University of Eastern Piedmont
"A. Avogadro"
Department of Medical Sciences

PhD thesis in Molecular Medicine

XXI cycle (2005-2008)

GENETIC AND MOLECULAR MECHANISMS OF AUTOIMMUNITY AND IMMUNODEFICIENCY

Dott.ssa Elisa Cerutti

Tutor: ***Prof. Umberto Dianzani***
Coordinator: ***Prof. Umberto Dianzani***

….to my family…..

Since the beginning of time, people have yearned to explore the unknown, chart where they have been, and contemplate what they have found. The maps we make of these treks enable the next explorers to push ever farther the boundaries of our knowledge, about the earth, the sea, the sky, and indeed, ourselves…..

INDEX

SUMMARY

There are different approaches to obtain an “insight” in the pathogenesis of human diseases. One is to study particular genetic variants and how they affect functioning of specific proteins and protein pathways, in order to understand about physiological processes in normal and disease states. A comprehensive understanding of genetic variation would facilitate to establish relationships between genotype and biological function.

Within this thesis I describe how genetic variations could be a susceptibility factor for the complex pathogenesis of autoimmune disease, focusing on Autoimmune Lymphoproliferative Syndrome (ALPS). ALPS is due to inherited defects decreasing function of the Fas death receptor, involved in the immune response switching off. It is characterized by autoimmune manifestations and accumulation of non malignant lymphocytes in the lymphoid organs with expansion of double negative (DN) T cells lacking CD4 and CD8; this expansion is absent in an ALPS incomplete variant named Dianzani Autoimmune Lymphoproliferative Disease (DALD). Mutations of the Fas gene (TNFR6) are the most frequent causes of ALPS. They are usually heterozygous and their penetrance depends on their effect on Fas function. Mutations hitting the intracellular domain of Fas (death domain) involved in recruitment of FADD and caspase-8/10 and initiating the death signal are generally severe since exert a dominant-negative effect and display high penetrance. By contrast, mutations hitting the extracellular portion or causing haploinsufficiency have weak penetrance and may cause disease development depending on concurrence of other genetic or environmental factors. The first part of this thesis shows that concurrent factors may be genetic variations hitting either the Fas pathway itself or other pathways co-involved in the immune response switching off.

The first report, describes two patients that are combined heterozygous for single nucleotide substitutions in the TNFRSF6 and CASP10 (caspase-10) genes. The first patient showed a heterozygous nucleotide substitution in TNFR6 (c334 -2a>g), located in the splicing-acceptor site in the third intron and determining the IVS3-2a>g splice site defect. The mutation results in skipping of exon 4, coding for an extracellular cysteine-rich domain, frameshift and premature termination after 38 codons. Sequencing of CASP10 detected a C>T substitution at nt1502 in exon 10 resulting in a

proline to leucine change (P501L) in the small catalytic subunit of the caspase. The mutation, was not detected in 80 healthy donors nor in 40 other ALPS patients. The second patient had a TNFR6 mutation in exon 2, causing a premature stop codon (Q47X). Sequencing of CASP10 detected a heterozygous nucleotide substitution (1337A>G) in exon 9 causing the Y446C amino acid change in the predicted protease domain of the small subunit. This variation has been previously associated to ALPS, but has been reported also in the healthy Caucasian population with allelic frequency ranging from 1.6 to 2%. Fas expression was reduced and caspase-10 activity was decreased in both patients. In both patients, the mutations were inherited from distinct healthy parents. Therefore, this work suggests that mutations of TNFR6 and CASP10 can cooperate in development of ALPS by hitting the Fas signaling pathway.

The second report shows that a concurrent factor favoring ALPS/DALD development may be perforin gene (PRF1) variations that decrease the function of this protein involved in cell-mediated cytotoxicity. Perforin is stored in the lytic granules of cytotoxic cells and is released on the target cell surface where it forms pores allowing entry of granzymes inducing target cell apoptosis. Cell-mediated cytotoxicity is crucial for clearance of viral infections, but plays also a role in switching off the immune response by killing of activated immune cells. Sequencing of PRF1 in 14 ALPS, 28 DALD, and 816 controls detected two variations: the N252S amino acid substitution in 2 ALPS, and the A91V amino acid substitution in 6 DALD. Frequency of N252S was higher in ALPS than in controls (7.1% vs 0.1%, p=0.0016) and conferred an OR=62.7 (95% CI: 6-654.9); frequency of A91V was higher in DALD than controls (12.5 % vs 4.6%, p=0.016) and conferred an OR=3 (95% CI: 1.2-7.1). In one N252S patient, NK activity was strikingly defective in early childhood, but became normal in late childhood. A91V patients displayed lower NK activity than controls. These data suggest that PRF1 variations are a susceptibility factor for development of ALPS and DALD in subjects with defective Fas function, possibly because both defects affect the immune response switching off system. Other data show that these variations may also be involved in development of type 1 diabetes mellitus and multiple sclerosis.

Involvement of NK defects in autoimmunity prompted my interest on molecules involved on release of lytic granules by cytotoxic cells, since they might be candidate molecules involved in diseases caused by defective cell-mediated cytotoxicity. The work was focused on the role of the kinase suppressor of Ras-1 (KSR1) and the third report shows that KSR1-deficient mice display reduction of NK cell cytotoxicity. The

defect was not mediated by defective cell-cell adhesion, since the absence of KSR1 did not affect the ability of NK cells to form conjugates with target cells. Instead, it might be mediated by defective polarization of lytic granules, which required KSR1 function. Search for the mechanism causing this defect showed that KSR1 recruitment to the membrane is required for recruitment of active ERK to the immunological synapse, which may be crucial to allow ERK to phosphorylate specific substrates at the plasma membrane. Indeed, ERK-dependent phosphorylation of the PXSP motif in Lck (crucial in enhancing T-cell activation) was diminished after KSR1 suppression. These data suggest that KRS1 may be crucial for cell-mediated cytotoxicity and may be a promising candidate molecule involved in diseases caused by defective cell-mediated cytotoxicity.

PUBBLICATIONS

1: Giurisato E, Lin J, Harding A, **Cerutti E**, Cella M, Lewis RE, Colonna M, Shaw AS. The mitogen-activated protein kinase scaffold KSR1 is required for recruitment of extracellular signal-regulated kinase to the immunological synapse. *Mol Cell Biol*. 2009 Mar;29(6):1554-64.

2: Orilieri E, Cappellano G, Clementi R, Cometa A, Ferretti M, **Cerutti E**, Cadario F, Martinetti M, Larizza D, Calcaterra V, D'Annunzio G, Lorini R, Cerutti F, Bruno G, Chiocchetti A, Dianzani U. Variations of the perforin gene in patients with type 1 diabetes. *Diabetes*. 2008 Apr;57(4):1078-83.

3: **Cerutti E**, Campagnoli MF, Ferretti M, Garelli E, Crescenzio N, Rosolen A, Chiocchetti A, Lenardo MJ, Ramenghi U, Dianzani U. Co-inherited mutations of Fas and caspase-10 in development of the autoimmune lymphoproliferative syndrome. *BMC Immunol*. 2007 Nov 13;8:28.

4: Castelli L, Comi C, Chiocchetti A, Nicola S, Mesturini R, Giordano M, D'Alfonso S, **Cerutti E**, Galimberti D, Fenoglio C, Tesser F, Yagi J, Rojo JM, Perla F, Leone M, Scarpini E, Monaco F, Dianzani U. ICOS gene haplotypes correlate with IL10 secretion and multiple sclerosis evolution. *J Neuroimmunol.* 2007 May;186(1-2):193-8.

5: Clementi R, Chiocchetti A, Cappellano G, **Cerutti E**, Ferretti M, Orilieri E, Dianzani I, Ferrarini M, Bregni M, Danesino C, Bozzi V, Putti MC, Cerutti F, Cometa A, Locatelli F, Maccario R, Ramenghi U, Dianzani U. Variations of the perforin gene in patients with autoimmunity/lymphoproliferation and defective Fas function. *Blood.* 2006 Nov 1;108(9):3079-84.

INTRODUCTION

Most inherited diseases are rare, but taken together, the more than 3,000 disorders known to result from single altered genes rob millions of healthy and productive lives. Today, little can be done to treat, let alone cure, most of these diseases. A better understanding of the etiology and pathogenesis of diseases and the development of better treatments have, therefore, important implications, not only for the individual patient but also for health care and the socioeconomic sector in general. As ‘experiments of nature’, human diseases and their study offer insight into basic mechanisms of the human immune system.

Similar to other highly prevalent diseases, for example cardiovascular diseases, Alzheimer's disease or psychiatric disorders such as schizophrenia or depression, autoimmune diseases are complex with respect to their genetic background and potential environmental risk factors, and consequently also the multiple pathogenic factors that contribute to tissue damage. With respect to clinical presentation, patients that have similar signs and symptoms have previously been bundled together under one disease heading, but it is becoming clear that such general disease labels might not adequately reflect the above complexity and could create difficulties when looking for biological markers or when testing new treatments.

The Human Genome Project helped scientists to better understand etiology and pathogenesis of disease. In fact, once a gene is located on a chromosome and its DNA sequence worked out, scientists can then determine which protein the gene is responsible for making and find out what it does in the body.

There are different approaches to obtain an “insight” in the pathogenesis of human diseases. One of these approaches is to analyze the phenotype of a patient and compare the features with which of other diseases, in order to identify the pathway, the proteins and the genes responsible for the pathological defect. Genetics seeks to correlate variations in DNA sequence with phenotypic differences. The greatest advances in human genetics have been made for traits associated with variations in a single gene. However, most phenotypes, including common diseases and variable responses to pharmacological agents, have a more complex origin, involving the interplay between

multiple genetic factors (genes and their products) and non-genetic factors (environmental influences). Unraveling such complexity will require both a complete description of the genetic variation in the human genome and the development of analytical tools for using that information to understand the genetic basis of disease.

A comprehensive understanding of genetic variation, both in humans and model organisms, would facilitate studies to establish relationships between genotype and biological function. The study of particular variants and how they affect the functioning of specific proteins and protein pathways will yield important new insights about physiological processes in normal and disease states. An enhanced ability to incorporate information about genetic variation into human genetic studies would introduce us in a new era for investigating the genetic bases of human diseases and drug responses.

On the other hand, genes and gene products do not function independently, but participate in complex, interconnected pathways, networks and molecular systems that, taken together, give rise to the workings of cells, tissues, organs and organisms. Defining these systems and determining their properties and interactions is crucial to understanding how biological systems function. Yet these systems are far more complex than any problem that molecular biology, genetics or genomics has yet approached. On the basis of previous experience, one effective path will begin with the study of relatively simple model organisms, such as bacteria and yeast, and then extend the early findings to more complex organisms, such as mice and humans. Alternatively, focusing on a few well-characterized systems in mammals will be a useful test of the approach. Understanding biological pathways, networks and molecular systems will require information from several levels. At the genetic level, the architecture of regulatory interactions will need to be identified in different cell types, requiring, among other things, methods for simultaneously monitoring the expression of all genes in a cell. At the gene-product level, similar techniques that allow in vivo, real-time measurement of protein expression, localization, modification and activity/kinetics will be needed. It’s important to develop and refine techniques that modulate gene expression, such as conventional gene-knockout methods, newer knock-down approaches and small-molecule inhibitors to establish the temporal and cellular expression pattern of individual proteins and to determine the functions of those proteins. This is a key first step towards assigning all genes and their products to functional pathways.

Within this thesis I'll describe how genetic variations could be a susceptibility factor for the complex pathogenesis of autoimmune disease, in particular for the Autoimmune Lymphoproliferative Syndrome (ALPS). I show that ALPS, that is regarded as a classic monogenic disease generally due to mutations hitting function of the Fas gene involved in switching off the immune response, may sometimes be the outcome of multiple mutations affecting different steps of the Fas signaling pathway or other pathways involved in the immune response switching off.

1-APOPTOSIS IN THE IMMUNE SYSTEM

The immune system is charged with the complex task of providing defense against a vast array of potential pathogens, whilst ensuring that those same protective mechanisms are not turned against the self. Mechanisms of physiological cell death (apoptosis) play key roles in the development, regulation and functioning of the immune system. Malfunctioning of the cell death process can cause autoimmune disease, immunodeficiencies, and lymphoid malignancies. Apoptosis is the physiological process of cell death that occurs in all multicellular organisms. The process of apoptosis may be divided into stages: the stimuli that trigger the cell death response; the pathway by which the message is transduced to the cell; and the effector mechanisms that implement the death program (Vaux et al, 1996). Diverse stimuli may trigger a death response in cells but the pathways converge upon the same, evolutionally conserved effector mechanisms, the key components of which are a family of cysteine proteases called caspases. Upon activation, these cysteine proteases directly or indirectly cause the morphological and biochemical changes characteristic of apoptosis, such as chromatin condensation and DNA fragmentation. Apoptotic cells are efficiently phagocytosed by neighbouring or inflammatory cells.

In the immune system, apoptosis plays an important role during positive and negative selection in lymphocyte development, in the "switching off" of the immune response, and in the effector function of cytotoxic cells.

1.1 Apoptosis of activated lymphocytes and homeostasis of lymphocyte numbers

Physiological elimination of activated T cells during an immune response can occur via an active extrinsic pathway triggered by the engagement of specialized transmembrane receptors called death receptors. These death receptors belong to the TNFR superfamily and include Fas (also called CD95, TNFRSF6, or APO-1), TNFR1 (also known as p60 or TNFRSF1A), death receptor 3 (also known as DR3, or TNFRSF25), death receptor 4 [also known as DR4, TNF related apoptosis-inducing ligand receptor 1 (TRAIL-R1), or TNFRSF10A], death receptor 5 (DR5, or TRAIL-R2 or TNFRSF10B), and death receptor 6 (DR6 or TNFRSF21). The TNFR superfamily is characterized by arrays of two to five extracellular cysteine-rich domains (CRDs) (Locksley et al, 2001). Members of the death receptor subgroup of TNFRs share a moderately conserved region of 80 amino acids in the cytoplasmic portion termed the death domain (DD), as it is required for death signaling. Fas, the prototypical death receptor, plays a critical role in lymphocyte regulation by apoptosis and, potentially, non-apoptotic death. Fas is activated by its cognate ligand, FasL, a type 2 transmembrane protein that can be released in soluble form by metallo proteinases (Suda et al, 1993). Both the ligand and the receptor can be potently upregulated by antigenic stimulation of T cells, especially if cells are activated and cultured in interleukin IL-2 (Zheng et al, 1998). Stimulation of B cells can induce expression of death receptors and TNFα, but not expression of FasL. Rather, B cells appear to be killed by conjugation to T cells that express FasL (Wang et al, 1997). The fundamental mode of signaling by death receptors is protein complex formation at two levels. First, the receptor assembly is triggered by ligand to form stoichiometric complexes with cytoplasmic signaling proteins. Second, higher order aggregates of receptors, ligands, and signaling proteins immediately follow and are crucial for signal transmission. In the Fas/FasL system, within seconds after FasL stimulation, Fas recruits the adapter molecule FADD and the initiator caspase-8 and -10 to form a large complex termed death-inducing signaling complex (DISC) (Kischkel et al, 1995). DISC formation is crucial for generating caspase activity and initiate the apoptotic process. Both Fas and FasL operate as homotrimers. Their interaction triggers changes in conformation and/or orientation of the receptor trimer that allow homotypic interactions between the DDs in Fas and FADD. In addition to its COOH terminal DD, FADD also possesses a NH2-terminal protein-protein-interaction domain of approximately 80 residues called the death effector domain (DED), which is also found

in the initiator (or apical) caspases-8 and -10. Remarkably, DD and DED are both hexahelical bundles with a similar overall topology that is also shared by the caspase-recruitment domain (CARD) (Fesik et al, 2000). Hence, these domains may be specializations of a common ancestor. It is generally the case, however, that each domain interacts in a homotypic manner, i.e., DD to DD, but do not cross bind one another (DD to DED, etc.). Therefore, FADD is brought to the DISC by its DD association with Fas and then recruits caspases-8 and -10 to the complex through DED interactions (Figure 1) (Tibbets et al, 2003; Zhang et al, 1998).

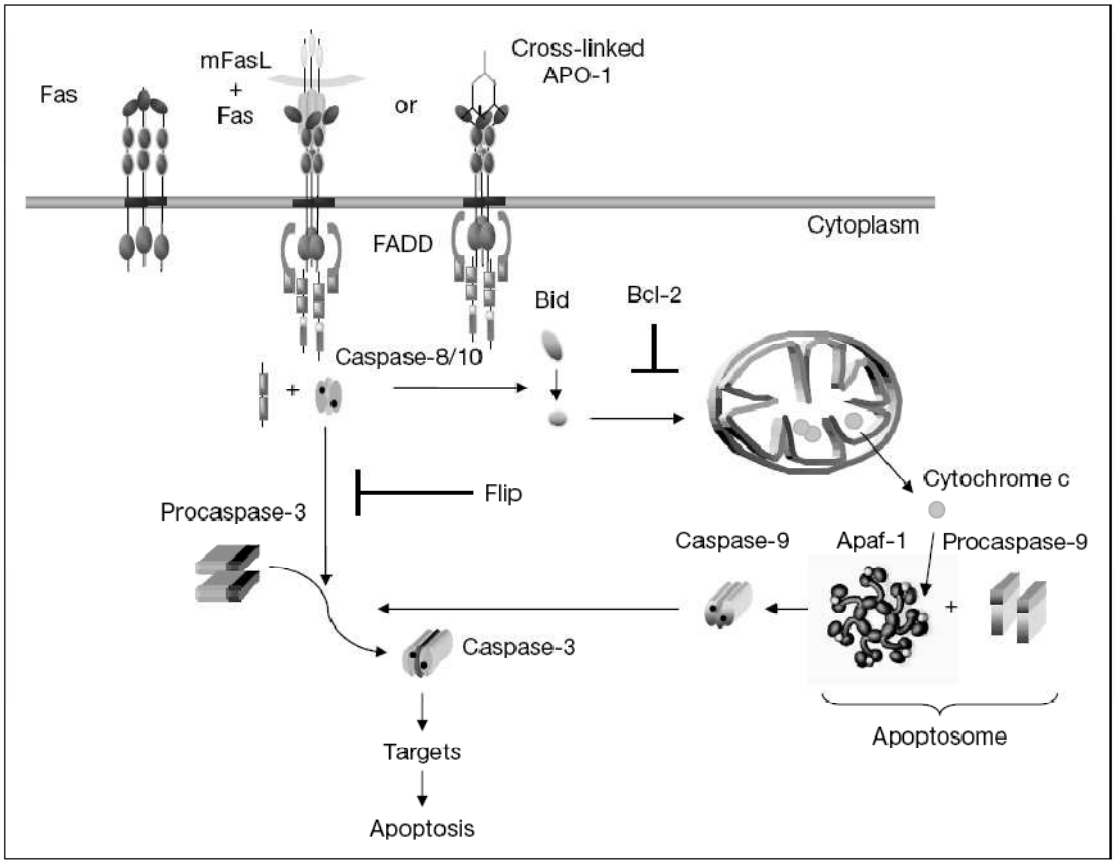


Figure 1. Fas signaling pathway. Fas is self-trimerized through interactions of the aminoterminal domain termed pre-ligand-associating domain (PLAD). Upon interaction with membrane FasL (mFasL), homophilic interactions of death domains allow the association of Fas with the cellular adapter called FADD. FADD contains another domain called the death effector domain allowing interactions with procaspase-8 (Mach/Flice) and 10 (Flice-2) in a death-inducing signaling complex (DISC), thereby connecting Fas to a proapoptotic pathway.

Within this multiprotein DISC aggregate, the increased local concentration of caspase-8/10 zymogens presumably promotes an altered conformation that leads to their autoprocessing and activation. Caspases-8 and -10 each contain three parts: a double-

DED containing prodomain, a large enzymatic subunit, and a small enzymatic subunit. Each part is flanked by aspartate residues that form caspase cleavage sites especially sensitive to its own activity. Initially, it was believed that autoprocessing and release of the two enzymatic subunits were essential for death signaling (Nicholson et al, 1997). However, recent data has suggested that apical caspases can, under certain conditions, transmit signals without cleavage by relying only on dimerization or other rearrangements of inactive unprocessed monomers (Boatright et al, 2003). Nevertheless, processing of apical caspases stabilizes the catalytic activity as heterotetramers (two each of the large and small subunits) and allows its release into the cytosol (Boatright et al, 2003). The released, highly active caspases cleave multiple downstream targets, including effector caspases that initiate a caspase amplification loop leading to the cell's demise. In the so-called type I cells, including thymocytes and peripheral T cells, a high quantity of active caspase-8/10 is generated at the DISC that directly activates effector caspases, i.e., caspases-3, -6, and -7. By contrast, in type II cells, including tumor cell lines such as Jurkat and CEM, the DISC is weaker, and caspase activation occurs more slowly and to a lesser extent than in type I cells. In type II cells, robust apoptosis is achieved by caspase-8 processing of Bid, a proapoptotic BH3-only Bcl-2 family member. The truncated active form of Bid causes cytochrome *c* release from the mitochondria and activation of the intrinsic apoptosis pathway (Jiang et al, 2004). Because of mitochondrial involvement, death receptor–mediated death in type II cells, but not type I cells, can be inhibited by anti-apoptotic Bcl-2 family members (Krueger et al, 2003). DISC formation is followed by the formation of Fas clusters recently designated as signaling protein oligomerization transduction structures (SPOTS) (Algeciras et al, 2002; Siegel et al, 2004). SPOTS are composed of perhaps thousands of individual receptor complexes (making them visible microscopically) that further increase the local concentration of caspase-8 and promote its autoprocessing and activation (Siegel et al, 2004). This receptor oligomerization depends on the Fas DD and FADD but does not require caspase activity. Once SPOTS are formed, a further caspase-dependent clustering and capping of Fas complexes is followed by receptor internalization in vesicles bearing endosomal markers. It is unclear whether internalization downregulates Fas-induced death or is a critical step for death signaling, as recently shown for the TNF receptosomes (Schneider-Brachert et al, 2004). Alternatively, since Fas internalization depends on caspase activation, it may be a post-lethal event that has no regulatory role at all. One potentially important regulator of Fas

at the DISC level is the cellular FLICE inhibitory protein (c-FLIP) that is present in two isoforms: c-FLIPS and c-FLIPL (Thorne et al, 2001). The c-FLIPL protein is very similar to caspases-8 and -10, and the genes for all three are found in a gene cluster on chromosome 2q33-34; this observation suggests that the genes may have arisen by tandem duplication of one ancestral gene (Fernandes-Alnemri et al, 1996; Irmler et al, 1997; Rasper et al, 1998). Although a double DED is intact in c-FLIPL, the part of the gene that encodes the enzymatic subunits contains numerous mutations that prevent caspase activity (Irmler et al, 1997). c-FLIPS is closer in structure to a viral counterpart, v-FLIP, which consists only of the double DED and a short COOH-terminal addition (Irmler et al, 1997). Both c-FLIPL and c-FLIPS can be recruited to the DISC, where they typically exert an inhibitory effect on caspase-8 activation and cell death. c-FLIPL has been observed to have different, sometimes contradictory functions. c-FLIPL in the DISC is processed by caspase-8 but cannot process caspase-8 in return, so that the latter is kept in an uncleaved form at the receptor level (Krueger et al, 2001). B cell receptor stimulation can induce c-FLIPL expression and block Fas- and TRAIL-mediated killing; this observation indicates a potential role in B cell selection and tolerance (Wang et al, 2000). On the other hand, small amounts of c-FLIPL can cause caspase-8 activation and cell death and have also been proposed to promote Fas-induced lymphocyte proliferation (Thorne et al, 2001).

1.2 Apoptosis in cell-mediated cytotoxicity

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are highly effective killers of virally infected and tumorigenic cells. Although the receptors involved in recognition of targets by these cells differ, the mechanisms by which they kill are essentially the same. The lytic activity of CTLs and NK cells is localized in specialized granules in their cytoplasm and, through the regulated secretion of these granules, killer cells can selectively induce target cell death.

In the 1980s, several groups identified the active proteins present in CTL and NK cell granules (Millard et al, 1984; Podack et al, 1985). The key soluble protein was called perforin (Figure 2).

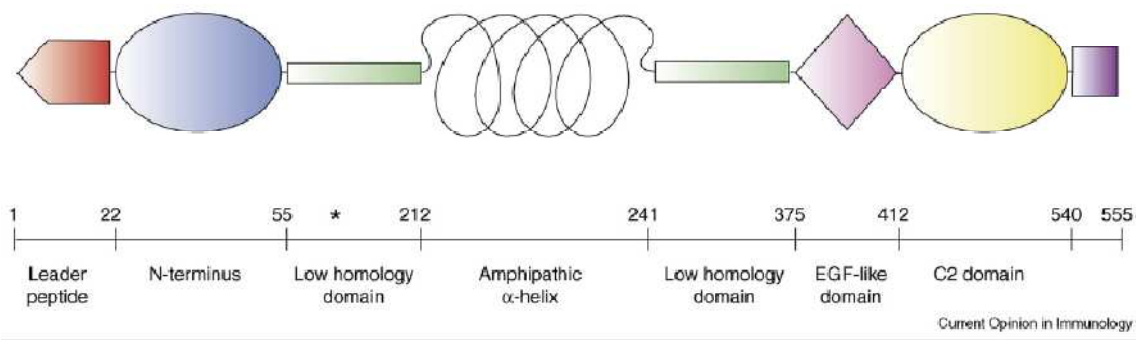


Figure 2. Perforin putative domain structure. The putative domains of the perforin monomer are shown. The leader peptide of human perforin contains an additional amino acid compared with mouse perforin. The amino terminus (N-terminus) is predicted to have lytic potential whereas the amphipathic α-helix is thought to insert into the target membrane and shares homology with terminal members of the complement membrane attack complex. The calcium-binding C2 domain confers the membrane binding property of perforin. Approximate amino acid boundaries of each domain are indicated by numbers.

