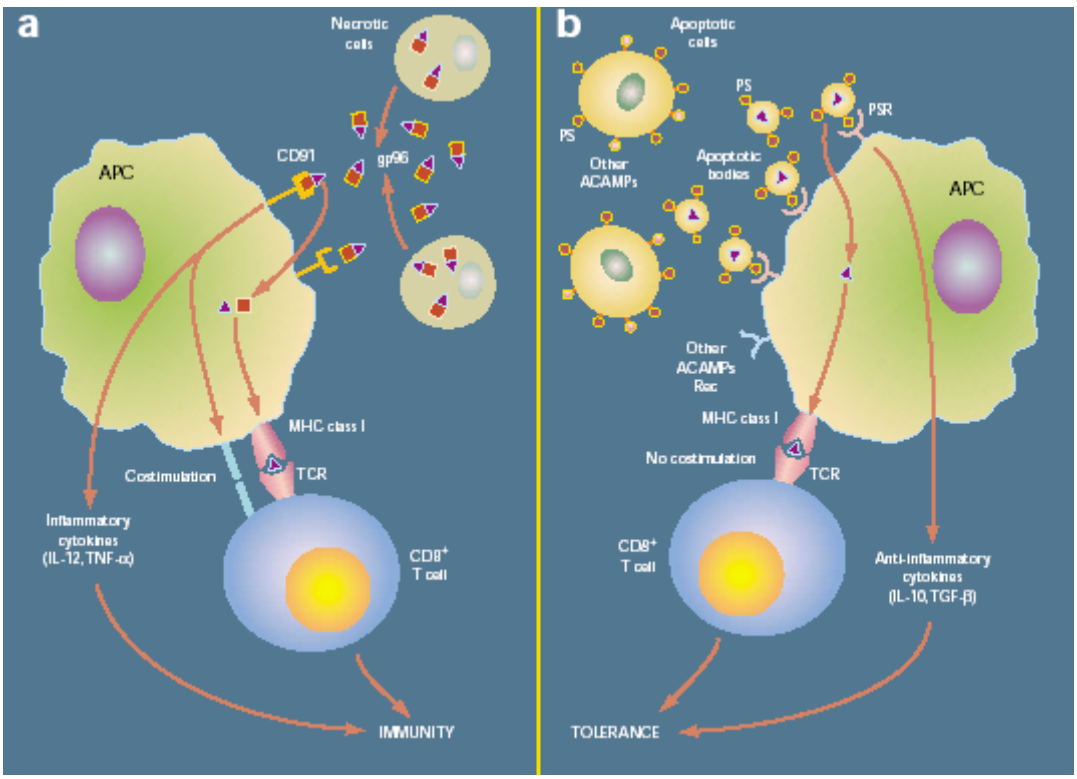


Abb. 3: aus Schild H. und Rammensee H.G., Nature Immunol., 1:100-101 (2000)

Entwicklung des Organismus' als regulärer Prozeß auftritt. Während die Nekrose häufig eine Folge von Infektionen ist und daher die Auslösung einer Immunantwort wünschenwert wäre, sollte Apoptose nicht zur Immunität führen. In der Tat finden sich in nekrotischen Zellüberständen Hitzeschockproteine wie Gp96, nicht jedoch in den Überständen von apoptotischen Zellen (siehe Kapitel 2). Solche nekrotisch entlassenen Gp96-Moleküle sollten in der Lage sein, Antigen aus der gestorbenen Zelle zu APCs in der Umgebung zu transferieren und gleichzeitig die APC zu aktivieren. Dies führt zur Präsentation der an Gp96 gebundenen Antigene, erhöhter kostimulatorischer Aktivität und zur Ausschüttung pro-inflammatorischer Zytokine, also zur Auslösung einer Immunantwort (Abb. 3, a). Hingegen werden bei der Apoptose apoptotische Vesikel gebildet. Auch diese können Antigen enthalten. Gleichzeitig können apoptotische Vesikel effektiv von APCs rezeptorvermittelt aufgenommen werden. Allerdings führt dies lediglich zur Kreuzpräsentation der in den apoptotischen Körperchen enthaltenen Antigenen, nicht aber zur Aktivierung der APC. Im Gegenteil, es werden sogar anti-inflammatorische Zytokine ausgeschüttet (siehe Kapitel 2), was zusammengenommen im allgemeinen zu immunologischer Toleranz führt (Abb. 3, b). Somit können Hitzeschockproteine wie Gp96 als nekrotische Signale oder Gefahrensignale (ein Begriff, den Polly Matzinger geprägt hat) aufgefaßt werden, die lokal wirken und systemisch reguliert werden. Weitere Hinweise zur Bestätigung dieses Modells ergeben sich aus Arbeiten unserer Gruppe: Wundflüssigkeiten, die *in vivo* als klassische nekrotische Körperflüssigkeiten aufgefaßt werden können, enthalten tatsächlich um ein vielfaches höhere Konzentrationen an Hitzeschockproteinen als im Blutserum. Im Laufe der Wundheilung nimmt die Konzentration von Gp96 in der Wundflüssigkeit ab, während sie im Serum konstant auf niedrigem Niveau verbleibt. (unveröffentlicht, Herter S., Singh-Jasuja H. et al.).

Hitzeschockproteine sind in der Zellbiologie als multifunktionale Proteine bekannt. Mit der hier vorliegenden Arbeit läßt sich diese Multifunktionalität erstmals auch auf das Gebiet der Immmunologie ausweiten. Gp96 kann Peptide durch den Extrazellulärraum transportieren, diese Peptide gezielt durch Bindung an CD91 zu dendritischen Zellen und anderen APC dirigieren und in den Kreuzpräsentationsweg einschleusen. Es kann gleichzeitig über die Toll-like Rezeptoren 2 und 4 die APC aktivieren, was zu vermehrter kostimulatorischer Aktivität und zur Ausschüttung von proinflammatorischen Zytokinen führt. Auf diese Weise reagiert Gp96 selbst wie ein Zytokin. Gleichzeitig kann es im endoplasmatischen Retikulum als

Aminopeptidase fungieren[1] und ist an der Faltung und damit an der Oberflächenexpression von Toll-like Rezeptoren beteiligt[2]. Jüngste Ergebnisse zeigen, daß Gp96 auch in der Lage ist, B-Zellen polyklonal zu aktivieren und daß dieser Prozeß unabhängig von Toll-like Rezeptoren zu funktionieren scheint (unveröffentlicht, Singh-Jasuja et al.). Der Autor dieser Disseratation arbeitet zur Zeit daran, den zuständigen Rezeptor auf B-Zellen zu identifizieren.

Wenn man die hier aufgezeigte Vielzahl der immunologischen Funktionen von Gp96 betrachtet, ist der Titel dieser Dissertation vielleicht nicht mehr so verwunderlich: "Gp96 – das Schweizer Taschenmesser des Immunsystems".


[1] Menoret A. et al., J. Biol. Chem., 276:33313-8 (2001)

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*Journal of Biological Chemistry,* in press

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