Initially, the structural and functional similarity of perforin to proteins associated with complement-mediated lysis suggested that this protein contributed to target cell death simply by damaging the cell membrane through pore formation (Tschopp et al, 1986; Sauer et al, 1991). However, a series of experiments in which perforin and granzymes were overexpressed in the mast cell line RBL, thus endowing them with cytotoxic potential, demonstrated that while overexpressing perforin in isolation induced target cell membrane permeabilization, it necessitated the combined expression of perforin and granzymes to induce further intracellular events associated with CTL/NK killing, such as DNA damage (Shiver et al, 1991 and 1992; Nakajima et al, 1995). This suggested that perforin might form pores in the target cell membrane through which

granzymes may pass; however, there is little experimental evidence for this at present. Plasma membrane disruption, however, is not the only feature of cell death inflicted by CTLs and NK cells. Unlike complement-mediated lysis, killer cell lysis was found to be accompanied by extensive cellular deterioration beyond damage to the plasma membrane, in particular nuclear disintegration, which suggested that additional factors in the lytic granules were contributing to cytolysis. Other components of the lytic granules were subsequently identified as serine proteases, called granzymes (Lowin et al, 1995). The granzymes are a family of serine proteases stored within CTL/NK cell granules, with humans and rodents possessing different granzyme genes in three linked chromosomal clusters. Granzymes A, B, C, D, E, F, G, K, L, M and N are found in the mouse, while humans possess a more restricted repertoire, namely granzymes A, B, H, K and M (Grossman et al, 2003).

Granzyme B is a major constituent of CTL/NK cell granules, promoting apoptosis through proteolysis of a relatively small number of substrates (Figure 3) (Masson et al, 1987). There are two main pathways of granzyme B-induced killing, one involving direct activation of caspases and the other mediated through granzyme B-initiated promotion of mitochondrial permeabilization. Like the caspases, granzyme B cleaves its substrates after aspartic acid residues, so it is not surprising that several caspases have been proposed to be direct granzyme B substrates including caspase-3, -6, -7, -8, -9 and -10 (Darmon et al, 1995; Martin et al, 1996; Medema et al, 1997; Duan et al, 1996; Fernanes-Alnemri et al, 1996; Gu et al, 1996; Muzio et al, 1996; Orth et al, 1996; Quan et al, 1996). However, recent work suggests that caspase-2, -6 and -9 are cleaved indirectly by granzyme B-activated caspase-3 (Adrain et al, 2005)

In many apoptosis pathways, apoptotic signals converge on mitochondria where they promote the oligomerization of the BCL-2 family members BAX and/or BAK in the outer mitochondrial membrane (Luo et al, 1998; Li et al, 1998). BAX/BAK oligomerization promotes mitochondrial permeabilization with the resulting escape of mitochondrial intermembrane space proteins including cytochrome *c* (Kuwana et al, 2002). Cytochrome *c* release into the cytosol facilitates the formation of a complex, known as the apoptosome, between APAF-1 and caspase-9 (Nijhawan et al, 1997; Jiang et al, 2000). Assembly of the apoptosome results in caspase-9 activation followed by a downstream caspase cascade, which ensures the rapid death of the cell (Slee et al, 1999; Hill et al, 2004). The antiapoptotic protein BCL-2 acts at mitochondria to inhibit cell death by heterodimerizing with and inhibiting the death promoters BAX and BAK,

thereby blocking the efflux of cytochrome *c* and other mitochondrial intermembrane space proteins (Kluck et al, 1997). Because caspase inhibitors are ineffective at preventing granzyme B-mediated cytochrome *c* release (Pinkoski et al, 2001), whereas the overexpression of BCL-2 is sufficient to abrogate killing (Davis et al, 2000), this strongly suggested that granzyme B utilized a caspase-independent, mitochondrial-mediated cell death pathway. The BH3-only protein BID was subsequently identified as a substrate for this granzyme. In a manner, analogous to caspase-8-mediated proteolysis of BID in the death receptor pathway, processing of BID by granzyme B is thought to expose a myristolyation sequence, targeting the latter protein to mitochondria where it can promote the oligomerization of BAX and/or BAK in the outer mitochondrial membrane (Sutton et al, 2000; Alimonti et al, 2000; Barry et al, 2000; Heibein et al, 2000). The resulting cytochrome *c* release, followed by assembly of the apoptosome and the ensuing caspase activation cascade ensures rapid cell death.

Several granzymes other than granzyme B are now also considered likely to contribute to perforin-dependent target cell death. Granzyme A, induces intense single-strand DNA breaks through cleavage of various components of the SET complex, including TREX1 (Chowdhury et al, 2006). Cytotoxic activity has also been described for granzyme M, however the mechanism and reliance on caspase activation is still debated (Kelly et al, 2004; Lu et al 2006).

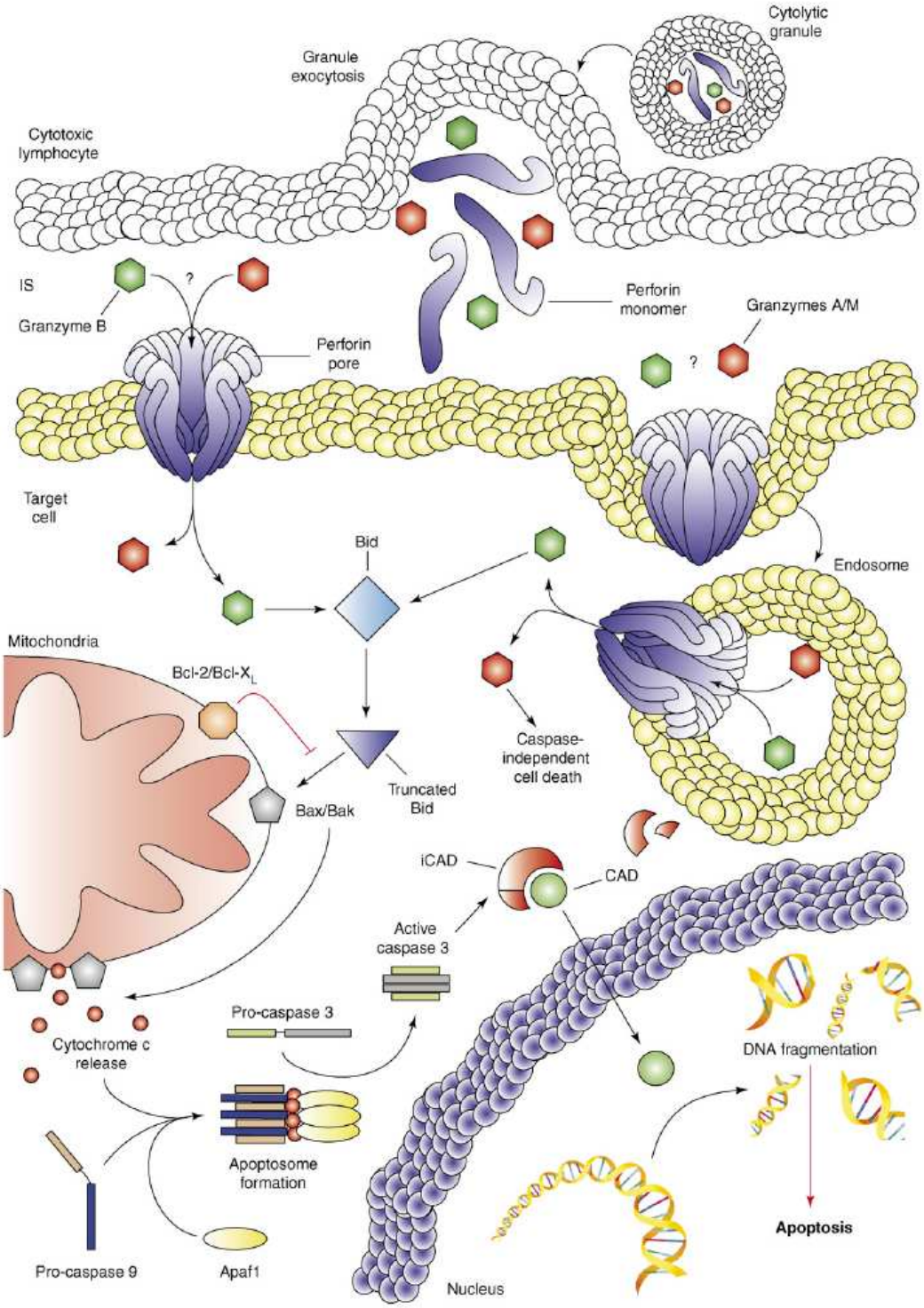


Figure 3. Delivery of granzymes by perforin and apoptosis. Contents of cytolytic granules such as perforin and granzymes are released into the extracellular space upon recognition and synapse formation between a cytotoxic lymphocyte and its target. Granzyme B induces caspase-dependent cell death by cleaving Bid, which then interacts with Bax and Bak on the mitochondrial membrane. This interaction can be inhibited by Bcl-2 family members, including Bcl-2 and Bcl-XL. Mitochondrial membrane integrity is then lost, resulting in release of cytochrome *c* into the cytoplasm. Pro-caspase 9, Apaf-1 and cytochrome *c* then assemble to form the apoptosome complex, which converts pro-caspase 3 into its active state. This executioner caspase mediates the release and subsequent translocation of caspase activated DNase (CAD) to the nucleus, resulting in DNA fragmentation and, finally, apoptosis of the target cell. Granzymes A and M induce caspase-independent cell death. iCAD, inhibitor of CAD; IS, immunological synapse.

The cytolytic process in CTLs and NK cells needs to be well regulated, and this involves additional or enhanced mechanisms for controlling the secretion of lytic-granule contents in NK cells compared with the process in CTLs.

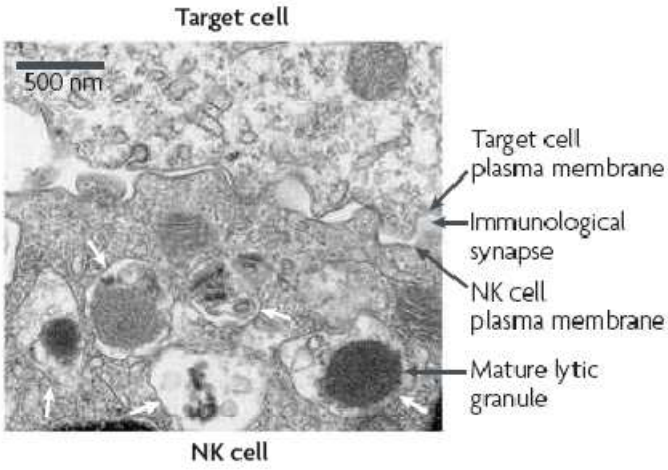
The induction of many NK-cell effector functions, including cytotoxicity, requires that the NK cell contacts its target cell. This ensures precise targeting of the cytolytic process to a single diseased cell in a tissue without affecting its neighbouring cells. The events that occur following the interaction between a cytolytic cell and its target cell have been well studied. They include the delivery and secretion of cytolytic effector molecules at the interface that is formed between the cytotoxic cell and its target cell through a process known as directed secretion. Our understanding of directed secretion for cytotoxicity has been advanced by the discovery of the immunological synapse. The immunological synapse was originally defined in the late 1990s (Grakoui et al, 1999; Monks et al, 1998) as the crucial junction between a T cell and an antigen-presenting cell (APC) at which T-cell receptors (TCRs) interact with MHC molecules. Subsequent studies extended these observations and identified relevant immunological synapses between different types of immune cell, as well as between immune cells and non-immune cells.

An immunological synapse can be defined as the orderly rearrangement of molecules in an immune cell at the interface with another cell. Numerous molecules have been identified as participating in the immunological synapse, including receptors, signalling molecules, cytoskeletal elements and cellular organelles. Some studies suggest that these molecules accumulate in distinct regions in an activating immunological synapse to form a supramolecular activation cluster (SMAC), which may be segregated into peripheral (pSMAC) and central (cSMAC) zones.

Although debate regarding the role of the immunological synapse in enabling immune responses continues, several of its potential functions seem relevant and worth considering. Lytic granules are hybrid organelles that are specialized for the secretion of the lytic effector molecules which reside in them.

For an NK cell to mediate cytotoxicity, lytic granules are emptied onto a target cell at a prototypical mature lytic synapse (Figure 4)

a)


Target cell
500 nm
Target cell plasma membrane
Immunological synapse
NK cell plasma membrane
Mature lytic granule
NK cell

b)

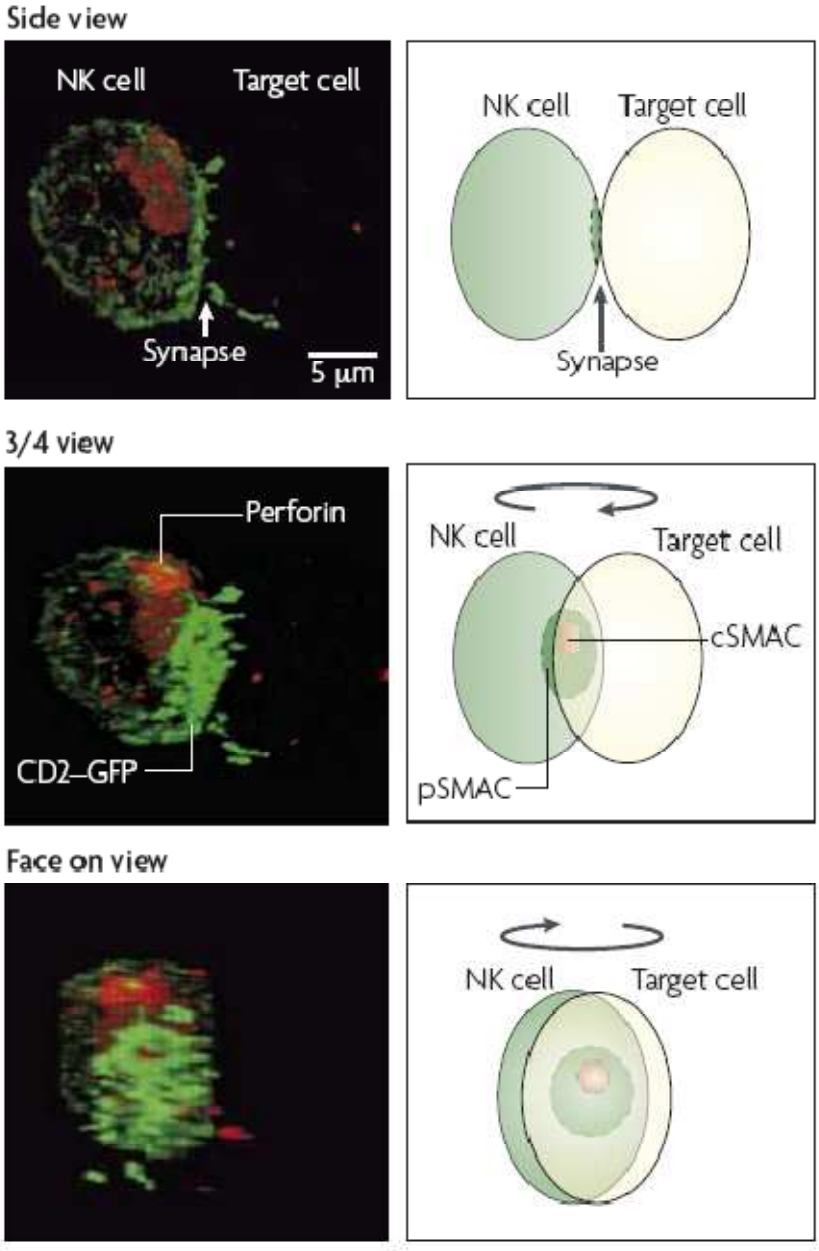

Side view
NK cell
Target cell
Synapse
5 μm
NK cell
Target cell
Synapse
3/4 view
Perforin
CD2–GFP
NK cell
Target cell
cSMAC
pSMAC
Face on view
NK cell
Target cell

Figure 4. Immunological synapse. a) Electron micrograph of the synapse between an *ex vivo* NK cell and a K562 target cell; arrows indicate lytic granules and their precursor forms. b) The mature natural killer (NK)-cell lytic synapse is defined by the formation of a supramolecular activation cluster (SMAC) at the interface between the NK cell and the target cell to which lytic granules polarize. The prototypical version of this synapse contains a central SMAC (cSMAC) that includes a secretory domain through which lytic granules may traverse. Confocal microscopy images show a human NK cell (YTS cell line) that expresses a CD2–GFP (green fluorescent protein) fusion protein making contact with a target cell (Epstein–Barr virus- transformed B-cell line). The cell–cell conjugates were fixed, permeabilized and evaluated for the presence of perforin (red) using a monoclonal antibody. Perforin is contained in lytic granules and therefore can be used as a marker for them; the distribution of CD2 under normal conditions parallels filamentous actin at the mature synapse.

In the mature synapse, filamentous actin (F-actin) and adhesion receptors accumulate and are thought to form a ring in the pSMAC through which perforin and other lytic-granule contents are secreted. Domains in the lytic synapse that contain specific signalling molecules or secretory machinery have been described in CTLs (Stinchcombe et al, 2001). Similar molecule distribution patterns have been observed in NK cells (Vyas et al, 2002; Almeida et al, 2006; Orange et al, 2006), but many aspects of NK-cell synapse organization have not been elucidated. However, lytic granules are large organelles and must traverse dense F-actin networks at the synapse, and actin reorganization is required for their release. Therefore, synapse formation has the specific function in NK cells of enabling cytotoxicity.

A variety of different signaling molecules are also involved, including calcium (Lyubchenko et al, 2001), phosphatidylinositol-3,4,5-triphosphate (Giurisato et al, 2007, Jiang et al, 2000) and activation of the ERK MAP kinase (Chen et al, 2006, Robertson et al, 2005; Wei et al, 1998). Recently, the recruitment of activated ERK to the immunological synapse has been shown to be a future of successful killing of a target by cytotoxic T lymphocytes, but the mechanism by which active ERK is recruited is not known.

1.2.1-MAPK pathway in granule exocytosis

As I said before, in a contact-dependent manner, NK cells ‘‘inject’’ cytolytic granules, containing perforin and granzymes, into target cells and induce target cell death (Trambas et al, 2003). The cytotoxicity process is orchestrated by two cytoskeletal elements: filamentous actin (FA) and microtubules (MT). Studies have shown that both of them undergo transient and dynamic structural remodeling during NK cell cytotoxicity (Wulfing et al, 2003). Filamentous actin accumulates in the NK cell immune synapse (NKIS), followed by the directional translocation of the microtubules organizing center (MTOC) toward the NKIS (Wulfing et al, 2003; Orange et al, 2003).

Perturbation of either actin or microtubules completely eliminates cytolytic activity of human NK cells. Studies on microtubules have suggested that the polarization of the MTOC is responsible for the polarization of cytolytic granules inside the cytotoxic lymphocyte, but dispensable for immune synapse formation (Kopcow et al, 2005). Granule polarization is a critical step in cytotoxicity. In NK cells, a pivotal signaling pathway from PI3K via Rac, PAK1, MEK to extracellular signal-regulated kinase (ERK) has been shown to be involved in triggering the polarization of cytolytic granules (Djeu et al, 2002). However, the mechanism by which ERK signaling regulates granule translocation was not investigated. Interestingly, the polarization of cytolytic granules in NK cells requires intact microtubules filaments (Trambas et al, 2003). Therefore, ERK signaling might be able to modulate the movement of the MTOC and thus granule transport in the cytotoxicity of NK cells.

How some of these signal molecules contribute to NK lysis of tumor cells was resolved when the sequence of events triggered by NK ligation of tumor cells was systematically investigated with one NK cell line. By use of biochemical analysis of kinase enzyme activation, gene transfer of dominant-negative and constitutively active signal molecules, accompanied by parallel examination of lytic function against 51Cr-labeled tumor cells, it became clear that a specific signal cascade was triggered in NK cells by exposure to tumor cells. NK cell ligation with tumor cells rapidly caused a transient activation of ERK, which apparently controls lytic granule movement (Porter et al, 2002). In known systems, ERK is downstream of a series of signal cascades, and of these, it immediately became apparent that the Ras/Raf/MEK/ERK pathway, which is crucial in gene expression and tumor cell growth, is not used in the killing process (Djeu et al, 2002). Instead, the utility of a specific phosphoinositide 3-kinase (PI3K)/ Rac/PAK/ MEK/ ERK pathway was identified in the NK92 cell line (Jiang et al, 2000). It is noteworthy that this same signal cascade for lytic function was also demonstrated in freshly isolated human NK cells, documenting its biological relevance (Jiang et al, 2000).

How active ERK is recruited to the synapse is not known. Since the kinase suppressor of Ras-1 (KSR1) is known to be recruited to the plasma membrane by Ras activation (Michaud et al, 1997) and since the immunological synapse is one of the major sites of Ras activation (Mo et al, 2006; Rechavi et al, 2007), it seemed plausible to test the hypothesis that KSR1 recruitment to the plasma membrane functions to recruit ERK to the immunological synapse and facilitate its activation.

KSR was originally described as a positive regulator of the RAS–MAPK signalling pathway in *Drosophila melanogaster* and *Caenorhabditis elegans* (Therrien et al, 1995; Kornfeld et al, 1995; Sundaram et al, 1995). KSR binds many proteins, including all three components of the ERK signalling pathway (RAF, MEK1 and ERK (Figure 5).

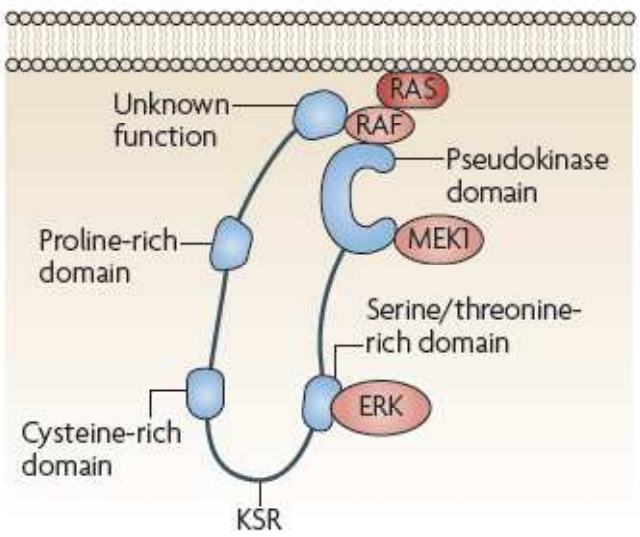


Figure 5. Mitogen-activated protein kinase scaffold proteins in immune signalling.| The general mitogen-activated protein kinase (MAPK) signaling cascade includes the activation of a MAPK kinase kinase (MAPKKK) that then activates a MAPKK, which activates the terminal MAPK. In the extracellular-signal regulated kinase (ERK) MAPK pathway, RAF acts as the MAPKKK, MAPK/ERK kinase 1 (MEK1) as the MAPKK and ERK as the MAPK. The scaffold protein kinase suppressor of RAS (KSR) acts to assemble these components at the plasma membrane to enhance ERK activation.

In mammals, KSR1 is highly expressed in the brain, thymus and spleen, and analysis of T cells from KSR1-deficient mice confirmed that KSR1 is required for efficient ERK activation (Nguyen et al, 2002). The defect in ERK activation that was observed in KSR1-deficient mice resulted in attenuated cytokine production and defective T-cell proliferation in the periphery. Surprisingly, despite significant expression of KSR1 in the thymus and a known role for ERK in thymopoiesis (Fisher et al, 2005), thymocyte development seemed to be normal in KSR1-deficient mice (Nguyen et al, 2003). KSR1 is also expressed by neutrophils and macrophages, and has been shown to be required for ERK activation in response to pro-inflammatory cytokines (Fusello et al, 2006).
We found that KSR1 was recruited to the immunological synapse and that KSR1 appeared to be required for the localization of active ERK at the contact site (Giurisato et al. 2009). As KSR1 deficient cells exhibit a defect in killing, this suggests that KSR1 recruitment to the synapse may be important in the cytolytic killing of target cells.

2-AUTOIMMUNITY

The concept of autoimmunity was first predicted by Nobel Laureate Paul Ehrlich at the start of the twentieth century, and he described it as ‘horror autotoxicus’. His experiments led him to conclude that the immune system is normally focused on responding to foreign materials and has an inbuilt tendency to avoid attacking self tissues. But when this process goes wrong, the immune system can attack self tissues resulting in autoimmune disease. The perplexing issue of what allows the immune system to attack self tissues is a continuing focus of research. In the past, autoimmune diseases have been studied on the basis of the organ affected, but in recent years the focus has switched to a more cross-disciplinary approach with a view to providing a better understanding of the common mechanisms underlying the pathogenesis of these diseases.

Autoimmune diseases are major causes of morbidity and mortality throughout the world. Many of these diseases tend to be difficult or impossible to cure, for the obvious reason that the focus of the immune response, self antigens, cannot be eliminated. The physical, psychological and economic burden of these diseases is especially devastating because they often attack young adults. The problem is also compounded by the failure of conventional cellular immunological analyses to shed much light on the pathogenic mechanisms. Recently developed therapies, such as tumor necrosis factor (TNF) antagonists, have had some remarkable successes, but these treatments target resulting organ damage and not the, usually unknown, underlying causes. The realization that the development of autoimmunity is strongly influenced by inherited polymorphisms brings hope that understanding the genetics of autoimmune diseases will teach us about the causal derangements, and perhaps lead to new therapeutic strategies.

2.1 Cellular mechanisms of self tolerance and autoimmunity

The mammalian immune system has an extraordinary potential for making receptors that sense and neutralize any chemical entity entering the body. Inevitably, some of these receptors recognize components of our own body, and so cellular mechanisms have evolved to control the activity of these ‘forbidden’ receptors and achieve immunological self tolerance. Many of the genes and proteins involved are conserved

between humans and other mammals. Our immune system is the body's sixth sense. It can react to any chemical structure imaginable to fight off every possible microorganism. The receptors coordinating this feat are antibodies expressed on the surface of B cells as B-cell receptors (BCRs) and T-cell receptors (TCRs) displayed on T cells. Huge receptor diversity is encoded in the mammalian genome by two processes of somatic genome modification that occur selectively in lymphocytes. First, V(D)J recombination assembles unique BCR and TCR genes from three separate gene segments, the variable (V), diversity (D) and joining (J) genes, during T-cell differentiation. This takes place in the 'central lymphoid tissues', which are principally the bone marrow for B cells and the thymus for T cells. Second, somatic hypermutation substitutes single nucleotides of BCR genes during a late phase of the immune response in peripheral lymphoid tissues (such as the spleen, lymph nodes and tonsils). A significant fraction of the receptors generated by both these processes bind to one or more self components in the body, a product of a deliberately random receptor-generating process.

Between 20 and 50% of TCRs and BCRs generated by V(D)J recombination bind with a potentially dangerous affinity to a self antigen (Ignatowitc et al, 1996, Zerrahn et al, 1997, Laufer et al, 1996, Wardermann et al, 2003).

Since only 3–8% of the population develops an autoimmune disease (Jacobson et al, 1997), it is remarkable that this enormous burden of self-reactive receptors is so well regulated in most of us.

Each lymphocyte usually produces only a single receptor out of the billions possible. Experiments have established that if this receptor is self reactive, then four cellular strategies are employed to deal with them (Figure 6). First, the cell displaying the 'forbidden', or self-reactive receptor can be triggered to die. Second, a cell bearing a forbidden receptor can 'edit' the offending receptor by further V(D)J recombination or somatic hypermutation to display a different receptor that is not self reactive (Nemazee et al, 2003). Third, intrinsic biochemical and gene-expression changes can reduce the ability of the cell to be triggered by self-reactive receptors. This is generally termed clonal anergy or tuning (Healy et al, 1998; Schwartz et al, 2003; Grossman et al, 2000) . Finally, even if the cells have evaded the three mechanisms above, collectively called 'immunological ignorance', extrinsic controls can limit the danger of self-reactive receptors. These extrinsic controls limit the supply of essential growth factors, costimuli, pro-inflammatory mediators and other factors, and also include active

suppression by regulatory T (Treg) cells, through a mechanism that is poorly understood. These four mechanisms act as checkpoints on the pathway leading to the production of secreted antibodies and effector T cells (Figure 6).

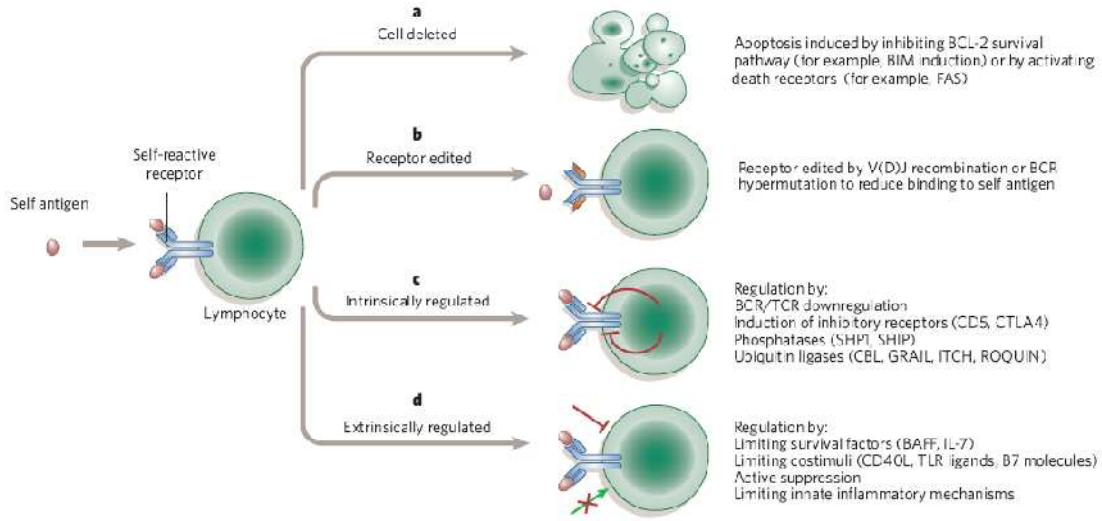


Figure 6. Four cellular strategies are used to regulate self-reactive receptors at different points during B- and T-cell differentiation. a, The cell is deleted through induction of cell death. **b**, The receptor is edited to one that is less self-reactive. **c**, Biochemical or gene-expression changes intrinsically dampen the self-reactive receptor's ability to activate the cell. **d**, The ability of self-reactive cells or antibody to cause autoimmunity is limited by using extrinsic suppression and by limiting essential growth factors, costimuli and inflammatory mediators.

2.1.1 Central Tolerance

The prevention of autoimmunity begins in the thymus with the process of central tolerance, whereby self-reactive T cells are deleted through positive and negative selection. Immature thymocytes undergo gene rearrangement of their T-cell receptor in the thymus such that each thymocyte displays a unique TCR capable of binding self-peptides from the periphery bound to the major histocompatibility complex (MHC) molecules. The strength of this interaction will determine the fate of the thymocyte. Those that fail to interact with MHC–peptide complexes undergo apoptosis in the positive selection process, whereas those that exhibit high affinity for such complexes are deleted in the negative selection process (Keir et al, 2005). The remaining population of the mature thymocytes are then released into the periphery.

An inherent problem of the central tolerance system is that not all peripheral self peptides are expressed in the thymus. It is estimated that medullary thymic epithelial cells express 10% of all known genes in addition to those basally expressed on such

cells (Kyewski et al, 2004). It is, therefore, necessary to have a second-line of defence against the onset of autoimmunity, namely peripheral tolerance.

2.1.2 Peripheral Tolerance

Mature T cells that enter the periphery from the thymus have low to intermediate affinity for self-antigens presented in the thymus. However, a small subset of T cells released will be autoreactive to self-antigens because of the absence of their expression in the thymus. Control and/or deletion of such T cells is essential if the autoimmune state is to be prevented. Peripheral tolerance mechanisms that perform this role can act directly on the T cell and include ignorance and anergy, phenotype skewing, and apoptosis (Walker et al, 2002), or they can act by activating other cells such as dendritic cells (DCs) and regulatory T cells (Treg).

Ignorance is probably the simplest mechanism of peripheral tolerance whereby a T-cell response to self-antigen does not occur because the antigens are either located in sites that are not accessible to the T cell (Alferink et al, 1998) or present in insufficient numbers to trigger a T-cell response (Kurts et al, 1998). However, if the T cell does interact with self-antigens, this can lead to anergy, which is a functional inactivation of the T cell itself. Two molecules that have been proposed as contributing to the anergic state are the costimulatory molecule, cytotoxic T-lymphocyte-associated antigen (CTLA)-4 and the programmed cell death (PD)-1 molecule. Expression of CTLA4, is induced at a high threshold of TCR self reactivity and inhibits T-cell activation by competing with CD28 for ligation with B7 molecules and by transmitting inhibitory signals (Waterhouse et al, 1995). Lack of CTLA4 causes massive accumulation of self-reactive T cells in peripheral lymphoid and nonlymphoid tissues, by disrupting intrinsic regulation of TCR-induced proliferation. PD-1 may act by inhibiting cytokine secretion or by causing cell-cycle arrest (Freeman et al, 2000; Latchman et al, 2001*)*.

Phenotypic skewing is also thought to be partially mediated by costimulatory molecules and involves alteration of the Th1/Th2 cytokine response by T cells in response to signals from these molecules received when the TCR is activated (Kuchroo et al, 1995; Dong et al, 2001). This can result in the development of a non-pathogenic phenotype, thus preventing autoimmune damage. Apoptosis is probably the most effective direct-acting mechanism to prevent autoimmunity from developing, as it involves deletion of the autoreactive T cell through interaction between Fas ligand and its receptor (Walker et al, 2002). However, in most cases, not all T cells are deleted but remain in the

periphery in an anergic state, suggesting that apoptosis only occurs until the levels of autoreactive T cells are such that they can be controlled by the process of anergy (Walker et al, 2002). In addition to these direct-acting mechanisms, activation of other cells can also aid peripheral tolerance. The major cells involved in this process are DCs and Tregs. DCs have been proposed to aid tolerance by two mechanisms. The first hypothesizes that DCs have receptors that recognize pathogen-associated molecules and initiate an immune response upon encountering such a molecule (Janeway et al, 1992; Matzinger et al, 1994). In the absence of such an encounter, any interaction with a T cell will lead to anergy or apoptosis. The second theory suggests that immune responses to pathogens only occur when the pathogen causes damage to the body. This is detected by the release of intracellular components of the cell and provides a signal to DCs to initiate an immune response (Matzinger et al, 1995). Absence of these "danger" signals would result in induction of tolerance upon interaction with a T cell.

Regulatory T cells are either naturally expressed in the thymus and express CD25 or are induced in the periphery. Absence of CD25+ T cells in mice has been shown to lead to multiple autoimmune diseases (Asano et al, 1996), indicating their importance in regulating peripheral tolerance. They represent 10% of CD4+ T cells in humans (O'Garra et al, 2004) and are thought to function by preventing T-cell proliferation to self-antigen (Thornton et al, 2000) through cell–cell contact (Kuniyasu et al, 2000). These cells express the forkhead/winged helix transcription factor Foxp3, with loss of function mutations of this gene leading to the absence of CD25+ Tregs and the development of multiple autoimmune diseases in both humans and mice (Ramsdell et al, 2003). Lack of regulatory CD25+ T cells has been shown to increase autoimmunity (McHugh et al, 2002), and, although the molecules required to enable Tregs to function are unknown, it can be hypothesized that downregulation of such molecules would affect the ability of Tregs to inactivate autoreactive T cells (Groux et al, 1997). Induced Tregs are termed TR1 cells (Sundstedt et al, 2003) and rely on interleukin 10 (IL)-10 and transforming growth factor (TGF)-β to function. They have been shown to inhibit T-cell proliferation in vitro, suppress autoimmune disease that has been experimentally induced and control CD4+ and CD8+ numbers in vivo (Groux et al, 1997; Sundstedt et al, 2003; Oida et al 2003). As can be seen, both central and peripheral tolerances are complex processes, involving the interaction of many components, defects of which could cause a breakdown in tolerance. However, other mechanisms that could cause

tolerance breakdown and lead to autoimmunity have been postulated. These include molecular mimicry, exposure of cryptic epitopes, or microbial superantigens.

2.1.3 Other factors causing tolerance breakdown

- *Molecular Mimicry*

One of the most intriguing hypotheses is that autoimmunity is the by-product of the immune response fighting infections. The initial demonstration that a virus-specific cytotoxic T lymphocyte (CTL) response can lead to selective damage of pancreatic β cells resulting in diabetes (Olstone et al, 1991) has opened a new axis of research. The basis for molecular mimicry lies on the intrinsic flexibility of the T-cell receptor (TCR), a molecule capable to interact with multiple ligands with a certain degree of degeneracy (Evavold et al, 1995; Ford et al, 2004). As a result, T cells can be triggered by peptides, which often have minimal homology to the primary immunogenic peptide, as long as they present a similar antigenic conformation (Quarantino et al, 1995). This is an important feature of T cells, which permits effective T-cell responses to the largest number of potential foreign peptide sequences complexed to MHC molecules. Furthermore, even a shift in the binding register can cause a dramatic change in the appearance of a peptide:MHC complex (Quarantino et al, 1995; Bankovich et al, 2004). By changing the MHC-binding residues into TCR contact residues, the TCR antigenic surface can be highly altered, converting two different peptide:MHC complexes into a cross-reactive pair. The observation that a single TCR can recognize quite distinct but structurally related peptides from multiple pathogens has important implications for understanding the pathogenesis of autoimmunity. Evidence for a role of molecular mimicry in autoimmunity has been reported in mouse and human. In human, both viral and bacterial peptides can efficiently activate MBP-specific T cells isolated from multiple sclerosis patients (Wucherpfenning et al 1995). Also in MS, a particular human T-cell clone can recognize peptide MBP (85–99) and an Epstein-Barr virus (EBV) DNA polymerase epitope (627–641) presented by DRB1∗1501 and DRB5∗0101, respectively, two MHC molecules contained in the MS associated DR2 haplotype (Lang et al, 2002). Viral infections play a role in shaping the peripheral T-cell repertoire and also in the initiation of autoimmunity through molecular mimicry. Thus, autoimmune responses provoked by molecular mimicry should occur when the foreign and self-determinants are similar enough to cross-react yet different enough to break immunological tolerance.

- *Cryptic Epitopes*

T cells can only be tolerized to epitopes that are presented to them in sufficient levels to be recognized. However, epitopes exist that are not recognized by T cells as they are either present at very low levels or inaccessible to the T cell. They are known as cryptic epitopes, and it has been proposed that they may play a major role in the autoimmune response if they increase in number or become visible to the immune system (Lanzavecchia et al, 1995). Several mechanisms have been proposed as to how this may occur. First, increased antigen may be processed, leading to levels of the epitope increasing above the threshold for recognition (Salemi et al, 1995). Second, the antigen may be processed in a different manner, thus revealing cryptic epitopes to the immune system (Simitsek et al, 1995), and, third, there may be an increase in human leukocyte antigen (HLA) class II or costimulatory molecule expression, leading to increased levels of the epitope being present to activate T cells (Lanzavecchia et al, 1995). These mechanisms may act together or independently to initiate an autoimmune response. These mechanisms could be triggered by presentation of self antigen containing cryptic epitopes on DCs, presentation of these epitopes by nonprofessional APCs such as B cells, or activation of autoreactive B cells by these epitopes (Lanzavecchia et al, 1995).

- *Superantigens*

Superantigens, of either viral or bacterial origin, activate T cells through the variable domain of the beta chain of the TCR. They are capable of binding to a large number of MHC class II molecules, thus activating a large population of T cells with a wide variety of MHC/peptide specificities (Wucherpfenning et al, 2001). It has been shown that they are capable of causing relapse and exacerbation of experimental autoimmune encephalomyelitis (EAE) in mice (Brocke et al, 1993) but cannot induce this disease in previously unaffected animals. However, in a similar scenario to molecular mimicry, it is difficult to prove a causative role for superantigens in autoimmune disease. The pathogen needs to be identified in patients with autoimmune disease and be isolated at the time of infection. This relies on the diagnosis of the autoimmune disease at the time of infection, an impossible task for most autoimmune disorders that do not exhibit an acute onset following infection (Wucherpfenning et al, 2001), therefore, although the hypothesis of microbial superantigens triggering autoimmunity is attractive, it remains to be proven for the majority of such disorders.

2.2 Genetic regions associated with autoimmune diseases

Owing to the observation that multiple autoimmune diseases can cluster within families, it has been postulated that such disorders will be caused by a combination of common and specific genes. To date, only three genes/gene regions have been consistently associated with multiple autoimmune conditions namely the HLA class II region on chromosome 6p21 (Awata et al, 1992; Badenhoop et al, 1995; Simmonds et al, 2005), the *CTLA-4* gene on chromosome 2q33 (Vaidya et al, 1999; Ueda et al, 2003), and the *PTPN22* gene encoding lymphoid tyrosine phosphatase (LYP) on chromosome 1p13 (Smyth et al, 2004; Criswell et al, 2005).

With the exception of the three genes mentioned, identification of novel susceptibility loci has been a slow process largely because of such loci only contributing small effects to disease susceptibility. To detect such effects, large data sets are needed to provide sufficient power to ensure that any association seen is a true association and not a false-positive result because of a small data set. A further confounding problem in this search is that some genes appear to confer susceptibility only to specific diseases or in specific populations, making replication studies difficult.

The pathways leading to the development of autoimmune disease are complex, involving defects in the immune system leading to the release of autoreactive T cells into the periphery, which can trigger disease. Many genes and gene products are involved in this process with a combination of both common and specific genes leading to the development of specific disorders. The recent advances in genotyping technology have vastly improved the ability of the geneticist to narrow down the search for primary etiological variants within these genes; however, further advances are necessary to identify rare variants that may contribute to these disorders and to elucidate the functional role that susceptibility loci play in disease development.

3- DEFECTIVE APOPTOSIS OF ACTIVATED LYMPHOCYTES: THE AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME (ALPS)

ALPS is a rare human disorder defined by lymphoproliferation, peripheral expansion of double-negative αβ T (DNT) cells, and impaired lymphocyte apoptosis. Autoimmune disease and an increased risk of lymphoma are also observed. The clinical presentation of ALPS in humans reflects impaired lymphocyte homeostasis, particularly of the DNT cells (Sneller et al 2003; Rieux-Laucat et al, 2003) (Table 1).

Criteria
Required features
Chronic nonmalignant lymphadenopathy, splenomegaly, or both
More than 1% circulating DNT cells
Demonstration of defective in vitro lymphocyte apoptosis (except ALPS Ib and Im)
Supporting features
Family history of ALPS
Typical findings on histopathologic analysis of lymph node or splenic tissue
Autoimmune disease
Mutation of genes encoding Fas or related apoptosis signaling molecules

Table 1. NIH criteria for the diagnosis of ALPS

ALPS is generally due to deleterious mutations of the Fas gene (TNFRSF6) and is classified as ALPS type-Ia (ALPS-Ia) (Fisher et al, 1995; Rieux-Laucat et al, 1995). Other mutations, namely of the FasL gene in ALPS-Ib (Wu et al, 1996; Del-Ray et al, 2006; Bi et al, 2007), and the caspase-10 gene (CASP10) in ALPS-II (Wang et al, 1999; Zhu et al, 2006; Cerutti et al, 2007) are occasionally detected, whereas some patients do not present any known mutations (ALPS-III) (Straus et al, 1999; Dianzani et al, 2003 and 1997; Sneller et al, 2003;Ramenghi et al, 2000; Campagnoli et al, 2006). Recently, mutations of the NRAS gene have been suggested to cause a further type of ALPS (ALPS-IV) (Oliveira et al, 2007).

Lymphoproliferation apparently results from the gradual accumulation of lymphocytes that have not undergone normal programmed cell death. This proliferation leads to chronic enlargement of the lymph nodes, thymus, liver, and/or spleen, beginning in

early childhood. Both B cells, including CD5+ B cells, and T cells are elevated. Not all lymphocyte subsets are affected, as CD4+CD25+ absolute numbers are reduced and CD4+CD25− absolute numbers are not increased. By contrast, expansion of an unusual population of peripheral CD4−CD8− T cells is striking. These peripheral DNT cells express αβ T cell receptor (TCR) chains as well as the CD45R isoform B220 (typically expressed on B cells); this expression distinguishes them from peripheral γδ T cells, which naturally lack CD4 and CD8 coreceptors (Blessing et al, 2001). DNTs also differ from double-negative thymocytes, an immature stage of T cells in which TCR genes have not yet completely rearranged (Zuniga et al, 1996). The DNT cells could be either previously activated, mature T cells that have lost CD8 or CD4 coreceptor expression, or a special minor cell lineage (Blessing et al, 2002). In the blood, DNT cells constitute less than 1% in normal individuals, but can reach up to 40% in patients with ALPS (Blessing et al, 2001). The DNT expansion is also evident histologically by nearly pathognomic abnormalities of the architecture of secondary lymphoid tissues, with paracortical and follicular hyperplasia. DNT cells appear to play a crucial role in disease development. In certain ALPS patients lacking germline mutations in the death receptor Fas, Holzelova et al. (Holzelova et al, 2004) found a population of DNT cells harboring somatic dominantly interfering Fas mutations. Thus, unregulated and excessive DNT cells are seemingly sufficient to cause disease. Two previously described characteristics of DNT cells may explain how. First, the DNT cells are primary producers of strikingly elevated IL-10 observed in ALPS patients. By contrast, DNT cells from normal individuals do not produce IL-10. Moreover, healthy relatives of ALPS patients bearing both Fas mutations and in vitro apoptotic defects have only modestly elevated DNTs and IL-10 (Lopatin et al, 2001). Besides elevated IL-10, ALPS patients also exhibit increased IL-4 and IL-5, but decreased IL-2 and IFN-γ (Fuss et al, 1997; Lopatin et al, 2001). This cytokine profile is characteristic of T helper type 2 (Th2) cells, which inhibit cell-mediated immunity and promote humoral immune responses. Thus, the overall cytokine environment may favor autoantibody production in ALPS. Second, DNT cells exhibit an unusual phenotype including B220 expression and altered cell surface O-glycans (Blessing et al, 2001). This phenotype could change the trafficking pattern and/or potential interactions of DNT cells with other cell types. There are some important and unresolved paradoxes regarding DNTs. First, they are very difficult to culture in vitro and almost immediately die despite an ostensible resistance to apoptosis in vivo. Their lifespan is not extended by IL-10. Second, although DNTs are believed to

play a role in hyperactive immune responses, they are generally unresponsive to proliferative and activating stimuli (Sneller et al, 1992). Finally, the antigen specificities recognized by the TCRs on DNTs have not been defined, but the general assumption is that they will include self-antigens.

Autoimmunity accompanies lymphoproliferation in ALPS. Most patients have elevated serum immunoglobulin levels and autoantibodies. Anti-cardiolipin antibodies are frequent but not usually associated with thromboembolic disease . Many patients exhibit Coombs' positive hemolytic anemia and/or immune thrombocytopenia (Sneller et al, 2003). Less common autoimmune manifestations in ALPS include anti-nuclear antibodies, rheumatoid factor, autoimmune neutropenia, glomerulonephritis, uveitis, autoimmune hepatitis, primary biliary cirrhosis, Guillain-Barrè, vasculitis, linear IgA dermopathy, and anti-Factor VIII antibodies with coagulopathy (Sneller et al, 2003; Wong et al, 2004). It is not known why, in contrast to other autoimmune diseases, antibody-mediated autoimmune disease is primarily directed against the hematopoietic system in ALPS patients. However, autoimmune phenotyping is imprecise, as illustrated by the discovery of a patient, initially diagnosed with systemic lupus erythematosus (SLE), who was later found to have a Fas ligand mutation with defective apoptosis (Wu et al, 1996). An interesting question is whether the reduced CD4+ CD25+ cells seen in ALPS patients might represent a defect in regulatory cells that contributes to autoimmune disease.

Although lymphoproliferation is initially nonmalignant, ALPS patients have a marked propensity to develop B or T cell malignancies with a 14-fold and 51-fold increased incidence of non-Hodgkin lymphoma and Hodgkin lymphoma, respectively (Straus et al, 2001). The lymphomas were found anywhere between 15 to 48 years after onset of ALPS symptoms (Straus et al, 2001). Analysis of lymphoma tissue from ALPS patients showed no loss of Fas heterozygosity or increased apoptosis resistance. Interestingly, the responsible mutations impair apoptosis, but not Fas-induced NF-κB and mitogen activated protein kinase (MAPK) signaling (Legembre et al, 2004). Hence, the contribution of Fas mutations to lymphomagenesis may involve blocking death and releasing growth-promoting effects. It is also possible that the increased number of lymphocytes in general may provide a larger pool for tumorigenesis (Davidson et al, 1998).

In an early clinical description of ALPS, Sneller and colleagues recognized that ALPS closely resembled the *lpr/lpr* (lpr) and *gld/gld* (gld) mouse strains. On certain inbred

genetic backgrounds, homozygosity of these alleles causes mice to develop profound lymphoproliferation involving DNT cells, hypergammaglobulinemia, autoantibodies, autoimmune disease, and lymphomas (Davidson et al, 1998; Cohen et al, 1991). Not surprisingly, these naturally arising mouse strains have molecular defects in some of the same genes as ALPS patients. The lpr and gld mice have autosomal recessive mutations in Fas and FasL, respectively, resulting in deficient expression, whereas the lprcg variant has a point mutation in Fas that renders it nonfunctional (Kimura et al, 1994; Nagata et al, 1998). Although the Fas locus is the primary genetic determinant, there is a strong effect of the inbred mouse strain background on the autoimmune phenotype. Severe disease occurs on the MRL background, whereas disease is substantially reduced on a B6 or BALB/c background. These mice do not develop the autoimmune hemolytic anemia and thrombocytopenia seen in human patients with ALPS. Instead, they display certain features resembling SLE (anti-dsDNA, anti-Sm, anti-immunoglobulin autoantibodies) with a predilection for developing glomerulonephritis, polyarteritis, sialoadenitis, and, with lesser frequency, arthritis or primary biliary cirrhosis (Nose et al, 2000; Tsuneyama et al, 2001). The Agnm3 locus associated with glomerulonephritis corresponds to allelic polymorphisms of the osteopontin gene. Polymorphisms of this gene, which is highly expressed in T cells, correspond to functional differences in activating macrophages and B cell antibody production (Miyazaki et al, 2005). Interestingly, we found that allelic polymorphisms of osteopontin may influence disease penetrance in humans who have a variant of ALPS termed DALD (Chiocchetti et al, 2004).

In all but the most severely affected patients, the clinical course of ALPS waxes and wanes over time and often improves with age. Thymic involution may decrease the output of T cells, and it is known that neonatal thymectomy in lpr mice prevents disease (Hang et al, 1984; Wofsy et al, 1982). Clinical complications relate mainly to the severity of autoimmune disease. Infections may occur after splenectomy or secondary to immunosuppressive treatment.

Finally, minor clinical variants of ALPS may reflect different and uncharacterized molecular defects in lymphocyte homeostasis mechanisms. A significant fraction of ALPS or ALPS-like patients has no mutations in the genes encoding proteins involved in the Fas signaling. Approximately half of these patients have no apoptosis defect following direct challenge with an agonist anti-Fas antibody. Although the ALPS diagnosis requires documentation of impaired apoptosis of mature lymphocytes, most

laboratories rely on assessment of apoptosis induced through the Fas death receptor. Defects in Fas-mediated apoptosis usually coincide with defects in TCR-restimulation-induced death; however, an ALPS patient with normal Fas mediated apoptosis but impaired apoptosis following stimulation with phytohemagglutin plus IL-2 has been described (Hundt et al, 2002). ALPS could also result from defects involving non–death receptor–induced intrinsic pathways of apoptosis. Patients can also exhibit the main clinical features as ALPS, with lymphoproliferation, apoptosis defects, autoimmune disease, and cancer, but lack DNT cell expansion (Ramenghi et al, 2000). This ALPS variation has been termed Dianzani autoimmune lymphoproliferative disease (DALD) by Makusic in the OMIM web site (OMIM reference #605233; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM). DALD patients likely share with ALPS patients a common affected pathway downstream of the Fas receptor. In summary, there are number of variant and overlapping clinical subtypes that will be extremely interesting to explore at the molecular level.

4-DEFECTIVE CELL-MEDIATED CYTOTOXICITY: IMMUNODEFICIENCY DISEASES

Primary immunodeficiency diseases in humans are characterized by genetic aberrations that impair immunological function, anti microbial defense or both. Several of these diseases affect NK cells (Orange et al, 2002 and 2006) and an informative subset is characterized by a specific block in the stages that lead to the formation of a functional lytic synapse (Table 2).

Disease	Gene	Protein	HLH phenotype	Effect on the lytic synapse
LAD-I	*ITGB2*	CD18	No	Decreased conjugation with target cell
WAS	*WAS*	WASP	Some	Decreased F-actin reorganization and integrin redistribution
CHS	*LYST*	LYST	Yes	Inability to generate normal lytic granules for trafficking to the synapse
HPS2	*AP3B1*	AP3 β-subunit	Yes	Inappropriate formation of lytic granules and movement along microtubules
GS2	*RAB27A*	RAB27A	Yes	Lytic granules move to the synapse but remain associated with microtubules
FHL3	*UNC13D*	MUNC13-4	Yes	Lytic granules move to the synapse but fail to dock and so do not achieve an intimate association with the NK-cell plasma membrane
FHL4	*STX11*	Syntaxin-11	Yes	Lytic granules polarize to and dock at the NK-cell plasma membrane but fail to fuse

Table 2. Defects of the lytic synapse in NK cells from patients with genetic disease. *AP3B1*, adaptor-related protein complex 3, β1 subunit; AP3, adaptor protein 3; CHS, Chediak–Higashi syndrome; F-actin, filamentous actin; FHL, familial haemophagocytic lymphohistiocytosis; GS2, Griscelli syndrome type 2; HLH, haemophagocytic lymphohistiocytosis; HPS2, Hermansky–Pudlak syndrome type 2; *ITGB2*, integrin-β2; LAD-I, leukocyte adhesion deficiency type I; *LYST*, lysosomal trafficking regulator; NK, natural killer; STX11, syntaxin-11; WAS, Wiskott–Aldrich syndrome; WASP, WAS protein.

So far, none of the diseases in this subset has been found to exclusively impair NK cells and should affect all cytotoxic lymphocytes. Insight into how the lytic synapse is formed in cells from patients with these diseases has been gained mostly from T-cell studies, but the functional defect has been established in NK cells too. Although cytotoxic lymphocytes have crucial roles in host defence and immune regulation, there are likely to be specific contributions of NK-cell deficiency to the clinical phenotypes.

Most of these diseases can result in haemophagocytic lymphohistiocytosis (HLH) (Henter et al, 2004). HLH represents an inappropriately robust immune response to infection (typically with herpes viruses), which results in persistent symptoms of septic shock and is also associated with the pathological finding of haematophagocytosis (the ingestion of red blood cells by phagocytes). The defect in cytotoxic lymphocytes is believed to contribute to this phenotype, as the infected cells and other activated cells that promote inflammation cannot be eliminated. NK cells may be most relevant to the HLH phenotype, given their localization to the marginal zone of lymphoid organs after viral infection, their innate function early in the course of infection (Biron et al, 1999) and their inherent ability to eliminate hyperactivated macrophages (Nedvetzki et al, 2007). Although the defective NK cells are unable to eliminate infected cells by direct cytotoxicity, they can still carry out other functions, including the production of cytokines and the stimulation of inflammatory responses. This is consistent with the idea that the requirements for secretion of cytokines and lytic-granule contents at the synapse are distinct. However, the inability of NK cells to eliminate infected cells early in the infection (before T cells would have expanded) may be an important cause of HLH.

Other primary immunodeficiency diseases that impair cytolytic cell function but do not disrupt the formation of the NK-cell synapse can also result in HLH. For example, HLH occurs in patients with a mutation in the gene encoding perforin (Stepp et al, 1999) and, in this setting, the disease is characterized by the normal secretion of lytic granules that are unable to mediate cytotoxicity owing to the absence of perforin (Mercenaro et al, 2006).

The diseases that provide specific insight into the NK-cell lytic synapse are considered in two groups. Diseases in the first group affect steps that are involved in the initiation stage or the activation steps of the effector stage of synapse formation. Diseases in the second group affect steps in lytic-granule trafficking to the synapse in the effector stage. For the aim of this thesis we can also consider the third group of disease in which there is a defect in perforin function itself.

4.1 Diseases affecting initiation or activation steps of NK‑cell lytic‑synapse formation.

Leukocyte adhesion deficiency type I (LAD-I) results from a defect in the CD18 (β2-integrin) component of leukocyte integrin heterodimers (Kishimoto et al, 1987). Leukocytes from patients with LAD-I do not adhere to inflamed or activated cells properly and cannot localize effectively to tissues and sites of inflammation. This leads to increased numbers of leukocytes in the blood and susceptibility to infectious diseases. Because early steps in NK-cell synapse formation, adhesion and activation signaling, depend on integrins, NK cells from patients with LAD-I do not adhere to their target cells, which results in defective cytotoxicity (Riteau et al, 2003; Krensky et al, 1985; Khol et al, 1984; Bryceson et al, 2006). LAD-I is distinguished from other diseases because it does not lead to HLH. This is presumably because NK cells from LAD-I patients do not form immunological synapses and are not activated through the synapse to produce cytokines.

Wiskott–Aldrich syndrome (WAS) results from a defect in actin reorganization and cell signalling in haematopoietic cells owing to WASP deficiency (Orange et al, 2004). Patients lacking WASP expression or expressing abnormal WASP have NK cells with decreased cytolytic capacity (Gismondi et al, 2004; Orange et al, 2002). Clinically, patients with WAS are susceptible to infection with herpes viruses (Sullivan et al, 1994) and can develop HLH (Snover et al, 1981; Pasic et al, 2003; Wang et al, 2005), which is consistent with a functional role for WASP in NK-cell lytic-synapse formation. Accordingly, formation of the lytic synapse is abnormal in NK cells from WAS patients and is associated with decreased F-actin accumulation and adhesion-receptor clustering at the synapse (Orange et al, 2003 and 2002; Gismondi et al, 2004).

4.2 Diseases affecting lytic‑granule traffic to the NK‑cell lytic synapse.

Chediak–Higashi syndrome (CHS) and Hermansky–Pudlak syndrome type 2 (HPS2) both affect the normal formation of lytic granules and lead to the presence of ‘giant’ lytic granules. both syndromes are also associated with albinism, which is caused by the aberrant function of melanocytes, the function of which is to pigment the skin through the secretion of melanosomes (an equivalent of lytic granules). CHS and HPS2 are

similar in that they both result from a block in the late effector stages of NK-cell lytic-synapse function owing to a failure in the migration of the abnormal lytic granules along microtubules to the MTOC. CHS results from a mutation in the *LYST* gene, which encodes lysosomal trafficking regulator (Introne et al, 1999).

Although not explored recently, older studies identified a defect in the cytolytic activity of NK cells from patients with CHS, despite normal NK-cell adhesion to target cells (Roder et al, 1982; Katz et al, 1982; Haliotis et al, 1980; Targan et al, 1983; Klein et al, 1980). These defective NK cells feature abnormal giant lytic granules (Abo et al, 1982), and studies in CTLs indicate that such granules arise from the fusion of individual lytic granules (Stinchcombe et al, 2000).

HPS2 is caused by a mutation in the *AP3B1* gene (Huizing et al, 2001), which encodes the β-subunit of adaptor protein 3 (AP3) and, unlike CHS, HPS2 is associated with excessive bleeding owing to the lack of the platelet storage pool and ensuing abnormal platelet aggregation. In addition, patients with HPS2 have defective NK-cell cytotoxicity (Fontana et al, 2006; Enders et al, 2006) and HLH (Enders et al, 2006). AP3 is required for the appropriate sorting of molecules from the Golgi into lytic granules. Similar to patients with CHS, CTLs from patients with HPS2 have enlarged granules that fail to move along microtubules (Clark et al, 2003).

Griscelli syndrome type 2 (GS2) is a third syndrome that combines albinism and immunodeficiency and is also associated with an accelerated phase of HLH. GS2 is caused by a mutation in the *RAB27A* gene, which encodes the RAb27A small GTPase (Menasche et al, 2000). NK-cell cytotoxicity is decreased in patients with GS2, but is not necessarily absent (Klein et al, 1994; Plebani et al, 2000). So, the mechanism underlying the defective lytic synapse in NK cells from patients with GS2 is also distinct from that in patients with CHS and HPS2. Studies carried out in mouse RAb27A-deficient CTLs show that lytic granules migrate and polarize towards the synapse but fail to dock at it (Stinchcombe et al, 2001).

Familial haemophagocytic lymphohistiocytosis (FLH) types 3 and 4 are similar to GS2 but are not associated with albinism, which indicates that the affected genes are not essential in melanocytes. FHL3 is caused by a mutation in the *UNC13D* gene, which encodes MUNC13-4. Initially defined in CTLs, the lytic granules in MUNC13-4-deficient cells polarize towards the synapse and dock at the plasma membrane but do not fuse with it (Feldmann et al, 2003). Defective cytolytic activity and decreased

granule fusion with the plasma membrane have also been observed in MUNC13-4-deficient NK cells (Mercenaro et al, 2006).

FHL4 is caused by mutations in the *STX11* gene, which encodes syntaxin-11 (zur Stadt et al, 2005). Studies indicated a direct role for syntaxin-11 in NK-cell degranulation (Arneson et al, 2007; Bryceson et al, 2007). Importantly, the polarization of lytic granules to the synapse of NK cells from FHL4 patients without subsequent degranulation has been directly observed. FHL4 patients have diverse clinical phenotypes, with some showing late onset of disease (zur Stadt et al, 2006), which implies that syntaxin-11 mutations are hypomorphic or that there is some redundancy of the protein in enabling lytic-granule fusion. In support of some level of redundancy, IL-2 stimulation of NK cells from patients with FHL4 can restore degranulation (Bryceson et al, 2007), which suggests an activation-induced synthesis of proteins that complement defective syntaxin-11 function.

4.3 Diseases affecting perforin function.

Loss of perforin function in mice is associated with immune dysregulation and impaired cytotoxicity, clearly demonstrating the crucial role perforin has as an immune effector molecule (Kagi et al, 1994 and 1996; van den Broek et al, 1996). Perforin-deficient mice are viable and healthy under specific pathogen-free conditions, however compromised cellular cytotoxicity is observed upon challenge with certain viruses and they display defects in tumor and transplant rejection. A recent study has suggested that a perforin-dependent immune surveillance process might exist in humans.

Amongst a group of 29 patients that presented with either non-Hodgkin lymphoma or Hodgkin disease, four had biallelic mutations and four others had monoalleleic mutations of the perforin gene (Clementi et al, 2005). Of the latter four patients, one also carried a mutation of the Fas gene. Notably, post-germinal centre B-cell malignancies have a higher incidence of mutation in the Fas gene (particularly in the death domain) compared with pre-germinal centre B-cell and non-B-cell malignancies (Muschen et al, 2002). Taken together, these data suggest that mutation and/or deficiencies in the perforin and/or death receptor pathways might be present in patients who have lymphoma and potentially support a mechanism of tumor immune surveillance in humans. A relatively common human perforin polymorphism, A91V, is not associated with increased risk of childhood acute lymphoblastic leukemia, but

A91V frequency was significantly increased in children with BCR-ABL positive acute lymphoblastic leukemia (Meheta et al, 2006). We demonstrated recently that mutations and polymorphism in perforin gene are involved in ALPS are susceptibility factors to development of ALPS, DALD, diabetes and multiple sclerosis (Clementi et al, 2006; Orilieri et al, 2008; Cappellano et al, 2008). Further information correlating perforin mutations and polymorphisms with prognosis in the context of human malignancies and graft-versus-leukemia, in which immune surveillance is operating, will be of importance.

In 1999, mutations in several FLH patients were mapped to the perforin locus at chromosome 10q22. Perforin mutations have been shown to account for between 15% and 60%(with an average of 30%) of all FLH cases, and many mutations throughout the gene have been identified (Molleran Lee et al, 2004; Katano et al, 2005; zur Stadt et al, 2006; Voskoboinik et al, 2006). These patients present with fever, hepatosplenomegaly and pancytopenia, have marked elevations of T-helper (Th) type 1 and type 2 cytokines, and have impaired NK cell and CTL cytotoxicity. Viral pathogens have been implicated as triggering the onset of disease, but no specific virus has been identified to date. Identification of mutations in perforin as the cause of FLH should allow prenatal diagnosis of the disorder.

AIMS OF THE STUDY

Autoimmune disease have a complex etiology, involving the interplay between multiple genetic (genes and their products) and non-genetic factors (environmental influences). A comprehensive understanding of the genetic variations predisposing to these diseases, in both animal models and humans, would facilitate to establish relationships between genotype and biological function. Moreover, functional characterization of these variations may help to understand the molecular mechanisms involved in the pathogenesis of these disease and detect novel molecular targets for therapeutic interventions.

Aim of this study was to search for variations of genes involved in the immune response switching off in Autoimmune Lymphoproliferative Syndrome, used as a prototypic autoimmune diseases with strong genetic component.

RESULTS

1 "CO-INHERITED MUTATIONS OF FAS AND CASPASE-10 IN DEVELOPMENT OF THE AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME"

1.1 Aim

ALPS is generally due to deleterious mutations of the Fas gene (TNFRSF6) and is classified as ALPS type-Ia . Fas mutations are usually heterozygous, their penetrance depends on their effect on Fas function. Mutations hitting the intracellular death domain of Fas, involved in recruitment of FADD and caspase-8/10 and initiating the death signal, are often the most severe. They exert a dominant-negative effect and display high penetrance. By contrast, mutations hitting the extracellular portion or causing haploinsufficiency have weaker penetrance. Most ALPS type-Ia patients are heterozygous for the Fas mutation, but the parent carrying the mutation is generally healthy. Other complementary factors may thus be required to the development of the disease. One possibility is that mild Fas mutations only induce ALPS when cooperate with mutations of other genes impairing function of the Fas system itself. In the past, Lenardo et al identified two CASP10 mutations involved in ALPS: the missense mutations causing the L285F and I406L amino acid substitutions. They were detected in 1 and 3 heterozygous patients respectively. Both mutations decreased caspase-10 activity and exerted a dominant negative effect on the wild type protein, but neither was sufficient to induce the overt disease, since several mutated familial components were healthy, and some displayed serum autoantibodies only.

Aim of the first paper was to demonstrate that mutations of TNFR6 and CASP10 can cooperate in development of ALPS by hitting the Fas signaling pathway. We described two unrelated patients carrying different double heterozygous mutations in the Fas and caspase-10 genes. Although these substitution affected the protein activities, in both patients, they were inherited from distinct healthy parents, which suggests that their coinheritance had been crucial for ALPS development in these patients.

1.2 Paper

BMC Immunology

BioMed Central

Research article

Open Access

Co-inherited mutations of Fas and caspase-10 in development of the autoimmune lymphoproliferative syndrome

Elisa Cerutti[†1], Maria F Campagnoli[†2], Massimo Ferretti[1], Emanuela Garelli[2], Nicoletta Crescenzio[2], Angelo Rosolen[3], Annalisa Chiocchetti[1], Michael J Lenardo[4], Ugo Ramenghi[2] and Umberto Dianzani*[1]

Address: [1]Interdisciplinary Research Center of Autoimmune Diseases (IRCAD) and Department of Medical Science, "A. Avogadro" University of Eastern Piedmont, Novara, Italy, [2]Department of Pediatrics, University of Turin, Turin, Italy, [3]Department of Pediatrics, University of Padua, Padua, Italy and [4]Molecular Development Section, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

Email: Elisa Cerutti - cerutti@med.unipmn.it; Maria F Campagnoli - campagnolifra@yahoo.com; Massimo Ferretti - ferretti@med.unipmn.it; Emanuela Garelli - emanuela.garelli@unito.it; Nicoletta Crescenzio - nicoletta.crescenzio@unito.it; Angelo Rosolen - rosolen@pediatria.unipd.it; Annalisa Chiocchetti - chiocche@med.unipmn.it; Michael J Lenardo - MLENARDO@niaid.nih.gov; Ugo Ramenghi - ramenghi@pediatria.unito.it; Umberto Dianzani* - dianzani@med.unipmn.it

* Corresponding author †Equal contributors

Published: 13 November 2007

BMC Immunology 2007, 8:28 doi:10.1186/1471-2172-8-28

Received: 26 September 2007
Accepted: 13 November 2007

This article is available from: http://www.biomedcentral.com/1471-2172/8/28

Abstract

Background: Autoimmune lymphoproliferative syndrome (ALPS) is a rare inherited disorder characterized by defective function of Fas, autoimmune manifestations that predominantly involve blood cells, polyclonal accumulation of lymphocytes in the spleen and lymph nodes with lymphoadenomegaly and/or splenomegaly, and expansion of TCRαβ+ CD4/CD8 double-negative (DN) T cells in the peripheral blood. Most frequently, it is due to Fas gene mutations, causing ALPS type Ia (ALPS-Ia). However, other mutations, namely of the FasL gene (ALPS-Ib) and the caspase-10 gene (ALPS-II) are occasionally detected, whereas some patients do not present any known mutations (ALPS-III). Recently, mutations of the NRAS gene have been suggested to cause ALPS-IV.

Results: This work reports two patients that are combined heterozygous for single nucleotide substitutions in the Fas and caspase-10 genes. The first patient carried a splice site defect suppressing allele expression in the Fas gene and the P501L substitution in caspase-10. The second had a mutation causing a premature stop codon (Q47X) in the Fas gene and the Y446C substitution in caspase-10. Fas expression was reduced and caspase-10 activity was decreased in both patients. In both patients, the mutations were inherited from distinct healthy parents.

Conclusion: These data strongly suggest that co-transmission of these mutation was responsible for ALPS.

Background

Autoimmune lymphoproliferative syndrome (ALPS) is a rare inherited disorder characterized by autoimmune manifestations that predominantly involve blood cells, polyclonal accumulation of lymphocytes in the spleen and lymph nodes with lymphoadenomegaly and/or splenomegaly, expansion of TCRαβ+ CD4/CD8 double-negative (DN) T cells in the peripheral blood and defective in vitro apoptosis of mature lymphocytes induced by the Fas death receptor [1-4]. Individuals with ALPS also have an elevated incidence of several types of lymphoma [5].

Fas belongs to the Tumor Necrosis Factor Receptor (TNFR) superfamily and induces cell death upon triggering by FasL [6,7]. It is highly expressed by activated effector lymphocytes in the immune response and switches it off by limiting clonal expansion of lymphocytes and favoring peripheral tolerance. Fas signaling starts from aggregation of Fas, the adaptor molecule FADD (Fas-associated death domain protein), and caspase-8 forming the Death Inducing Signaling Complex (DISC) which triggers caspase-8 activation and induces cell apoptosis through two partly interconnected pathways; the extrinsic pathway involves caspase-8-mediated direct activation of the cascade, whereas the intrinsic pathway proceeds through mitochondrial release of cytochrome c and activation of caspase-9. Both pathways converge in the activation of effector caspases, such as caspase-3, -6 and -7. In humans, but not in mice, the extrinsic pathway also involves caspase-10, that is recruited into the DISC and cooperates with caspase-8 in activation of the caspase cascade [8-10].

ALPS is generally due to deleterious mutations of the Fas gene (TNFRSF6) and is classified as ALPS type-Ia (ALPS-Ia) [11,12]. Other mutations, namely of the FasL gene in ALPS-Ib [13-15], and the caspase-10 gene (CASP10) in ALPS-II [16,17], are occasionally detected, whereas some patients do not present any known mutations (ALPS III)[1-3,18-20]. Recently, mutations of the NRAS gene have been suggested to cause a further type of ALPS (ALPS-IV) [21]. ALPS does not behave as a classical monogenic disease. Most ALPS type-Ia patients are heterozygous for the Fas mutation, but the parent carrying the mutation is generally healthy. Other complementary factors may thus be required in function of the severity of the mutation [22]. One possibility is that mild Fas mutations only induces ALPS when cooperate with mutations of other genes impairing function of the Fas system itself or other systems involved in similar functions. In line with this possibility, we have described osteopontin and perforin gene variations that predispose to ALPS [23,24]. The osteopontin gene variation correlated with production of increased amounts of this cytokine, which is involved in inflammation and also inhibits activation-induced cell death. The perforin gene variations were associated with decreased function of cytotoxic cells, which may switch off the immune response by fratricide of effector lymphocytes.

This work describes two unrelated patients that are double heterozygous for mutations of the Fas and the caspase-10 gene. Since the two mutations were inherited from distinct healthy parents, their co-transmission probably resulted in ALPS.

Results

Analysis of TNFRSF6 and CASP10

Pt.1 showed a heterozygous nucleotide substitution in TNFRSF6 (c334 -2a>g, [Genbank NM_000043.3]) located in the splicing-acceptor site in the third intron and determining the IVS3-2a>g splice site defect. The mutation results in skipping of exon 4, coding for an extracellular cysteine-rich domain, frameshift and premature termination after 38 codons. The mutated allele produces no protein. This mutation had already been described in a homozygous ALPS patient, whose heterozygous parents were healthy [25,26].

Sequencing of CASP10 detected a C>T substitution at nt1502 in exon 10 [Genbank NM_032977.2] resulting in a proline to leucine change (P501L) in the small catalytic subunit of caspase. The mutation, not previously described, was not detected in 80 healthy donors nor in 40 other ALPS patients. Family analysis showed that the CASP10 mutation was inherited from the apparently healthy mother, who did not carry the TNFRSF6 mutation. This was presumably inherited from the father, who was not available for analysis, or was a de novo mutation.

Pt.2 carried a heterozygous C>T substitution at nt139 in TNFRSF6. It was located in exon 2 coding for an extracellular domain and created a premature stop codon (Q47X). The mutation was predicted to cause haploinsufficiency due to nonsense mediated decay of the aberrant mRNA; alternatively, a truncated soluble Fas fragment might be produced. Sequencing of CASP10 detected a heterozygous nucleotide substitution (1337A>G) in exon 9 causing the Y446C amino acid change in the predicted protease domain of the small subunit. This variation has been previously associated to ALPS, but has been reported also in the healty Caucasian population with allelic frequency ranging from 1.6 to 2% [17,20]. Family analysis showed that the Fas mutation was inherited from the apparently healthy mother; the CASP10 variation was possibly inherited from the father, who was not available for the study.

The pedigrees of these families are shown in the Figure 1.

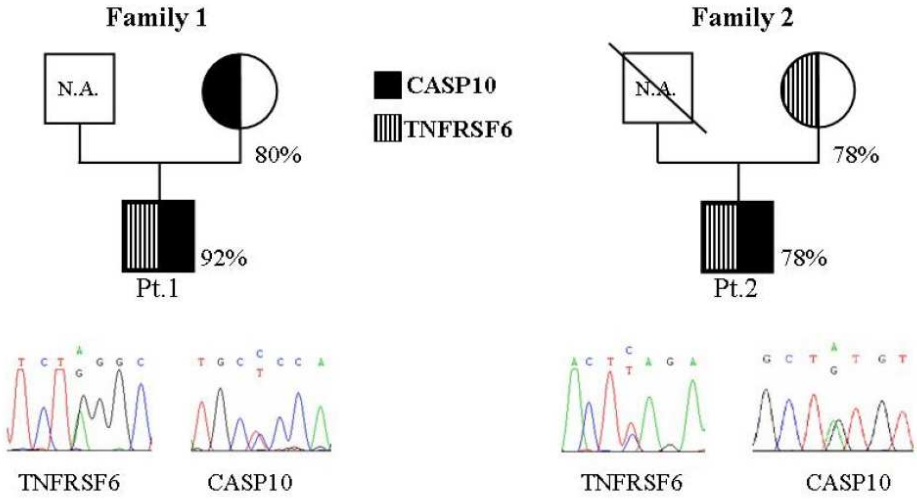


Figure 1
Pedigrees of Family 1 and Family 2. Inheritance of the CASP10 and TNFRSF6 mutations and electropherograms of the sequences performed on the genomic DNA of Pt.1 and Pt.2. Circles represent females: squares, males: subjects carrying a CASP10 mutation are marked in black, those with a TNFRSF6 are marked with striped lines. Numbers indicate the cell survival upon Fas triggering by mAb in T cell lines generated form each subject; Fas function was defective in Pt.1 and borderline in the other subjects (normal values of cell survival: median 60%, 95th percentile 82%)

Functional analysis of the mutations

Analysis of Fas function in PHA-activated T cells from the patients and their mothers showed that it was defective in Pt.1 and borderline in Pt.2 since cell survival upon triggering of Fas was 92% in the former and 78% in the latter (normal values: median 60%, 95th percentile 82%). Moreover, Fas function was borderline in both mothers since cell survival was 80% in the Pt.1's mother and 78% in the Pt.2 mother (Fig. 1).

To asses whether the IVS3-2a>g and Q47X TNFRSF6 mutations affected Fas expression, activated T cells from Pt.1 and Pt.2 were stained by direct immunofluorescence with an anti-Fas mAb and analyzed by flow cytometry. Results showed that both patients displayed decreased Fas expression (Fig. 2a).

To assess whether the P501L and Y446C caspase-10 amino acid substitutions affected the enzyme function, we evaluated caspase-10 activity induced by triggering of Fas with anti-Fas mAb in activated T cells from available mutated subjects, i.e. Pt.1, his mother, and Pt.2. All subjects showed a caspase-10 activity that was lower than that of the controls (Fig. 2b). These data suggest that both substitution decrease caspase-10 activity. Data on Y446C are in line with those previously reported [17] showing that the cloned Y446C-caspase-10 is less effective than the wild type form in restoring Fas-mediated apoptosis in cells lacking endogenous caspases-8 and -10, but does not exert dominant negative activity on the wild type.

To further assess the activity of the novel P501L-caspase-10, the cDNAs coding for it or the wild-type protein (isoform d) were cloned into the pcDNA3.1 Myc-His vector, fused to HA- or FLAG-tag sequences respectively (P501L^{HA} and WT^{FLAG} plasmids). Both were transiently transfected into 293T cells, expressing minimal levels of endogenous caspase-10. Moreover, 293T cells were cotrasfected with the P501L^{HA} and WT^{FLAG} plasmids to determine whether the mutated form exterted a dominant negative activity on the wild type form. Western blot analysis showed that both constructs were expressed at comparable levels in all trasfectants and both proteins were spontaneously cleaved, the mutated form even more efficiently than the wild type form (Fig. 3). However, analysis of the caspase-10 enzyme activity on the lysates by a fluorimetric assay

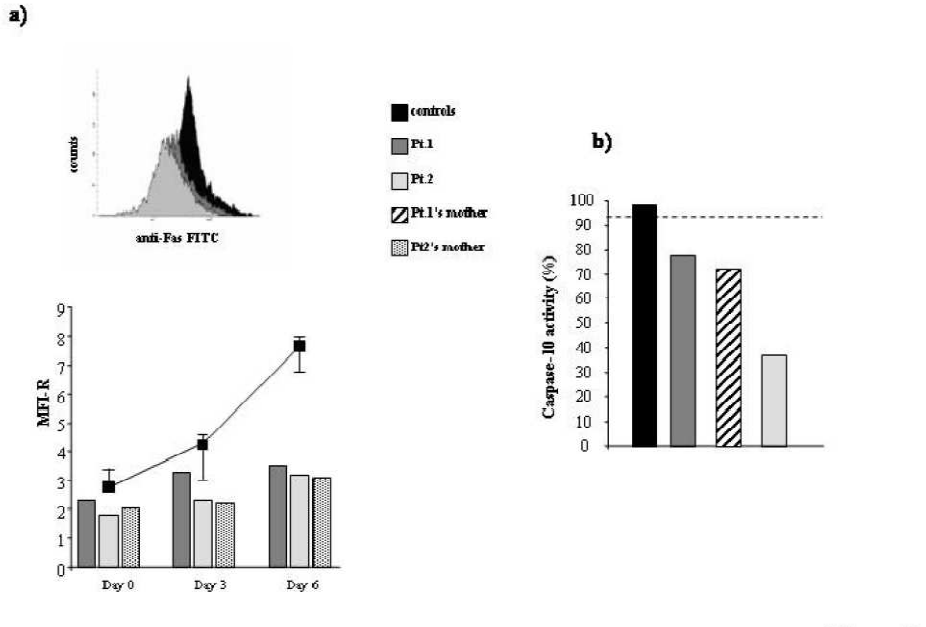


Figure 2

Fas expression and caspase-10 activity in subjects carrying the TNFRSF6 and CASP10 mutation. a) Fas expression was evaluated in T cell lines obtained by activating PBMC with PHA (1 μg/ml) and cultured for 6 days in RPMI 1640 +10% FCS+rIL-2 (2 U/mL). Before activation (day 0), and at day 3 and day 6 of culture, cells were stained with a FITC-conjugated anti-Fas mAb and analyzed with a cytofluorimeter. The *upper panel* shows the cytofluorimetric staining of cells from Pt.1, Pt.2, and a control donor after 6 days of culture. The *lower panel* shows the MFI ratio calculated for each subject at different times of culture. Control data are the medians ± interquartile ranges (25–75% range) from 5 control donors; their 5th percentile value at day 6 was MFI-R = 6.48. b) Caspase-10 activity was evaluated in PHA-activated T cells cultured for 12 days (see Methods) in RPMI 1640 +10% FCS+rIL-2 (10 U/mL) and then treated or not with an anti-Fas mAb for 3 hours. Results are expressed as relative caspase activity % calculated as follows: (result displayed by each subject/mean of the results displayed by the 2 controls run in the same experiment) × 100; 100% indicates the mean of the results obtained with the 2 control donors run in parallel with the patient samples in each experiment; the dotted horizontal lines indicate the 5th percentile of the activity displayed by all normal controls. The color code is the same in all panels.

showed that the P501L-caspase-10 displayed about 50% of the activity displayed by the wild type. Cells cotransfected with the P501L^{HA} and WT^{FLAG} plasmids showed levels of caspase-10 activity intermediate between those of cells transfected with each plasmid alone, which indicates that the mutated form does not exert a dominant negative activity on the wild type.

Discussion

This work reports that mutations of TNFRSF6, characterizing ALPS type Ia, and CASP10, characterizing ALPS type II, can cooperate in the development of ALPS.

Fas mutations are the most frequent in ALPS. Usually heterozygous, their penetrance depends on their effect on Fas function. Mutations hitting the intracellular death domain of Fas, involved in recruitment of FADD and caspase-8 and initiating the death signal, are often the most severe. They exert a dominant-negative effect and display

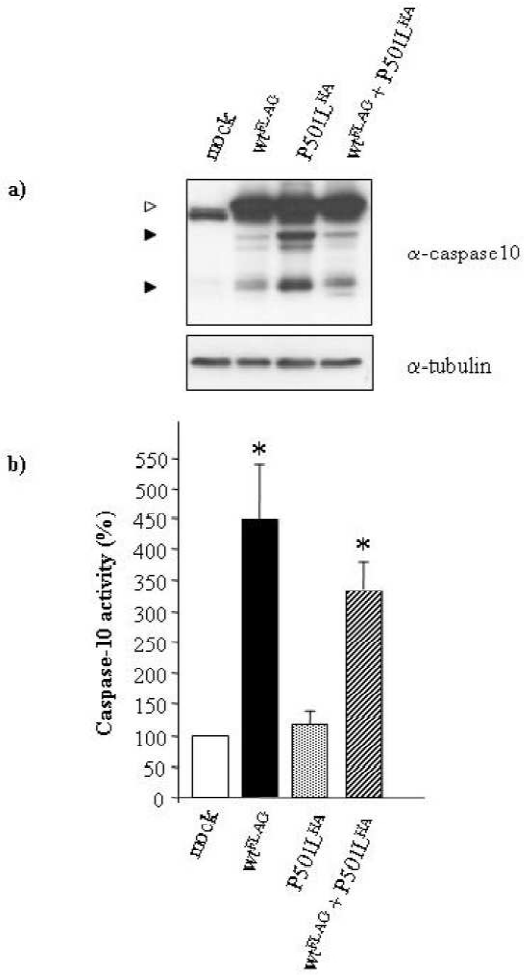


Figure 3

Caspase-10 activity in 293T cells transfected with P501L-caspase-10. Analysis of 293T cells transiently transfected with the mock, WT^{FLAG}, $P501L^{HA}$, or $P501L^{HA}$+WT^{FLAG} plasmids, as indicated in the panel. a) Western blot analysis of cell transfectant lysates performed with anti-caspase-10 antibody; expression of the transfected molecules was confirmed using anti-HA and -FLAG antibodies (data not shown). The white arrow shows the pro-caspase-10; black arrows indicate the cleaved forms. b) Fluorimetric enzyme assay of caspase-10 activity evaluated in the cell transfectant lysates 24 h after transfection; data are relative to those displayed by mock-transfected cells (indicated as 100%) and are the means ± SE of data from 6 independent experiments. The asterisks mark the data significantly different from those obtained with $P501L^{HA}$-transfected cells ($p < 0.01$, Mann Whitney test).

high penetrance. By contrast, mutations hitting the extracellular portion or causing haploinsufficiency have weaker penetrance [22,27].

Both our patients carried mutations predicted to cause haploinsufficiency. In line with this prediction, T cells from both Pt.1 and Pt.2 expressed low levels of Fas. Penetrance of these mutations is presumably weak. This is suggested for the nonsense Q47X mutation by the lack of any sign of ALPS in the Pt.2's carrier mother. By contrast, the absence of Pt.1's father meant that clinical effect of IVS3-2a>g splice site defect could not be determined by a pedigree analysis, since it might have been a de novo mutation. However, this mutation has been previously described in a different family, where its heterozigosity was not sufficient to cause ALPS [26].

Only two CASP10 mutations have so far been unequivocally involved in ALPS. They are the missense mutations causing the L285F and I406L amino acid substitutions detected in 1 and 3 heterozygous patients respectively. Both mutations decreased caspase-10 activity and exerted a dominant negative effect on the wild type protein, but neither was sufficient to induce the overt disease, since several mutated familial components were healthy, and some displayed serum autoantibodies only [16,17].

Both CASP10 mutations carried by our patients are mild heterozygous missense mutations decreasing caspase-10 activity without exerting a dominant negative effect on the wild type protein. It is therefore intriguing that these patients also displayed mild Fas mutations that were presumably required to worsen the apoptotic defect and cause ALPS development. Pt.2 carried the Y446C mutation, previously reported in one heterozygous patient displaying a mild form of ALPS [17]. This mutation decreased caspase-10 function, without inducing a dominant negative effect on the wild type protein. The mutation is not sufficient to induce ALPS since it is also detected in 1–2% of the healthy Caucasian population [17,20]. Pt.1 carried the novel P501L mutation, located, like Y466C, in the small subunit of caspase-10. P501L, too, decreased caspase-10 function without inducing a dominant negative effect on the wild type protein. Decreased activity was clearly detected in lymphocytes from both this patient, who also carried the Fas mutation, and his mother, who only carried the CASP10 mutation. Moreover, 293T cells transfected with the mutated form displayed about 50% of the enzyme activity displayed by the wild type. Lack of negative dominance was shown when cotransfection with both the mutated and the wild type forms produced an additive and not an antagonistic effect on caspase-10 enzyme activity. It is noteworthy that western blot analysis of transfected cells showed that P501L did not affect cleavage of caspase-10, which is often interpreted as an evidence of activation. However, the decreased activity detected by the in vitro caspase-10 enzyme assay supports the model proposed by Boatright et al. that cleavage is neither sufficient or necessary for activation of initiator caspases, that mainly depends on dimerization [28].

The effect of the interaction between these Fas and caspase-10 mutations seems opposite to those reported for interactions between Fas mutations and caspase-10 missense variation V410I [16,17]. This variation was initially posited as a cause of ALPS since it had been detected in one homozygous patient. It has since been found as an "innocent" polymorphism carried by 3–5% of the Caucasian population [16,17,29]. An association analysis, indeed, suggested that it gave protection against severe ALPS in 63 families with ALPS-Ia caused by severe dominant mutations of Fas [17]. It is intriguing that the first ALPS patient homozygous for V410I harbored a heterozygous missense mutation in the tumor necrosis factor receptor-1 gene (TNFRSF1A), which is mutated in the TNF receptor-associated periodic fever syndrome (TRAPS) and may explain its clinical pattern [30]. This suggests that the caspase-10 variation also influenced the clinical phenotype due to the TNFRSF1A mutation.

Conclusion

This work suggests that ALPS may sometimes be caused by the concurrent effect of mutations hitting different genes involved in Fas function and hence that it may be both a classic monogenic disease, as occurs in the presence of severe mutations hitting the intracellular portion of Fas, and the outcome of digenic or even oligogenic mutations affecting different steps of the Fas signalling pathway.

Methods

Patients

Patient 1 (Pt.1) was a 27-year-old Caucasian male. At the age of 23, he presented fever of unknown origin associated with mucositis, weight loss and nocturnal sweating. Laterocervical, axillary and inguinal lymphadenopathies with hepatomegaly and splenomegaly were clinically disclosed. Blood analyses showed reduced white blood cell and platelet counts, borderline hemoglobin levels, positive Coombs test and hypergammaglobulinemia, and expansion of DN T cells in the peripheral blood (6%). Histopathologic examination of left axillary lymph nodes showed reactive follicular hyperplasia with regular distribution of T- and B-dependent areas. Defective Fas-induced apoptosis of lymphocytes in cell death assays pointed to ALPS. Oral steroids improved the clinical picture, reduced lymph nodes and spleen size, and increased the platelet count and hemoglobin level.

Patient 2 (Pt.2) was a 12-year-old Caucasian male who displayed an ALPS phenotype at the age of 3, with laterocervical lymphadenopathy, mild hepatosplenomegaly, immune neutropenia and thrombocytopenia. Histopathologic examination of a submaxillary lymph node demonstrated follicular hyperplasia. DN T cells were 3.6% and Fas-induced lymphocyte apoptosis was borderline. Blood analyses also showed increased IgM and IgA levels and lupus anticoagulant. At the age of 4, he developed transient mild hemolytic anemia; hematological alterations spontaneously remitted at the age of 5, but laterocervical adenopathy increased to bulky masses. The left submaxillary and cervical lymph nodes began to compress the upper airways and were excised when he was ten years old; he is now in hematological remission, with persisting bulky laterocervical lymphadenopathies. The patient's father died of peritoneal carcinosis at 33. The paternal grandfather had died of gastric cancer.

Sequence analysis

Genomic DNA was extracted from PBMCs by standard methods (Pure Gene DNA Isolation Kit, Gentra Biosystem Inc, Minneapolis, Minnesota) after informed consent. All coding exons and intron-exon boundaries of TNFRSF6 [Genbank AY450925.1] and CASP10 [Genbank NT_005403.14] were amplified by polymerase chain reaction (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA); primer sequences and annealing temperatures for each primers pair used for amplification were previously reported [20,31]. The PCR products were sequenced with the BigDyeTM Terminator Kit (Applied Biosystems,) on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using the same primers. The family segregation of mutations was ascertained by enzymatic digestion.

Fas function assay

Fas-induced cell death was evaluated as previously reported on T cell lines obtained by activating PBMCs with PHA at days 0 (1 μg/ml) and 15 (0.1 μg/ml) and cultured in RPMI 1640 + 10% FCS + rIL-2 (2 U/ml) (Biogen, Geneva, Switzerland). Fas function was assessed 6 days after the second stimulation (day 21) [18,19]. Cells were incubated with control medium or anti-Fas mAb (CH11, IgM isotype) (1 μg/ml) (UBI, Lake Placid, NY) in the presence of rIL-2 (1 U/ml) to minimize spontaneous cell death. Cell survival was evaluated after 18 h by counting live cells in each well by the trypan blue exclusion test and by flow cytometry of cells excluding propidium iodide and unstained by annexin V-FITC; the two methods gave overlapping results. Assays were performed in duplicate. Cells from two normal donors were included in each experiment as positive controls. Results were expressed as specific cell survival %, calculated as follows: (total live cell count in the assay well/total live cell count in the control well) × 100. Fas function was defined as defective when cell survival was >82 % (the 95th percentile of data obtained from 200 normal controls).

Immunophenotype analysis

Expression of surface molecules was evaluated by direct immunofluorescence and flow cytometry (FACScan. Becton Dickinson, San Jose, CA). The following mAb were used: anti-CD4 (Leu-3a), -CD8 (Leu-2a), -TCRαβ (Becton Dickinson), and -Fas (Immunotech, Marseilles, France). CD4 and CD8 DN TCRαβ-positive cells were detected by 2-color immunofluorescence with fluorescein isothiocyanate (FITC)-conjugated anti-TCRαβ mAb and phycoerythrin (PE)-conjugated anti-CD4 and anti-CD8 mAbs. Fas was detected by 2-color immunofluorescence on resting or activated T cells, using PE-conjugated anti- TCRαβ mAb and FITC-conjugated anti-Fas mAb (Chemicon, Temecula, CA). Nonspecific background fluorescence was established with the appropriate isotype-matched control mAb (Becton Dickinson)

Caspase-10 activity

PBMCs were activated with PHA at days 0 (1 μg/ml) and 8 (0.1 μg/ml) and cultured in RPMI 1640 + 10% FCS + rIL-2 (10 U/ml). Four days after the second stimulation, 6 × 10^6 T cells were treated or not with anti Fas mAb (CH11, IgM isotype) (1 μg/ml) on ice for 30 min, then moved to 37°C for 3 h and centrifuged. At least 2 control samples, using T cells from different healthy donors, were always run in parallel. Caspase-10 activity of T-cells and transfected 293T was assessed in cell lysates using a fluorimetric assay (MBL, Watertown, MA).

Caspase-10 cloning

cDNA coding for wild type caspase-10 was obtained as previously reported [16]. To obtain the cDNA of mutated caspase-10, we amplified the fragment containing the P501L mutation, from 1055 bp to 1569 bp [Genbank NM_032977.2] by RT-PCR. Briefly, total RNA extracted from patient's PBMC was reverse-transcribed with the ThermoScript RT-PCR system (Invitrogen, Milan, Italy) and amplified with primers C10 NcoI-fw and C10 XhoI-rev (Table 1). The fragment from ATG to 1055 bp was obtained from the wild type clone by PCR using a 5'-primer adding the HA TAG (C10HA-fw/C10 NcoI-rev). In order to discriminate between the wild type and the mutated form, we added the FLAG TAG to the wild type clone by PCR (C10FLAG-fw/NcoI-rev). Both cDNAs were then cloned into the pcDNA3.1 Myc-His vector (Invitrogen) and sequenced.

Transient Transfection and Western Blotting

Human embryonic kidney 293T cells (ATCC #CRL-11268) were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% FCS at 37°C.

Table 1: Primers used for caspase-10 cloning

C10HA-*fw*	5'-ccgaattccatgtaccttatgatgtgccagattatgcctctaaatctcaaggtcaacattgg-3'
C10FLAG-*fw*	5'-ccgaattccatggactacaaggacgacgatgacaagaaatctcaaggtcaacattgg-3'
C10NcoI-*fw*	5'-gttctgtattctgaccccatgggag-3'
C10NcoI-*rev*	5'-tccaaatctccccatgggtc-3'
C10XhoI-*rev*	5'-ccgctcgagtaatgaaagtgcatccag-3'

3×10^6 cells were plated in 10 cm dishes and transfected with 24 μg of the empty vector, the WT^{FLAG} vector, the $P501L^{HA}$ vector, or a mix of them by Lipofectamine 2000 kit (Invitrogen). After 24 h, adherent and floating cells were harvested in lysis buffer (MBL) for 30 min. Cell debris were removed by centrifugation and equal amounts of the cleared lysates were heated for 5 min at 95°C. Protein extracts were then separated by SDS-PAGE, transferred to Hybond-C extra membranes (Ge Healthcare, Piscataway, NJ, USA), blotted with anti-caspase-10 (1 μg ml^{-1}) (MBL), anti-HA (1 μg ml^{-1}) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-FLAG (1 μg ml^{-1}) (Sigma, Milan, Italy), anti-tubulin (1 μg ml^{-1}) (Sigma) and a peroxidase-conjugated anti-mouse or rabbit antibodies (Ge Healthcare), and revealed by chemiluminescence.

Authors' contributions

UD, EC and AC drafted the manuscript, all authors contributed to the revision. EC, MFC, MF, EG, NC performed experiments. AR diagnosed one patient and contributed to the manuscript writing. LG was responsible for data collection and analysis. ML critically revised the paper and is responsible for important intellectual content. UD and UR were involved in the conception and design of the study. All authors edited and approved the written manuscript.

Acknowledgements

This work was partially supported by grants from "Banca del Piemonte" and University of Torino to UR, by grants from Compagnia di San Paolo (Turin), Regione Piemonte (Turin), Telethon grant E1170 (Rome), FISM grant 2003/R/20 (Genoa), PRIN Project (MIUR, Rome), AIRC (Milan), Fondazione CARIPLO (Milan) to UD. We wish to thank dr Claudio Favre and Dr Margherita Nardi, Pediatric Onco-Hematology Dept, University of Pisa for their help in following the patients.

References

1. Straus SE, Sneller M, Lenardo MJ, Puck JM, Strober W: **Inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome.** *Ann Int Med* 1999, **130**:591-601.
2. Dianzani U, Chiocchetti A, Ramenghi U: **Role of inherited defects decreasing Fas function in autoimmunity.** *Life Sciences* 2003, **72**:2803-2824.
3. Sneller MC, Dale JK, Straus SE: **Autoimmune lymphoproliferative syndrome.** *Curr Opin Rheum* 2003, **15**:417-21.
4. Rieux-Laucat F, Le Deist F, Fischer A: **Autoimmune lymphoproliferative syndromes: genetic defects of apoptosis pathways.** *Cell Death Diff* 2003, **10**:124-133.
5. Straus SE, Jaffe ES, Puck JM, Dale JK, Elkon KB, Rosen-Wolff A, Peters AMJ, Sneller M, Hallahan CW, Wang J, Fischer RE, Jackson CM, Lyn AY, Baumler ES, Marx A, Vaishnaw AK, Grodzicky T, Fleisher TA, Lenardo MJ: **The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis.** *Blood* 2001, **98**:194-200.
6. Nagata S: **Apoptosis by death factor.** *Cell* 1997, **88**:355-365.
7. Krammer PH: **CD95's deadly mission in the immune system.** *Nature* 2000, **407**:789-95.
8. Wang J, Chun HJ, Wong W, Spencer DM, Lenardo MJ: **Caspase-10 is an initiator caspase in death receptor signaling.** *Proc Natl Acad Sci USA* 2001, **98**:13884-13888.
9. Kischkel FC, Lawrence DA, Tinel A, LeBlanc H, Virmani A, Schow P, Gazdar A, Blenis J, Arnott D, Ashkenazi A: **Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8.** *J Biol Chem* 2001, **276**:46639-46646.
10. Sprick MR, Rieser E, Sthal H, Grosse-Wilde A, Weigand MA, Walczak H: **Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signaling complexes in a FADD-dependent manner but can not functionally substitute caspase-8.** *EMBO J* 2002, **21**:4520-4530.
11. Fisher GH, Rosenberg FJ, Straus SE, Dale JK, Middleton LA, Lin AY, Strober W, Lenardo MJ, Puck JM: **Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome.** *Cell* 1995, **81**:935-46.
12. Rieux-Laucat F, Le Deist F, Hivroz C, Roberts IA, Debatin KM, Fischer A, deVillartay JP: **Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity.** *Science* 1995, **268**:1347-9.
13. Wu J, Wilson J, He J, Xiang L, Schur PH, Mountz JD: **Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease.** *J Clin Invest* 1996, **98**:1107-13.
14. Del-Rey M, Ruiz-Contreras J, Bosque A, Calleja S, Gomez-Rial J, Roldan E, Morales P, Serrano A, Anel A, Paz-Artal E, Allende LM: **A homozygous Fas ligand gene mutation in a patient causes a new type of autoimmune lymphoproliferative syndrome.** *Blood* 2006, **108**:1306-12.
15. Bi LL, Pan G, Atkinson TP, Zheng L, Dale JK, Makris C, Reddy V, McDonald JM, Siegel RM, Puck JM, Lenardo MJ, Straus SF: **Dominant Inhibition of Fas Ligand-Mediated Apoptosis due to a Heterozygous Mutation Associated with Autoimmune Lymphoproliferative Syndrome (ALPS) Type Ib.** *BMC Med Genet* 2007, **8**:41.
16. Wang J, Zheng L, Lobito A, Chan FK, Dale J, Sneller M, Yao X, Puck JM, Straus SE, Lenardo MJ: **Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II.** *Cell* 1999, **98**:47-58.
17. Zhu S, Hsu AP, Vacek MM, Zheng L, Schaffer AA, Dale JK, Davis J, Fischer RE, Straus SE, Boruchov D, Saulsbury FT, Lenardo MJ, Puck JM: **Genetic alterations in caspase-10 may be causative or protective in autoimmune lymphoproliferative syndrome.** *Hum Genet* 2006, **119**:284-94.
18. Dianzani U, Bragardo M, DiFranco D, Alliaudi C, Scagni P, Buonfiglio D, Redoglia V, Bonissoni S, Correra A, Dianzani I, Ramenghi U: **Deficiency of the Fas apoptosis pathway without Fas gene mutations in pediatric patients with autoimmunity/lymphoproliferation.** *Blood* 1997, **89**:2871-2879.
19. Ramenghi U, Bonissoni S, Migliaretti G, DeFranco S, Bottarel F, Gambaruto C, DiFranco D, Priori R, Conti F, Dianzani I, Valesini G, Merletti F, Dianzani U: **Deficiency of the Fas apoptosis pathway without Fas gene mutations is a familial trait predisposing to development of autoimmune diseases and cancer.** *Blood* 2000, **95**:3176-82.
20. Campagnoli MF, Garbarini L, Quarello P, Garelli E, Carando A, Baravalle V, Doria A, Biava A, Chiocchetti A, Rosolen A, Dufour C, Dian-

zani U, Ramenghi U: **The broad spectrum of autoimmune lymphoproliferative disease: molecular bases, clinical features and long-term follow-up in 31 patients.** *Haematologica* 2006, **91**:538-41.
21. Oliveira JB, Bidere N, Niemela JE, Zheng L, Sakai K, Nix CP, Danner RL, Barb J, Munson PJ, Puck JM, Dale J, Straus SE, Fleisher TA, Lenardo MJ: **NRAS mutation causes a human autoimmune lymphoproliferative syndrome.** *Proc Natl Acad Sci USA* 2007, **104**:8953-8.
22. Jackson CE, Fischer RE, Hsu AP, Anderson SM, Choi Y, Wang J, Dale JK, Fleisher TA, Middelton LA, Sneller MC, Lenardo MJ, Straus SE, Puck JM: **Autoimmune lymphoproliferative syndrome with defective Fas: genotype influences penetrance.** *Am J Hum Gen* 1999, **64**:1002-1014.
23. Chiocchetti A, Indelicato M, Bensi T, Mesturini R, Giordano M, Sametti S, Castelli L, Bottarel F, Mazzarino MC, Garbarini L, Giacopelli F, Valesini G, Santoro C, Dianzani I, Ramenghi U, Dianzani U: **High levels of Osteopontin associated to polymorphisms in its gene are a risk factor for development of autoimmunity/lymphoproliferation.** *Bloo* 2004, **103**:1376-1382.
24. Clementi R, Chiocchetti A, Cappellano G, Cerutti E, Ferretti M, Orilieri E, Dianzani I, Ferrarini M, Bregni M, Danesino C, Bozzi V, Putti MC, Cerutti F, Cometa A, Locatelli F, Maccario R, Ramenghi U, Dianzani U: **Variations of the perforin gene in patients with autoimmunity/lymphoproliferation and defective Fas function.** *Blood* 2006, **108**:3079-84.
25. Liu C, Cheng J, Mountz JD: **Differential expression of human Fas mRNA species upon peripheral blood mononuclear cell activation.** *Biochem J* 1995, **310**:957-63.
26. Kasahara Y, Wada T, Niida Y, Yachie A, Seki H, Ishida Y, Sakai T, Koizumi F, Koizumi S, Miyawaki T, Taniguchi N: **Novel Fas (CD95/APO-1) mutations in infants with a lymphoproliferative disorder.** *Int Immunol* 1998, **10**:195-202.
27. Rieux-Laucat F, Blachere S, Danielan S, De Villartay JP, Oleastro M, Solary E, Bader-Meunier B, Arkwright P, Pondarè C, Bernaudin F, Chapel H, Nielsen S, Berrai M, Fischer A, Le Deist F: **Lymphoproliferative syndrome with autoimmunity: A possible genetic basis for dominant expression of the clinical manifestations.** *Blood* 1999, **94**:2575-82.
28. Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, Ricci JE, Edris WA, Sutherlin DP, Green DR, Salvesen GS: **A unified model for apical caspase activation.** *Mol Cell* 2003, **2**:529-41.
29. Gronbaek K, Dalby T, Zeuthen J, Ralfkiaer E, Guldberg P: **The V410I (G1228A) variant of the caspase-10 gene is a common polymorphism of the Danish population.** *Blood* 2000, **95**:2184-2185.
30. Hull KM, Drewe E, Aksentijevich I, Singh HK, Wong K, McDermott EM, Dean J, Powell RJ, Kastner DL: **The TNF receptor-associated periodic syndrome (TRAPS): emerging concepts of an autoinflammatory disorder.** *Medicine (Baltimore)* 2002, **81**:349-68.
31. Clementi R, Locatelli F, Dupre L, Garaventa A, Emmi L, Bregni M, Cefalo G, Moretta A, Danesino C, Comis M, Pession A, Ramenghi U, Maccario R, Arico M, Roncarolo MG: **A proportion of patients with lymphoma may harbor mutations of the perforin gene.** *Blood* 2005, **105**:4424-8.

1.3 Conclusions

Both patients described in this paper carried TNFR6 mutations predicted to cause haploinsufficiency. In line with this prediction, their T cells expressed low levels of Fas. This type of mutations have been shown to have weak penetrance. In fact, the nonsense Q47X mutation was not sufficient to cause ALPS in the Pt.2's carrier mother. Similarly in the case of Pt.1, the IVS3-2a>g splice site defect was probably carried by father who did not display ALPS, but his unavailability does not allow to rule out the possibility that this was a de novo mutation. However, this mutation has been previously described in a different family, where its heterozigosity was not sufficient to cause ALPS (Kasahara et al, 1998).

Pt.2 carried also the Y446C mutation in CASP10. This mutation has been previously reported in one heterozygous patient displaying a mild form of ALPS (Zhu et al, 2006) and codes for a caspase-10 displaying defective activity but no dominant negative effect on the wild type protein. The mutation is not sufficient to induce ALPS since it is carried by 1–2% of the healthy Caucasian population (Zhu et al, 2006; Campagnoli et al, 2006).

Pt.1 carried the novel P501L mutation, located, like Y466C, in the small subunit of caspase-10. P501L, too, decreased caspase-10 function without inducing a dominant negative effect on the wild type protein as shown by analyzing cells from the patient and the mother and 293T cells transfected with the mutated and or the wild type forms of caspase-10. Interestingly the western blot analysis of transfected cells showed that P501L did not affect cleavage of caspase-10, which is often interpreted as an evidence of activation. However, the decreased activity detected by the in vitro caspase-10 enzyme assay supports the model proposed by Boatright et al. that cleavage is neither sufficient or necessary for activation of initiator caspases, that mainly depends on dimerization (Boatright et al, 2003).

In conclusion this work suggests that ALPS may sometimes be caused by the concurrent effect of mutations hitting different genes involved in Fas function and hence that it may be both a classic monogenic disease, as occurs in the presence of severe mutations hitting the intracellular portion of Fas, and a digenic or even oligogenic disease caused by mutations affecting different steps of the Fas signalling pathway.

2 “VARIATIONS OF THE PERFORIN GENE IN PATIENTS WITH AUTOIMMUNITY/LYMPHOPROLIFERATION AND DEFECTIVE FAS FUNCTION”

2.1 Aim

ALPS-like disorders , like DALD, do not behave as classic monogenic diseases. Most ALPS type-Ia patients are heterozygous for the Fas mutation, but the parent carrying the mutation is generally healthy. Other complementary factors may thus be required in function of the severity of the mutation. In line with this hypothesis, our group previously demonstrated that a concurrent factor in the development of the disease is a polymorphic variant of the osteopontin gene (OPN), inducing production of high levels of this cytokine that inhibits activation-induced cell death (AICD) of lymphocytes (OPN^{high} variations).

A previous work from our group described a patient affected with ALPS type Ia who carried a heterozygous mutation of the perforin gene (PRF1) together to a heterozygous mutation of the Fas gene (Clementi et al, 2004). These mutations were inherited from distinct parents who were healthy, which suggested that their co-transmission was responsible for the son's ALPS. Interestingly, both Fas and perforin are involved in cell-mediated cytotoxicity and in control of lymphocyte homeostasis.

The aim of the second paper was to extend the analysis of PRF1 gene to a larger number of patients with ALPS or DALD and to evaluate its role in the development of the diseases.

2.2 Paper

blood

2006 108: 3079-3084
Prepublished online May 23, 2006;
doi:10.1182/blood-2006-02-001412

Variations of the perforin gene in patients with autoimmunity/lymphoproliferation and defective Fas function

Rita Clementi, Annalisa Chiocchetti, Giuseppe Cappellano, Elisa Cerutti, Massimo Ferretti, Elisabetta Orilieri, Irma Dianzani, Marina Ferrarini, Marco Bregni, Cesare Danesino, Valeria Bozzi, Maria Caterina Putti, Franco Cerutti, Angela Cometa, Franco Locatelli, Rita Maccario, Ugo Ramenghi and Umberto Dianzani

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published semimonthly by the American Society of Hematology, 1900 M St, NW, Suite 200, Washington DC 20036.

Variations of the perforin gene in patients with autoimmunity/lymphoproliferation and defective Fas function

Rita Clementi, Annalisa Chiocchetti, Giuseppe Cappellano, Elisa Cerutti, Massimo Ferretti, Elisabetta Orilieri, Irma Dianzani, Marina Ferrarini, Marco Bregni, Cesare Danesino, Valeria Bozzi, Maria Caterina Putti, Franco Cerutti, Angela Cometa, Franco Locatelli, Rita Maccario, Ugo Ramenghi, and Umberto Dianzani

Mutations decreasing function of the Fas death receptor cause the autoimmune lymphoproliferative syndrome (ALPS) with autoimmune manifestations, spleen/lymph node enlargement, and expansion of CD4/CD8-negative T cells. Dianzani Autoimmune Lymphoproliferative Disease (DALD) is a variant lacking this expansion. Perforin is involved in cell-mediated cytotoxicity and its biallelic mutations cause familial hemophagocytic lymphohistiocytosis (HLH). We previously described an ALPS patient carrying heterozygous mutations of the Fas and perforin genes and suggested that they concurred in ALPS. This work extends the analysis to 14 ALPS, 28 DALD, and 816 controls, and detects an N252S amino acid substitution in 2 ALPS, and an A91V amino acid substitution in 6 DALD. N252S conferred an OR = 62.7 (P = .0016) for ALPS and A91V conferred an OR = 3 (P = .016) for DALD. Copresence of A91V and variations of the osteopontin gene previously associated with DALD conferred an OR = 17 (P = .0007) for DALD. In one N252S patient, NK activity was strikingly defective in early childhood, but became normal in late childhood. A91V patients displayed lower NK activity than controls. These data suggest that perforin variations are a susceptibility factor for ALPS/DALD development in subjects with defective Fas function and may influence disease expression. (Blood. 2006;108:3079-3084)

Introduction

Fas is a death receptor belonging to the tumor necrosis factor receptor (TNFR) superfamily and induces cell death upon triggering by FasL.[1-3] In the immune response, it is highly expressed by activated effector lymphocytes and is involved in switching off the immune response, limiting clonal expansion of lymphocytes, and favoring peripheral tolerance. Moreover, FasL is expressed by cytotoxic T cells and NK cells and is involved in killing of target cells expressing Fas. Fas induces cell apoptosis by triggering a cascade of caspases through 2 partly interconnected pathways: the extrinsic pathway involves caspase-8–mediated direct activation of the cascade, whereas the intrinsic pathway proceeds through mitochondrial release of cytochrome c and activation of caspase-9. Both pathways converge in the activation of effector caspases, such as caspase-3, -6, and -7.[1-3]

Defective Fas function leads to the unwanted accumulation of lymphocytes and favors autoimmunity possibly by impairing the switching off of autoreactive lymphocytes. This has been shown in the autoimmune lymphoproliferative syndrome (ALPS), an inherited disease characterized by (1) defective function of Fas, (2) autoimmune manifestations that predominantly involve blood cells, (3) polyclonal accumulation of lymphocytes in the spleen and lymph nodes with lymphoadenomegaly and/or splenomegaly, and (4) expansion of TCRαβ + CD4/CD8 double-negative (DN) T cells in the peripheral blood. Moreover, ALPS patients are predisposed to develop lymphomas in adulthood.[3-11] ALPS is generally due to deleterious mutations of the Fas gene *(TNFRSF6)* and is classified as ALPS type-Ia, but rare mutations of other genes have been detected, for instance, the FasL genes in ALPS-Ib, and the caspase-10 gene in ALPS type-IIa, whereas the mutated gene is not known in other patients. Mutations of the Fas and the FasL gene detected in MLR *lpr/lpr* and *gld/gld* mice, respectively, give rise to a disease that overlaps ALPS. We described an ALPS variant that fulfils the first 3 criteria but lacks expansion of DN T cells and mutations of the Fas, FasL, or caspase-10 genes.[12,13] Since the complete paradigm of ALPS could not be demonstrated, this disease has been provisionally named Dianzani Autoimmune Lymphoproliferative Disease (DALD) by McKusick (OMIM reference #605233; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = OMIM).

ALPS-like disorders do not behave as classic monogenic diseases.[3-8] This is true in *lpr/lpr* and *gld/gld* mice and even more evident in ALPS and DALD. The lpr and gld mutations cause the disease in homozygosity, but its expression greatly depends on the genetic background, since it is much milder in BALB/c than in MLR mice. Most ALPS type-Ia patients are heterozygous for the Fas mutation, but the parent carrying the mutation is generally healthy. Other complementary factors may thus be required in

From the Pediatric Haematology-Oncology, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, University of Pavia, Pavia, Italy; Interdisciplinary Research Center of Autoimmune Diseases (IRCAD) and Department of Medical Sciences, "A. Avogadro" University of Eastern Piedmont, Novara, Italy; Laboratory of Tumor Immunology and Department of Oncology, Scientific Institute H. S. Raffaele, Milano, Italy; Division of Hematology-Bone Marrow Transplantation, Scientific Institute H. S. Raffaele, Milano, Italy; Department of Biology and Medical Genetics, University of Pavia and IRCCS Policlinico San Matteo, Pavia, Italy; Pediatric Haematology-Oncology, Department of Pediatrics, University of Padua, Padova, Italy; and Department of Pediatrics, University of Turin, Torino, Italy.

Submitted February 3, 2006; accepted May 9, 2006. Prepublished online as *Blood* First Edition Paper, May 23, 2006; DOI 10.1182/blood-2006-02-001412.

R.C. and A.C. contributed equally to this work.

Reprints: Umberto Dianzani, IRCAD-Department of Medical Sciences, Via Solaroli, 17, I-28100, Novara, Italy; e-mail: umberto.dianzani@med.unipmn.it.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

function of the severity of the mutation.[14] The same observation is true in DALD patients, since both parents generally display defective Fas function, but are healthy.[13] We suggested that a concurrent factor may be production of high levels of osteopontin (OPN), a cytokine involved in inflammation that inhibits activation-induced cell death (AICD) of lymphocytes. We found that DALD patients display increased frequency of polymorphic variants of the OPN gene (OPN^{high} gene variants) that cause increased production of OPN by stabilizing its mRNA and increase the risk of DALD by about 8-fold.[15,16]

A second concurrent factor may be inherited alterations of the perforin gene *(PRF1)* that decrease the function of this protein, which is stored in the lytic granules of cytotoxic cells and plays a crucial role in cell-mediated cytotoxicity by forming pores in the target-cell membrane.[17] Biallelic mutations of *PRF1* cause about 30% of cases of familial hemophagocytic lymphohistiocytosis (HLH), a rare life-threatening immune deficiency ascribed to decreased capacity of CD8+ T cells and NK cells to kill virus-infected cells.[17-22] A further 25% of patients display mutations of the *MUNC 13-4* gene involved in perforin storage in the lytic granules and exocytosis.[23] HLH is a recessive disease and subjects carrying heterozygous PRF1 mutations are generally healthy.

We have identified a heterozygous mutation of *PRF1* in an ALPS patient who also carried a heterozygous mutation of the Fas gene. Since these mutations were inherited from distinct parents who were healthy, we suggested that their cotransmission was responsible for the son's ALPS.[24]

Systematic evaluation of the role of *PRF1* in ALPS was not undertaken on that occasion. The present study has thus been performed to extend the analysis of *PRF1* to a larger number of patients and evaluate its role in the development of ALPS and DALD.

Patients, materials, and methods

Patients

We analyzed 14 ALPS and 28 DALD Italian patients (some have already been presented in Dianzani et al,[12] Ramenghi et al,[13] and Campagnoli et al[25]). Diagnosis of ALPS was based on the presence of all the following criteria: (1) autoimmune manifestations; (2) chronic nonmalignant lymphadenopathy (2 or more lymph nodes enlarged over 2 cm in diameter) and/or splenomegaly; (3) defective Fas-induced apoptosis in vitro; and (4) mutations in the Fas, FasL, or caspase-10 genes and/or expansion of DN T cells in the peripheral blood. The Fas, FasL, caspase-10, and OPN genes were sequenced from genomic DNA, as previously reported.[12,13] Seven ALPS patients (patients 1-7) carried heterozygous mutations of the Fas gene.

Diagnosis of DALD was based on the presence of the first 3 criteria, but lack of the fourth one. Two DALD patients (DALD-2 and -24) carried a heterozygous variation of the caspase-10 gene, causing a V410I amino acid substitution, initially associated with ALPS in homozygosity,[10] but then recognized as a polymorphism.[25,26]

No patients displayed the diagnostic criteria for HLH. Controls (n = 816) were ethnically matched, healthy individuals. All patients and controls were unrelated, white and Italian. Peripheral-blood specimens and serum were obtained from patients and healthy controls with written informed consent, which was obtained in accordance with the Declaration of Helsinki. The study was performed according to the guidelines of the local ethics committee of the Ospedale Maggiore of Novara (Novara, Italy).

Flow cytometry

Analysis of lymphocyte subpopulations in peripheral-blood mononuclear cells (PBMCs) was performed by direct immunofluorescence and flow cytometry. Perforin expression was evaluated in fixed and permeabilized cells (Cytofix-Cytoperm; BD PharMingen, San Diego, CA) using a phycoerythrin (PE)–conjugated antiperforin antibody (BD PharMingen) and flow cytometry.

Fas function assay

Fas-induced cell death was evaluated as previously reported on T-cell lines obtained by activating PBMCs with phytohemagglutinin at days 0 (1 μg/mL) and 15 (0.1 μg/mL) and cultured in RPMI 1640 + 10% fetal calf serum (FCS) + rIL-2 (2 U/mL) (Biogen, Geneva, Switzerland). Fas function was assessed 6 days after the second stimulation (day 21).[12,13] Cells were incubated with control medium or anti-Fas MAb (CH11, IgM isotype) (1 μg/mL) (UBI, Lake Placid, NY) in the presence of rIL-2 (1 U/mL) to minimize spontaneous cell death. Cell survival was evaluated after 18 hours by counting live cells in each well by the trypan blue exclusion test and by flow cytometry of cells excluding propidium iodide and unstained by annexin V–FITC; the 2 methods gave overlapping results. Assays were performed in duplicate. Cells from 2 healthy donors were included in each experiment as positive controls. Results were expressed as specific cell-survival percent, calculated as follows: (total live-cell count in the assay well/total live-cell count in the control well) × 100.

Fas function was defined as defective when cell survival was less than 82% (the 95th percentile of data obtained from 200 healthy controls).

Amplification of *PRF1* and mutation detection

Genomic DNA was isolated from PBMCs, and exon 2 and 3 of the perforin coding region were amplified in standard polymerase chain reaction (PCR) conditions. The primers used for amplification have been previously described. PCR products were purified with the EXO/SAP kit.[24] Sequencing was performed with the ABI PRISMR BigDye Terminator kit (Applied Biosystems, Foster City, CA) on an automatic sequencer (Applied Biosystems 3100 Genetic Analyzer) according to the manufacturer's instructions with the amplification primers plus 2 internal primers (forward 5′-CAGGTCAACATAGGCATCCACG-3′; reverse 5′-GAACAGCAG-GTCGTTAATGGAG-3′) for exon 3. OPN gene variants were typed as previously reported.[15]

Cytotoxicity assays

NK activity of PBMCs was assessed by a standard 4-hour ^{51}Cr release assay with K562 cells as the target. Results are expressed as specific lysis percent calculated as follows: (sample ^{51}Cr release-spontaneous release)/(maximal release-spontaneous release) × 100.

Statistical analysis

Comparisons of NK activity, perforin expression, and NK-cell distribution were performed with the nonparametric Mann-Whitney *U* test. Genotype distributions were analyzed with the χ^2 test or the Fisher exact test as reported. All *P* values are 2-tailed, and the significance cut-off was *P* below .05.

Results

The work involved 14 patients with ALPS and 28 with DALD. Both groups displayed autoimmune manifestations, lymphadenopathy and/or splenomegaly, and defective Fas function; ALPS patients alone also displayed mutations of the Fas gene and/or peripheral-blood expansion of DN T cells. Figure 1 shows Fas function of T cells from all patients and available parents. That of ALPS-4 and -7 was borderline, but they were included in the ALPS group because they also carried a Fas gene mutation and expansion of DN T cells. Most parents, too, displayed defective Fas function.

The coding region of *PRF1* was sequenced from genomic DNA in all patients and 816 random controls in the search for variations previously associated with HLH. Only 2 HLH-associated missense variations were detected, a C/T substitution in position 272 of the cDNA (numerations are referred to cDNA clone M28393,

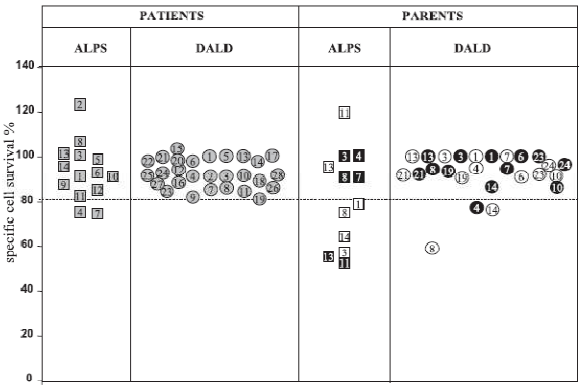


Figure 1. Fas-induced T-cell death in patients with ALPS or DALD and several of their parents. Data from ALPS patients' families are marked with squares, those from DALD patients' families with circles; gray symbols mark patients; black symbols, the fathers; and white symbols, the mothers. Numbers correspond to the code assigned to each patient. Long-term T-cell lines were treated with anti-Fas Mab, and survival was assessed after 18 hours. Results are expressed as relative cell-survival percent. The horizontal lines indicate the upper limit of the normal range, calculated as the 95th percentile of data obtained from 200 healthy donors (median cell survival of controls was 60%; the 5th-95th percentile range was 38%-82%). In the control wells (ie, in the absence of apoptotic stimuli), spontaneous cell loss was always less than 10% of the seeded cells and similar in cultures from the patients and healthy donors. ALPS patients 1 to 7 carried heterozygous mutations of the Fas gene.

ATG = +1) and an A/G substitution in position 755, which caused an A91V and an N252S amino acid substitution at the protein level, respectively. The genotypic distributions of these variations did not deviate significantly from the Hardy-Weinberg equilibrium in either group.

The N252S substitution was found in 2 ALPS patients (ALPS-5 and ALPS-11), 2 controls, and no DALD patients. The overall genotype distributions (Table 1) were significantly different in ALPS and controls ($P = .0016$). The N252S allelic frequency was significantly higher in the ALPS patients (7.1% vs 0.1%, $P = .0016$) and conferred an OR = 62.7 (95% CI: 6-654.9). This variation had been previously reported by some of us in ALPS-5.[24]

The A91V variation was carried by 6 DALD patients only: it was heterozygous in 5 (DALD-3, -6, -9, -14, -25) and homozygous in one (DALD-10). Moreover, it was detected in 72 controls: heterozygous in 69 and homozygous in 3. The overall genotype distributions (Table 1) were significantly different in DALD and controls ($P = .01$). The A91V allelic frequency was significantly higher in the DALD patients (12.5% vs 4.6%, $P = .016$) and conferred an OR = 3 (95% CI: 1.2-7.1).

Table 1. Frequency distribution of the A91V and N252S PRF1 variations and the *OPN*^{high} genotypes in 14 ALPS and 28 DALD patients

Genotypes according to variation	ALPS* N	ALPS* %	DALD† N	DALD† %	Controls N	Controls %
PRF1 N252S						
NN	12	85.7	28	100	814	99.8
NS	2	14.3	0	0	2	0.2
SS	0	0	0	0	0	0
PRF1 A91V						
AA	14	100	22	78.5	744	91.2
AV	0	0	5	17.9	69	8.4
VV	0	0	1	3.6	3	0.4
***PRF1* and *OPN* susceptibility genotypes‡**						
None	4	28.6	6	21.4	51	36.1
PRF1 alone	2	14.3	0	0	10	7.5
OPN alone	8	57.1	16	57.2	70	52.2
PRF1+*OPN*	0	0	6	21.4	3	2.2

*The overall genotypic distribution of N252S was significantly different from controls ($P = .0016$, Fisher exact test).

†The overall genotypic distribution of A91V was significantly different from controls ($P = .01$, Chi square test); frequency of *PRF1*+*OPN* susceptibility genotypes was significantly higher than in controls ($P = .0009$, Fisher exact test).

‡Genotypes with the N252S (in ALPS) or A91V (in DALD) PRF1 variations (either homozygous or heterozygous) and/or the 282C-750T-1083A-1239C or 282C-750T-1083G-1239C *OPN*^{high} haplotypes.

Four other nucleotide variations were detected, but were not further evaluated since they did not change the amino acid nor influence the splicing sites. Two (C822T and T900C) had been previously reported as common polymorphisms not associated with HLH. Their frequency was similar in the patients and the controls. The other 2 (G435A and A462G) were in perfect linkage disequilibrium with the A/G substitution in position 755 (N252S) and were in fact only detected in the 4 subjects carrying this variation.

We had previously found that DALD development is favored by the 282C-750T-1083A-1239C and 282C-750T-1083G-1239C single nucleotide polymorphism haplotypes of the OPN gene (*OPN*^{high} gene variants).[15] To determine whether *PRF1* and *OPN* variations have a cooperative effect on ALPS/DALD development, we typed the OPN gene in all patients and 134 controls and evaluated the frequency of copresence of the *PRF1* and *OPN* genotypes conferring susceptibility to ALPS/DALD (Table 1). Copresence was displayed by 6 (21.4%) of 28 DALD patients, but only 3 (2.2%) of 134 controls, and increased the risk of DALD by 17-fold relatively to the absence of both factors (OR = 17; 95% CI: 2.7-122; $P = .0007$) and by 9-fold relatively to the presence of only one (OR = 8.8, 95% CI: 1.7-50.5; $P = .004$). By contrast, this cooperation was not detected in ALPS patients since none of them carried both factors.

We had previously shown that ALPS-5 carried a heterozygous mutation of the Fas gene.[24] His PRF1 N252S variation was inherited from the mother, whereas the Fas mutation also was carried by the father and a brother, and cosegregated with defective Fas function (Figure 2A). Since all 3 relatives were healthy, we suggested that co-inheritance of the Fas and perforin gene variations played a role in ALPS development in this patient. The mutated gene was not known in ALPS-11, and his inheritance pattern was determined by analyzing Fas function and sequencing *PRF1* in his parents. N252S was carried by his father only, whereas Fas function was defective in his mother only (Figure 2A). Once again, therefore, Fas and perforin alterations were inherited from different parents, who were both healthy. These data indicate that association of defective Fas function with the N252S variation strongly favors ALPS development.

A family analysis also was conducted for 3 DALD patients carrying the A91V variation (DALD-3, -6, -10, Figure 2B). In

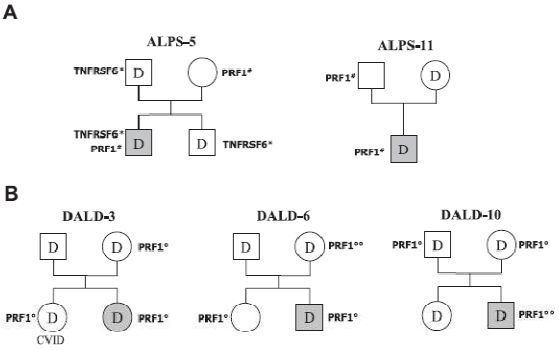


Figure 2. Pedigrees of patients ALPS-5 and -11 and DALD-3,-6, and -10. (A) Inheritance of the N252S PRF1 mutation (PRF1#) and defective Fas function (D) in ALPS-5 and -11; inheritance of the Fas mutation (TNFRSF6*) is also shown for ALPS-5. (B) Inheritance of the A91V PRF1 variation (PRF1°) and defective Fas function (D) in the 3 DALD patients. Subjects with ALPS/DALD are marked in gray; the sister of DALD-3 displayed CVID. PRF1°° marks A91V homozygotes. Fas function was evaluated as reported in Figure 1.

DALD-3's family, Fas function was defective in both parents and the sister, whereas A91V was carried by the mother and the sister: both parents were healthy, whereas the sister presented common variable immune deficiency (CVID). Intriguingly, DALD-3 also developed hypogammaglobulinemia some years after disease onset. In DALD-6's family, Fas function was defective in both parents, whereas A91V was carried by the mother (homozygous) and the sister; all these relatives were healthy. In DALD-10's family, Fas function was defective in both parents and the sister, whereas A91V was carried by both parents; all these relatives were healthy. Analysis of the OPN gene showed that all subjects in these 3 families also carried the OPN^{high} gene variants (data not shown). Association of defective Fas function and the A91V variation is thus not sufficient to induce DALD, even in the presence of the *OPN* susceptibility alleles, since 4 of 8 subjects with this association were healthy.

To assess whether N252S and A91V correlated with altered function and/or expression of perforin, NK activity was evaluated by a standard ^{51}Cr-release assay and perforin expression by flow cytometry in 5 patients: ALPS-5 and -11 with N252S, and DALD-3, -6, and -10 with A91V. Perforin expression was slightly decreased in ALPS-5, DALD-6, and DALD-10, but normal in the other subjects, whereas the proportion of NK cells, detected as $CD3^-CD56^+$ or $CD3^-CD16^+$ cells, was in the normal range in all subjects. NK activity was significantly lower in the patients with A91V than in the controls ($P = .015$), but not decreased in those with N252S (Table 2). However, in ALPS-11, previous analyses showed that NK activity was almost undetectable at the age of 3 (ie, at diagnosis), extremely low but detectable at the age of 5, and normal at the age of 12 (Table 2). In ALPS-5, NK activity was assessed at the age of 30, and no previous analyses were available.

Discussion

This paper follows a description of an ALPS patient (ALPS-5) with variations of both the Fas gene and *PRF1*.[24] It shows that his N252S variation is significantly more frequent in ALPS patients than in healthy controls. Moreover, the frequency of a second HLH-associated PRF1 variation, A91V, is significantly increased in DALD patients, who display an incomplete ALPS pattern. N252S was detected in 2 of 14 ALPS patients (ALPS-5 and -11). It increased susceptibility to ALPS by about 63-fold and was found only in 2 of 816 ethnically matched controls, as in other studies.[27]

Table 2. NK activity, perforin expression, and proportion of NK cells in PBMCs of ALPS/DALD patients carrying the N252S and A91V perforin variations

Subjects according to PRF1 variation*	Effector-target ratios for NK activity†			Perforin expression‡		Peripheral-blood NK cells, %	
	100:1	30:1	10:1	%	MFI	$CD3^-CD16^+$	$CD3^-CD56^+$
N252S							
ALPS-5	58	49	38	6§	9§	11	16
ALPS-11 (12 years)	35	26	18	24	50	13	15
(5 years)	7§	4§	2§	nd	nd	nd	nd
(3 years)	3§	1§	0§	nd	nd	nd	nd
A91V							
DALD-3	13	12	3	45	62	8	8
DALD-6	28	12	3	15§	61	13	13
DALD-10	18	11	4	36	17§	9	13
Median	18	12	3	36	61	9	13
Controls‖	31 (13-56)	23 (6-35)	12 (2-31)	23 (17-26)	47 (28-74)	11 (5-31)	17 (4-27)

nd indicates not determined.

*All patients were heterozygous for the indicated variation except for DALD-10, who was homozygous.

†NK activity is expressed as specific cell-lysis percent, and it is the mean of triplicate assays, whose standard deviation was always < 10% of the mean. Spontaneous cell lysis was always < 10% of maximal cell lysis.

‡Perforin expression is shown as percentage of positive cells and mean fluorescence intensity (MFI) in arbitrary units.

§< 5th percentile of controls.

‖Median (5th-95th percentile range) from 10 controls. The NK activity of these DALD patients was significantly lower than that of the controls ($P = .015$, Mann-Whitney test).

N252S occurs within the membrane-attack-complex, a region critically involved in the pore-forming activity of perforin, but its functional significance has been debated since it has been associated with normal NK activity[17,28,29] as in both ALPS-5 and -11 at the time of this study. The finding that ALPS-11 displayed a striking defect of NK activity when he was 3 and 5 years old suggests that N252S is here associated with other factors decreasing NK function in early childhood, followed by normalization on the part of unknown compensatory mechanisms.

A91V was detected in 6 of 28 DALD patients. It increased susceptibility to DALD by 3-fold and was relatively frequent (4.6%) in the controls, as in other studies.[27,30,31] This variation decreases perforin function by altering its conformation, decreasing its cleavage to the active form and increasing its degradation.[28-30] In line with this view, our patients displayed lower NK activity than the controls, especially at low effector-to-target ratios.

These data suggest that some PRF1 variations that cause HLH when combined with a second PRF1 variation may favor ALPS/DALD development if inherited defects hitting Fas function also are present. Fas function is normal in HLH and hence is not a contributory of this disease.[21]

The family analyses showed that combination of A91V with the Fas defect was not sufficient to induce DALD since several healthy family members carried both alterations. This risk was significantly increased by copresence of the OPN^{high} gene variants, but even their combination with A91V and the Fas defect was not sufficient to induce DALD in 4 of 8 DALD family members. Combination of N252S with the Fas defect seems to have a stronger effect than A91V, since we found it in patients only. However, even this combination may not be sufficient for ALPS development, since Rieux-Laucat et al[32] have described an ALPS patient and his healthy father carrying both N252S and a Fas gene mutation. The observation that N252S is in perfect linkage disequilibrium with G435A and A462G raises the possibility that these variations or others included in the ancestral haplotype play a role in ALPS development. A second possibility is that concurrent roles are played by other factors. The fact that *OPN* did not cooperate with N252S in ALPS development may be related to the stronger effect displayed by N252S in ALPS than by A91V in DALD, and to the possibility that the genetic hit of Fas function is more severe in ALPS than in DALD.

Fas and perforin alterations may cooperate in affecting both the antiviral response and the switching off of the immune response. Both molecules are used by cytotoxic cells to kill virus-infected cells. Moreover, Fas is highly expressed by effector lymphocytes that are switched off by several $FasL^+$ cell types, but a regulatory activity also has been ascribed to perforin-mediated killing of effector lymphocytes and antigen-presenting cells.[33-43] In this connection, it is noteworthy that OPN inhibits lymphocyte AICD, another mechanism involved in switching off the immune response. Lymphocyte accumulation and autoimmunity displayed by ALPS and DALD patients may be favored by both defective immune response switching off and decreased virus clearance that would prolong the immune response. This possibility opens the way to the view that ALPS/DALD may overlap both HLH and other inherited diseases characterized by lymphoproliferation and defective control of viral infections, such as the X-linked lymphoproliferative syndrome (XLP) due to mutations of the SAP gene altering function of the 2B4 NK coreceptor.[44] Intriguingly, both ALPS and XLP are associated with high susceptibility to lymphoid neoplasia, which also seems favored by inherited PRF1 variations.[45]

Acknowledgments

This work was partially supported by Telethon grant E1170 (Rome), Fondazione Italiana Sclerosi Multipla (FISM) grant 2003/R/20 (Genoa), Programma di Rilevante Interesse Nazionale (PRIN) Project (Ministero Istruzione Universita Ricerca [MIUR], Rome), Fondazione Cariplo (Milan), Compagnia di San Paolo (Turin), Regione Piemonte (Turin), and Associazione Italiana Ricerca sul Cancro (AIRC) (Milan).

References

1. Nagata S. Apoptosis by death factor. Cell. 1997; 88:355-365.
2. Krammer PH. CD95's deadly mission in the immune system. Nature. 2000;407:789-795.
3. Dianzani U, Chiocchetti A, Ramenghi U. Role of inherited defects decreasing Fas function in autoimmunity. Life Sciences. 2003;72:2803-2824.
4. Fisher GH, Rosenberg FJ, Straus SE, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell. 1995;81:935-946.
5. Rieux-Laucat F, Le Deist F, Hivroz C, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. Science. 1995;268:1347-1349.
6. Straus SE, Sneller M, Lenardo MJ, Puck JM, Strober W. An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. Ann Int Med. 1999;130:591-601.
7. Sneller MC, Dale JK, Straus SE. Autoimmune lymphoproliferative syndrome. Curr Opin Rheum. 2003;15:417-421.
8. Rieux-Laucat F, Fischer A, Le Deist F. Cell-death signaling and human disease. Curr Opin Immunol. 2003;15:325-331.
9. Straus SE, Jaffe ES, Puck JM, et al. The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis. Blood. 2001;98:194-200.
10. Wang J, Zheng L, Lobito A, et al. Inherited human caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. Cell. 1999; 98:47-58.
11. Rieux-Laucat F, Le Deist F, Fischer A. Autoimmune lymphoproliferative syndromes: genetic defects of apoptosis pathways. Cell Death Diff. 2003;10:124-133.
12. Dianzani U, Bragardo M, DiFranco D, et al. Deficiency of the Fas apoptosis pathway without Fas gene mutations in pediatric patients with autoimmunity/lymphoproliferation. Blood. 1997;89:2871-2879.
13. Ramenghi U, Bonissoni S, Migliaretti G, et al. Deficiency of the Fas apoptosis pathway without Fas gene mutations is a familial trait predisposing to development of autoimmune diseases and cancer. Blood. 2000;95:3176-3182.
14. Jackson CE, Fischer RE, Hsu Ap, et al. Autoimmune lymphoproliferative syndrome with defective Fas: genotype influences penetrance. Am J Hum Gen. 1999;64:1002-1014.
15. Chiocchetti A, Indelicato M, Bensi T, et al. High levels of osteopontin associated to polymorphisms in its gene are a risk factor for development of autoimmunity/lymphoproliferation. Blood. 2004;103:1376-1382.
16. Chiocchetti A, Comi C, Indelicato M, et al. Osteopontin gene haplotypes correlate with multiple sclerosis development and progression. J Neuroimmunol. 2005;63:172-178.
17. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science. 1999;286: 957-959.
18. Clementi R, zur Stadt U, Savoldi G, et al. Six novel mutations in the PRF1 gene in children with haemophagocytic lymphohistiocytosis. J Med Genet. 2001;38:643-646.
19. Ueda I, Morimoto A, Inaba T, et al. Characteristic perforin gene mutations of haemophagocytic lymphohistiocytosis patients in Japan. Br J Haematol. 2003;121:503-510.
20. Feldmann J, Le Deist F, Ouachee-Chardin M, et al. Functional consequences of perforin gene mutations in 22 patients with familial haemophagocytic lymphohistiocytosis. Br J Haematol. 2002; 117:965-972.
21. Fadeel B, Orrenius S, Henter JI. Familial hemophagocytic lymphohistiocytosis: too little cell death can seriously damage your health. Leuk Lymphoma. 2001;42:13-20.
22. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nature Rev Immunol. 2002;2:735-747.
23. Feldmann J, Callebaut I, Raposo G, et al. Munc13–4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). Cell. 2003; 115:461-473.

24. Clementi R, Dagna L, Dianzani U, et al. Inherited perforin and Fas mutations in a patient with autoimmune lymphoproliferative syndrome and lymphoma. N Engl J Med. 2004;351:1419-1424.
25. Campagnoli MF, Garbarini L, Quarello P, et al. The broad spectrum of autoimmune lymphoproliferative disease: molecular bases, clinical features and long-term follow-up in 31 patients. Haematologica. 2006;91:538-541.
26. Zhu S, Hsu AP, Vacek MM, et al. Genetic alterations in caspase-10 may be causative or protective in autoimmune lymphoproliferative syndrome. Hum Genet. 2006;119:284-294.
27. Molleran Lee S, Villanueva J, Sumegi J, et al. Characterisation of diverse PRF1 mutations leading to decreased natural killer cell activity in North American families with haemophagocytic lymphohistiocytosis. J Med Genet. 2004;41:137-144.
28. Voskoboinik I, Thia MC, Trapani JA. A functional analysis of the putative polymorphisms A91V and N252S and 22 missense perforin mutations associated with familial hemophagocytic lymphohistiocytosis. Blood. 2005;105:4700-4706.
29. Risma KA, Frayer RW, Filipovich AH, Sumegi J. Aberrant maturation of mutant perforin underlies the clinical diversity of hemophagocytic lymphohistiocytosis. J Clin Invest. 2006;116:182-192.
30. Trambas C, Gallo F, Pende D, et al. A single amino acid change, A91V, leads to conformational changes that can impair processing to the active form of perforin. Blood. 2005;106:932-937.
31. Busiello R, Fimiani G, Miano MG, et al. A91V perforin variation in healthy subjects and FHLH patients. Int J Immunogen. 2006;33:123-125.
32. Rieux-Laucat F, Le Deist F, De Saint Basile G. Autoimmune lymphoproliferative syndrome and perforin. N Engl J Med. 2005;352:306-307.
33. Su MW, Pyarajan S, Chang JH, et al. Fratricide of CD8+ cytotoxic T lymphocytes is dependent on cellular activation and perforin-mediated killing. Eur J Immunol. 2004;34:2459-2470.
34. Badovinac VP, Hamilton SE, Harty JT. Viral infection results in massive CD8+ T cell expansion and mortality in vaccinated perforin-deficient mice. Immunity. 2003;18:463-474.
35. Badovinac VP, Tvinnereim AR, Harty JT. Regulation of antigen-specific CD8+ T cell homeostasis by perforin and interferon-gamma. Science. 2000;290:1354-1358.
36. Marks L, Levy RB. The cytotoxic potential of regulatory T cells: what has been learned from gene knockout model systems? Transplantation. 2004;77:S19-S22.
37. de Saint Basile G, Fischer A. Defective cytotoxic granule-mediated cell death pathway impairs T lymphocyte homeostasis. Curr Opin Rheumatol. 2003;15:436-445.
38. Voskoboinik I, Thia MC, De Bono A, et al. The functional basis for hemophagocytic lymphohistiocytosis in a patient with co-inherited missense mutations in the perforin (PFN1) gene. J Exp Med. 2004;200:811-816.
39. Zhou S, Ou R, Huang L, Moskophidis D. Critical role for perforin-, Fas/FasL-, and TNFR1-mediated cytotoxic pathways in down-regulation of antigen-specific T cells during persistent viral infection. J Virol. 2002;76:829-840.
40. Beilke JN, Kuhl NR, Van Kaer L, Gill RG. NK cells promote islet allograft tolerance via a perforin-dependent mechanism. Nat Med. 2005;11:1059-1065.
41. Bose A, Inoue Y, Kokko KE, Lakkis FG. Cutting edge: perforin down-regulates CD4 and CD8 T cell–mediated immune responses to a transplanted organ. J Immunol. 2003;170:1611-1614.
42. Ferlazzo G, Munz C. NK cell compartments and their activation by dendritic cells. J Immunol. 2004;172:1333-1339.
43. Della Chiesa M, Vitale M, Carlomagno S, Ferlazzo G, Moretta L, Moretta A. The natural killer cell–mediated killing of autologous dendritic cells is confined to a cell subset expressing CD94/NKG2A, but lacking inhibitory killer Ig-like receptors. Eur J Immunol. 2003;33:1657-1666.
44. Nichols KE, Ma CS, Cannons JL, Schwartzberg PL, Tangye SG. Molecular and cellular pathogenesis of X-linked lymphoproliferative disease. Immunol Rev. 2005;203:180-199.
45. Clementi R, Locatelli F, Dupre L, et al. A proportion of patients with lymphoma may harbor mutations of the perforin gene. Blood. 2005;105:4424-4428.

2.3 Conclusions

This report shows that a concurrent factor favoring ALPS/DALD development may be perforin gene (PRF1) variations that decrease the function of this protein involved in cell-mediated cytotoxicity. We detected the N252S amino acid substitution in 2 ALPS (frequency 7.1% vs 0.1% controls), and the A91V amino acid substitution in 6 DALD (12.5 % vs 4.6%). Several works have shown that A91V decreases perforin function by altering its conformation, decreasing its cleavage to the active form, and increasing its degradation (Molleran Lee et al, 2004; Trambas et al, 2005; Voskoboinik et al, 2005). By contrast, the functional significance of N252S has been debated because it occurs within the membrane attack complex, a region critically involved in the pore forming activity of perforin, but several works have associated it with normal NK function and perforin expression (Stepp et al, 1999; Trambas et al, 2005; Voskoboinik et al, 2005; Risma et al, 2006). Interestingly, in one of our N252S patients, NK activity was strikingly defective in early childhood, but became normal in late childhood. Instead A91V patients displayed lower NK activity than controls. These data suggest that some PRF1 variations that cause HLH when combined with a second PRF1 variation may favor ALPS/DALD development if inherited defects hitting Fas function also are present. The family analyses showed that combination of A91V with the Fas defect was not sufficient to induce DALD since several healthy family members carried both alterations. This risk was significantly increased by copresence of the OPN^{high} gene variations, but even their combination with A91V and the Fas defect was not sufficient to induce DALD in 4 of 8 DALD family members. Combination of N252S with the Fas defect seems to have a stronger effect than A91V, since we found it in patients only. However, even this combination may not be sufficient for ALPS development, since Rieux-Laucat et al (Rieux-Laucat et al, 2005) have described an ALPS patient and his healthy father carrying both N252S and a Fas gene mutation. The observation that N252S is in perfect linkage disequilibrium with G435A and A462G raises the possibility that these variations or others included in the ancestral haplotype play a role in ALPS development.

These data suggest that Fas and perforin alterations may cooperate in affecting both the antiviral response and the switching off of the immune response. Both molecules are used by cytotoxic cells to kill virus-infected cells. Moreover, Fas is highly expressed by effector lymphocytes that are switched off by several FasL-positive cell types, but a

regulatory activity also has been ascribed to perforin-mediated killing of effector lymphocytes and antigen-presenting cells. Defects of both of these functions may predispose to autoimmunity by prolonging the immune response and increasing the risk of cross-reactions between viral and self antigens by molecular mimicry. In line with a role of the perforin gene in the pathogenesis of autoimmune diseases, we recently demonstrated the association variations of this gene with type 1 diabetes mellitus and multiple sclerosis respectively.

3 “THE MITOGEN-ACTIVATED PROTEIN KINASE SCAFFOLD KSR1 IS REQUIRED FOR RECRUITMENT OF EXTRACELLULAR SIGNAL-REGULATED KINASE TO THE IMMUNOLOGICAL SYNAPSE”

3.1 Aim

KSR1 was originally described as a positive regulator of the RAS–MAPK signalling pathway in *Drosophila melanogaster* and *Caenorhabditis elegans* (Therrien et al, 1995; Kornfeld et al, 1995; Sundaram et al, 1995). KSR functions as a MAP kinase scaffold because binds many proteins, including all three components of the ERK signalling pathway (RAF, MEK1 and ERK).While the exact function of KSR is unknown, preassembling the three components of the ERK MAP kinase cascade could function to enhance the efficiency of ERK activation, potentially regulate the subcellular location of ERK activation, and promote access to specific subcellular substrates.

KSR1-deficient mice develop without any obvious defects (Nguyen et al, 2002), but KSR1-deficient cells exhibit an attenuation of ERK activation with defects in cell proliferation.

Aim of the third paper was to investigate the role of KSR1 in NK cell-mediated cytolysis. The killing of a target cell by a cytolytic T cell or NK cell is a complicated process that involves cell polarization with microtubule-dependent movement of cytolytic granules to the immunological synapse. A variety of different signaling molecules are also involved in this process, including calcium, phosphatidylinositol-3,4,5-triphosphate and activation of the ERK MAP kinase. Recently, the recruitment of activated ERK to the immunological synapse has been shown to be a feature of successful killing of a target by cytotoxic T lymphocytes (Yachi et al, 2006).

How active ERK is recruited to the synapse is not known. Since KSR1 is known to be recruited to the plasma membrane by Ras activation (Michaud et al, 1997), it seemed plausible to test the hypothesis that KSR1 recruitment to the plasma membrane functions to recruit ERK to the immunological synapse and facilitate its activation.

3.2 Paper

MOLECULAR AND CELLULAR BIOLOGY, Mar. 2009, p. 1554–1564 Vol. 29, No. 6
0270-7306/09/$08.00+0 doi:10.1128/MCB.01421-08

The Mitogen-Activated Protein Kinase Scaffold KSR1 Is Required for Recruitment of Extracellular Signal-Regulated Kinase to the Immunological Synapse▽

Emanuele Giurisato,[1] Joseph Lin,[1] Angus Harding,[1]† Elisa Cerutti,[1] Marina Cella,[1] Robert E. Lewis,[2] Marco Colonna,[1] and Andrey S. Shaw[1,3]*

Department of Pathology & Immunology, Washington University School of Medicine, St. Louis, Missouri 63110[1]; Department of Pathology, University of Nebraska Medical Center, Eppley Cancer Institute, Omaha, Nebraska 68198-7696[2]; and Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri 63110[3]

Received 10 September 2008/Returned for modification 8 October 2008/Accepted 19 December 2008

KSR1 is a mitogen-activated protein (MAP) kinase scaffold that enhances the activation of the MAP kinase extracellular signal-regulated kinase (ERK). The function of KSR1 in NK cell function is not known. Here we show that KSR1 is required for efficient NK-mediated cytolysis and polarization of cytolytic granules. Single-cell analysis showed that ERK is activated in an all-or-none fashion in both wild-type and KSR1-deficient cells. In the absence of KSR1, however, the efficiency of ERK activation is attenuated. Imaging studies showed that KSR1 is recruited to the immunological synapse during T-cell activation and that membrane recruitment of KSR1 is required for recruitment of active ERK to the synapse.

Kinase suppressor of Ras was originally identified in *Drosophila melanogaster* (53) and *Caenorhabditis elegans* (19, 32, 52) as a positive regulator of the extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase signaling pathway. It is thought to function as a MAP kinase scaffold because it can bind to Raf, MEK, and ERK (18, 19, 27, 28, 44, 59). While the exact function of KSR is unknown, preassembling the three components of the ERK MAP kinase cascade could function to enhance the efficiency of ERK activation, potentially regulate the subcellular location of ERK activation, and promote access to specific subcellular substrates (16, 45, 46).

While only one isoform of KSR is expressed in *Drosophila* (53), two KSR isoforms have been identified in *C. elegans* (19, 32, 52) and most higher organisms. They are referred to as KSR1 and KSR2 (32, 43). While KSR1 mRNA and protein are detectable in a wide variety of cells and tissues, including brain, thymus, and muscle (10, 11, 29), little is known about the expression pattern of KSR2.

We previously reported the phenotype of KSR1-deficient mice (30). These mice are born at Mendelian ratios and develop without any obvious defects. Using gel filtration, we showed that KSR1 promotes the formation of large signaling complexes containing KSR1, Raf, MEK, and ERK (30). Using both primary T cells stimulated with antibodies to the T-cell receptor as well as fibroblasts stimulated with growth factors, we showed that KSR1-deficient cells exhibit an attenuation of ERK activation with defects in cell proliferation.

Here we explored the role of KSR1 in NK cell-mediated cytolysis. The killing of a target cell by a cytolytic T cell or NK cell is a complicated process that involves cell polarization with microtubule-dependent movement of cytolytic granules to an area that is proximal to the contact surface or immunological synapse (7, 33, 34, 48–50, 54). A variety of different signaling molecules are also involved, including calcium (23), phosphatidylinositol-3,4,5-triphosphate (13, 17), and activation of the ERK MAP kinase (6, 42, 56). Recently, the recruitment of activated ERK to the immunological synapse (IS) has been shown to be a feature of successful killing of a target by cytotoxic T lymphocytes (58).

How active ERK is recruited to the synapse is not known. Since KSR1 is known to be recruited to the plasma membrane by Ras activation (24), and since the immunological synapse is one of the major sites of Ras activation (26, 41), it seemed plausible to test the hypothesis that KSR1 recruitment to the plasma membrane functions to recruit ERK to the immunological synapse and facilitate its activation. We found that KSR1 was recruited to the immunological synapse and that KSR1 appeared to be required for the localization of active ERK at the contact site. As KSR1-deficient cells exhibit a defect in killing, this suggests that KSR1 recruitment to the synapse may be important in the cytolytic killing of target cells.

MATERIALS AND METHODS

Mice. KSR1-deficient mice (*KSR1*$^{-/-}$) have been described previously (30). All mice were housed under specific-pathogen-free conditions in the Washington University animal facilities in accordance with institutional guidelines.

Cell cultures and antibodies. Jurkat E6.1 T cells, Daudi lymphoma B cells, YAC-1 lymphoma cells, human K562 eritroblastoma cells, and RMAs and RMAs-Rae1e cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Human interleukin-2 (hIL-2)-dependent cell line NK92 cells (15) were grown in RPMI 1640 medium supplemented with 10% FBS and 100 U/ml of hIL-2. Mouse NK cells were purified by DX5^{+} magnetic-activated cell sorting enrichment (Miltenyi) and grown in hIL-2-containing medium (5). Polyclonal rabbit anti-Grb2, rabbit anti-ERK2, rabbit anti-KSR1, and mouse anti-Lck were obtained from Santa Cruz Biotechnology. Polyclonal rabbit anti-phospho-ERK [pERK1/2 (Thr202/Tyr204)] and rabbit anti-phospho-

* Corresponding author. Mailing address: Department of Pathology & Immunology, Washington University School of Medicine, St. Louis, MO 63110. Phone: (314) 362-6311. Fax: (314) 362-8888. E-mail: shaw@pathology.wustl.edu.
† Present address: Queensland Brain Institute, University of Queensland, QBI Building 79, St. Lucia, QLD 4072, Australia.
▽ Published ahead of print on 12 January 2009.

MAPK/CDK substrates (PXSP) were obtained from Cell Signaling Technology. Monoclonal anti-MAP kinase (diphosphorylated ERK1/2) and mouse anti-α-tubulin were purchased from Sigma. Fluorescein isothiocyanate-labeled CD3ε and phycoerythrin (PE)-NK1.1-labeled antibody were obtained from BD Biosciences.

Generation of DNA constructs. Murine KSR1 (mKSR1) full-length cDNA was subcloned into a pEYFP-N1 vector (Clontech). After EcoRI and NotI digestion, mKSR1-YFP was cloned into a pMX retrovirus vector (31). The C359 and C362 mutants in the CA3 domain of mKSR1 (CCSS mutant) were generated using PCR site-directed mutagenesis (Stratagene). The primers used for C359S were 5′-G ATT TTT GGC GTG AAG AGC AAA CAC TGC AGG-3′ and 5′-CCT GCA GTG TTT GCT CTT CAC GCC AAA AAT C-3′; for C362S they were 5′-GTG AAG AGC AAA CAC AGC AGG TTA AAA TGC CAT AAC-3′ and 5′-GTT ATG GCA TTT TAA CCT GCT GTG TTT GCT CTT CAC-3′. The integrity of all constructs was confirmed by automated sequencing.

Retroviral transduction. The Phoenix amphotropic retroviral packaging cell line was kindly provided by Garry Nolan. After transfection using Lipofectamine 2000 (Life Technologies), cells were transferred to 32°C to allow accumulation of virus in the supernatant. Virus-containing supernatant was harvested at 24 and 48 h after transfection and filtered through 0.45-μm syringe filters (Millipore). Jurkat cells were incubated with viral supernatant in the presence of 8 μg/ml of Polybrene (Sigma) and then centrifuged at 900 × *g*. This step was repeated after 4 h. Cells expressing yellow fluorescent protein (YFP) were sorted 3 to 5 days later on a Beckton Dickinson FACSVantage SE at the Flow Cytometry Core Facility (Dept. of Pathology and Immunology, Washington University, St. Louis, MO).

RNA interference and lentivirus production. KSR1 small hairpin RNA (shRNA) and luciferase shRNA (control) constructs were generated using the multifunctional lentivirus system (pFLRu lentivector; provided by Y. Feng and G. D. Longmore). To generate human KSR1 shRNA fragments, two sequences corresponding to nucleotides 1507 to 1530 and 2139 to 2157 were selected. Primers (sequence 1 forward, GTG GAA AGG ACG AAA CAC CGC CTA CTT CAT TCA TCA TAG ATA GCA TTC AAG AGA TGC TG; sequence 2 forward, GTG GAA AGG ACG AAA CAC CGC AGA CGT CTC TGG ACG TCA ATT CAA GAG ATT GAT GT) were designated together with their complementary counterparts and annealed by PCR to obtain shRNA fragments. Joint PCR was carried out by using human U6 promoter forward primer (ACA GAA TTC TAG AAC CCC AGT GGA AAG ACG CGC AG), shRNA reverse primer, and mixed template (1 μl of purified human U6 promoter and 2 μl of purified shRNA fragment). The PCR products were purified, digested with XhoI/XbaI, and subcloned into pFLRu lentivector [pFLRu-(KSR1-shRNA)]. To reconstitute KSR1 expression, mKSR1-YFP wild type and CCSS mutant were subcloned into the pFLRu-KSR1#1-shRNA vector to create pFLRu-(KSR1#1-shRNA)-mKSR1(WT)-YFP and pFLRu-(KSR1#1-shRNA)-mKSR1(CCSS)-YFP, respectively. The integrity of all constructs was verified by automated sequencing. For lentivirus production, subconfluent cultures of 293T cells were transfected with packaging plasmid (pHR′8.2ΔR/pCMV-VSV-G at a ratio of 8:1) and pFLRu-derived plasmid (Y. Feng, H. Zhao, B. Wang, and G. D. Longmore, submitted for publication) using Lipofectamine 2000. The lentivirus infection of Jurkat cells was performed using the protocol described above, and cells expressing the same level of YFP were sorted 3 to 5 days later.

Confocal imaging and immunofluorescence staining. To study the recruitment of phosphorylated ERK (pERK) into the immunological synapse, purified NK cells from wild-type (WT) or *KSR1*$^{-/-}$ mice were mixed with equal amount of carboxyfluorescein succinimidyl ester (CFSE)-preloaded YAC-1 target cells. After centrifugation, cells were gently resuspended and placed onto poly-D-lysine-coated glass slides for 10 min at 37°C. After aspiration of the medium, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. Cells were permeabilized with 90% methanol for 30 min at −20°C, washed with PBS containing 4% FBS, and incubated with mouse anti-pERK for 45 min at room temperature. After washing, cells were incubated with Cy3-conjugated anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at room temperature. To image the lytic granule polarization, the indicated mouse NK cells were loaded with 100 nM Lysotracker (Molecular Probes) for 20 min at 37°C. After washing, loaded mouse NK cells were mixed with RMAs-Rae1ε target cells at a 1:1 ratio and flowed onto a parallel plate of flow cells in a temperature-controlled chamber at 37°C. Images were taken over 0 to 20 min using a Zeiss LSM 510 laser-scanning confocal microscope (Oberkochen, Germany) with 63× objective lenses. To study the recruitment of KSR1 and pERK into the T-cell immunological synapse, transduced Jurkat cells were mixed with an equal amount of Daudi B cells preloaded with or without staphylococcal enterotoxin E (SEE). After centrifugation, cells were gently resuspended, placed onto poly-L-lysine-coated glass slides for 5 min at 37°C, fixed, permeabilized, and stained as described above. To quantitate the recruitment of KSR1-YFP or *YFP* to the contact site, boxes were drawn at the contact area between the effector and target cells, at the cytosol, and in a background area outside the cell by using the Image J software program (NIH). The relative recruitment index (RRI) was calculated as follows: (mean fluorescence intensity [MFI] at synapse − background)/(MFI at regions in the cytosol − background). For each experiment, the percentage of Jurkat cells with an RRI of more than 1.1 was calculated. For quantification of pERK translocation to the cell-cell contact area, the ratio of MFI at the contact area versus an equivalent in the cytosol was calculated and a ratio of more than 1.1 was scored as protein accumulation. At least 50 conjugates were examined for each experiment, and three different experiments were performed.

Cytotoxicity assays. Cytotoxic activity of mouse NK cells was tested against YAC-1 or RMAs or RMAs-Rae1ε target cells using standard 4-h ^{51}Cr release assays (5). Where indicated, NK cells were preincubated with 10 μM specific MEK inhibitor (UO126; Calbiochem) at 37°C for 30 min. In all experiments, spontaneous release did not exceed 10% of maximum release.

CFSE labeling and in vivo NK killing assay. The in vivo NK cell cytolytic experiments were performed essentially as previously described (3). RMAs and RMAs-Rae1ε cells (10^7) were labeled with 1 μM (low peak) and 10 μM (high peak) CFSE (Molecular Probes) for 15 min at 37°C in RPMI 1640 medium supplemented with 5% FBS. Labeling was blocked with 1:1 (vol/vol) FBS, and cells were washed several times with RPMI complete medium. CFSE-loaded cells were counted, mixed at a 1:1 ratio, and injected intraperitoneally (8×10^6 to 10×10^6 cells/mouse in a 300-μl volume) in WT and *KSR1*$^{-/-}$ mice. A small sample of injection mix was acquired at the zero time point to record the ratio between RMAs and RMAs-Rae1ε cells. At 24 h after injection, cells were recovered by peritoneal lavage. After washing, the Rae1ε expression was monitored by binding with a specific antibody (anti-Rae1ε antibody conjugated to biotin; provided by Marina Cella) followed by the appropriate secondary antibody. The ratio between RMAs and RMAs-Rae1ε cells from CFSE-labeled cells was determined by flow cytometry.

Cell conjugation assay. Target cells were loaded with 10 μM CFSE for 15 min at 37°C. NK cells from WT or *KSR1*$^{-/-}$ mice were stained with PE-labeled NK1.1 antibody (BD Biosciences). After washing, stained NK cells were mixed with CFSE-loaded target cells at a 1:1 ratio, centrifuged, and incubated for 20 min at 37°C to form conjugates. After fixation in 4% paraformaldehyde in PBS for 10 min, cells were analyzed by flow cytometry.

Flow cytometric measurement of intracellular ERK activation. T cells transduced with the indicated lentivectors were mixed with SEE-pulsed Daudi B cells at a 1:1 ratio, spun at 350 × *g* for 10 s, and placed at 37°C for 5 min. T-cell–antigen-presenting cell (APC) conjugates were then separated with ice-cold PBS–2.5 mM EDTA and fixed with 4% paraformaldehyde for 10 min on ice. Cells were permeabilized with 90% methanol for 30 min at −20°C, washed with PBS containing 3% fetal bovine serum, and incubated with mouse anti-pERK for 45 min at room temperature. After washing, cells were incubated with phycoerythrin-labeled F(ab′)$_2$ anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at room temperature. Staining was measured by flow cytometry after gating for YFP-positive T cells and analyzed using FlowJo.

Cell stimulation, immunoprecipitation, and immunoblotting. Jurkat cells transduced with the indicated lentivectors were starved for 1 h in RPMI 1640. Daudi cells were loaded with 100 ng/ml of SEE (Toxin Technology, Inc.) for 30 min before mixing 2:1 (T cells:B cells) with T cells in RPMI medium. Cells were gently centrifuged for 30 s and placed at 37°C for the indicated times. After stimulation, the pellet was resuspended in ice-cold lysis buffer (0.1 M Tris base, 140 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mg/ml apoprotein, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 50 mM sodium fluoride). After centrifugation, proteins from cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting with the indicated primary antibodies followed by incubation with anti-mouse immunoglobulin G or anti-rabbit immunoglobulin G coupled to horseradish peroxidase. To quantified the level of KSR1, whole-cell lysates from Jurkat or NK92 cells transduced with the indicated lentivector were resolved by SDS-PAGE. KSR1 protein bands were detected and quantified by immunoblotting with the Odyssey system (Li-Cor). NK92 cells (5×10^6/sample) transduced with the indicated lentivectors, after sorting, were IL-2 starved for 4 h in RPMI 1640 containing 5% FBS. Cells were incubated with an equal number of K562 target cells at 37°C for the indicated times. Cells were resuspended in ice-cold lysis buffer and were centrifuged as described above. Cell lysates were analyzed by immunoblotting with antibodies specific for phosphorylated ERK. In the immunoprecipitation experiments, nucleus free supernatant was incubated with 2 μg/ml of monoclonal anti-Lck at 4°C for 60 min and then incubated with protein A-Sepharose beads (Pharmacia) at 4°C for 90 min. After washing, Lck

immunoprecipitates were resolved by SDS-PAGE, transferred to a membrane, and analyzed by immunoblotting with the indicated antibodies.

Statistics. Statistical analyses were performed using a paired Student's *t* test. Differences that were statistically significant are noted in the figures below.

RESULTS

KSR1 is required for NK lytic activity. Previously we showed that thymic and peripheral T-cell populations in *KSR1*$^{-/-}$ mice were similar to wild type (30). To determine the role of KSR1 in NK cell development, we measured the numbers of NK cells by using antibodies to CD3 and NK1.1 in the spleens of wild-type and *KSR1*$^{-/-}$ mice (Fig. 1A). Flow cytometric analysis showed that NK cells (NK1.1$^+$ CD3$^-$ cells) were normally represented in spleens of *KSR1*$^{-/-}$ mice (Fig. 1B). We also observed that KSR1 deficiency did not affect the numbers of NKT cells (NK1.1$^+$ CD3$^+$). Altogether, these data suggest that NK cell development is normal in *KSR1*$^{-/-}$ mice.

We then tested the role of KSR1 in NK cell killing. Splenic NK cells from wild-type and KSR1-deficient mice were purified and NK lytic activity was tested by incubation with YAC-1 target cells. While wild-type NK cells efficiently killed YAC-1 cells (Fig. 2A), there was a significant reduction of killing when using KSR1-deficient NK cells.

Since YAC-1 cell recognition is complex and involves several different receptors (5), we also tested NK lytic activity mediated by the NK receptor NKG2D. For these experiments we used RMAs cells transfected with the mouse NKG2D ligand Rae1ε (RMAs-Rae1ε) as targets. NK cells from KSR1-deficient mice showed a significant reduction in cytolytic activity (Fig. 2B) that was specific to NKG2D, as there was no killing of RMAs cells lacking Rae1ε expression. Importantly, the reduction of NK cell cytotoxicity was not mediated by the decreased cell-cell adhesion, since the absence of KSR1 did not affect the ability of NK cells to form conjugates with the indicated target cells (Fig. 2C).

We confirmed the NK cell killing defect in vivo by injecting wild-type and KSR1-deficient mice with RMAs and RMAs-Rae1ε cells and monitoring tumor growth as previously described (3). In this system, elimination of RMAs-Rae1ε cells is mediated by NK cells in an NKG2D-dependent manner. RMAs and RMAs-Rae1ε cells (10^7) were distinguished by labeling with either 1 μM (low staining) or 10 μM (high staining) CFSE, respectively. Tumor cells were mixed at a 1:1 ratio and injected intraperitoneally (8×10^6 to 10×10^6 cells/mouse in a 300-μl volume). A sample was measured before injection to document the starting ratio between RMAs and RMAs-Rae1ε cells (Fig. 2D and G). Twenty-four hours after injection, cells were recovered by peritoneal lavage and stained with anti-Rae1ε, and the ratio between RMAs and RMAs-Rae1ε cells in the CFSE-labeled cells was determined by flow cytometry. As expected (12), RMAs-Rae1ε cells were preferentially eliminated in wild-type mice (Fig. 2E and G). In contrast, elimination of RMAs-Rae1ε cells in *KSR1*$^{-/-}$ mice was impaired (Fig. 2F and G). This demonstrates that KSR1 is required to mediate NK cell lytic activity in vivo.

NKG2D-induced lytic granule polarization is impaired in *KSR1*$^{-/-}$ mice. NK killing of the target cells is mediated by the polarized release of lytic granules (13, 34). Since NK lytic activity was impaired in the absence of KSR1, we investigated whether KSR1 was required for lytic granule polarization. Purified NK cells from wild-type and *KSR1*$^{-/-}$ mice were incubated with Lysotracker to label lytic granules and then imaged before and after conjugate formation with YAC-1 cells. Prior to conjugation, lytic granules were randomly distributed in the cytosol (Fig. 3A). After interaction with YAC-1 cells, lytic granules of wild-type NK cells moved to a location near the site of contact with the target cell (Fig. 3A and C). This was spe-

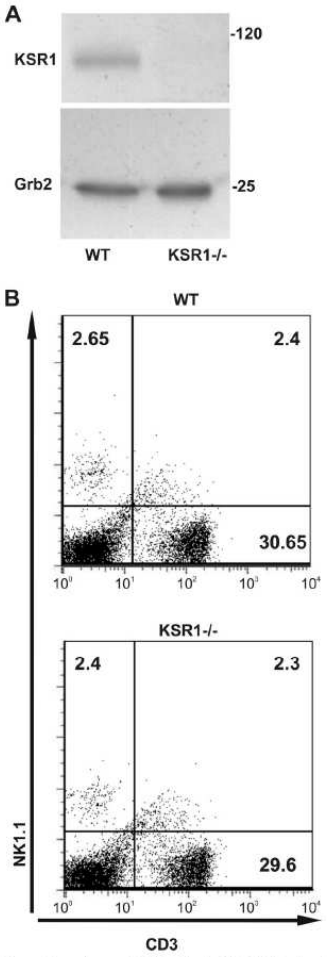


FIG. 1. Normal numbers of NK cells in *KSR1*$^{-/-}$ mice. (A) Expression of KSR1 in splenocytes of WT and *KSR1*$^{-/-}$ mice. Total cell lysates of WT and *KSR1*$^{-/-}$ mice were analyzed by immunoblotting with anti-KSR1 antibody. Grb2 antibody was used as a loading control. (B) Splenocytes of WT and *KSR1*$^{-/-}$ mice were stained with NK1.1-PE and CD3ε-fluorescein isothiocyanate antibody and analyzed by flow cytometry. The dot blot is representative of three different experiments (two mice/experiment).

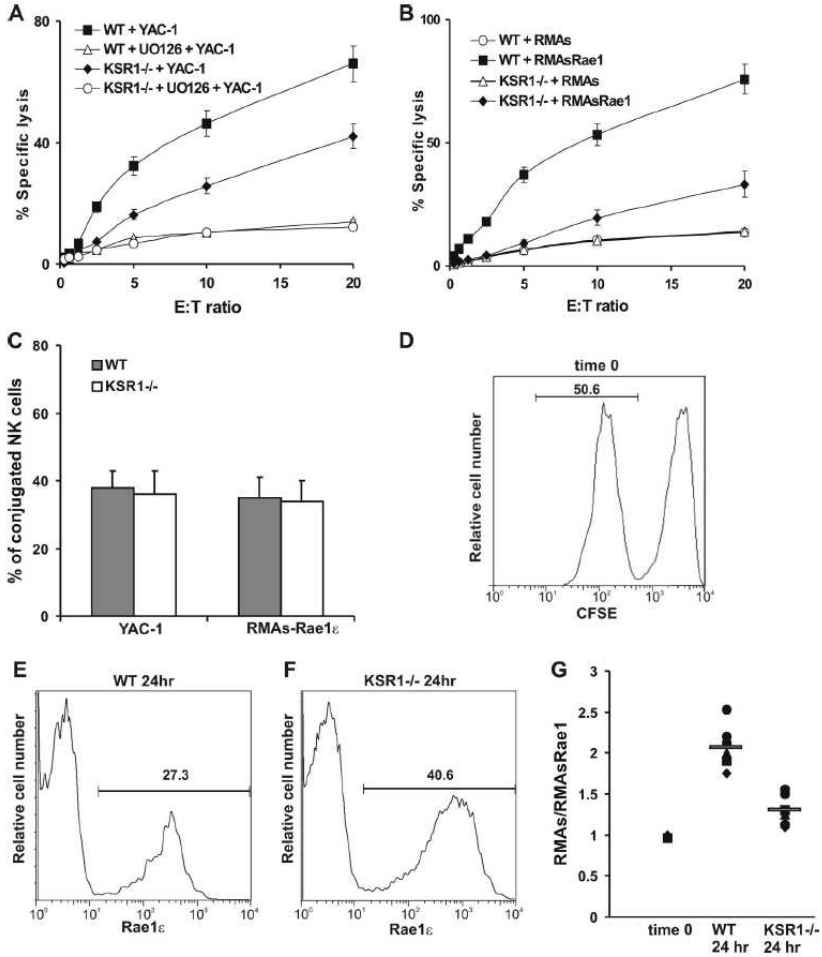


FIG. 2. KSR1 is required for NK lytic activity in vitro and in vivo. (A and B) Cytotoxicity of WT and *KSR1*$^{-/-}$ NK cells was tested against YAC-1 cells (A) or RMAs and RMAs-Rae1ε target cells (B) in vitro. Purified NK cells (>96% NK1.1$^+$ CD3ε$^-$) were IL-2 starved in RPMI medium for 4 h before incubation with the indicated target cells. Where indicated, NK cells were preincubated with the MEK inhibitor UO126 (10 μM). Data are representative of three independent experiments. E:T ratio, effector:target ratio. (C) Conjugate formation is normal in KSR1-deficient cells. NK cells from WT or *KSR1*$^{-/-}$ mice were stained with NK1.1-PE antibody and mixed with CFSE-loaded target cells. Cells were allowed to form conjugates for 20 min at 37°C, fixed, and analyzed by flow cytometry. The bar graphs represent the percentages of NK1.1$^+$ CFSE$^+$ double-positive cells from the total pool of NK1.1$^+$ cells. Data are represented as averages ± standard errors of the means of at least three separate experiments. (D to G) NK killing assay in vivo. RMAs and RMAs-Rae1ε cells were labeled with different concentrations of CFSE and mixed at a 1:1 ratio. (D) An aliquot of the cell mixture was analyzed before injection (time zero). (E and F) The cell mixture was injected intraperitoneally into WT and *KSR1*$^{-/-}$ mice. Twenty-four hours after injection, cells were recovered by peritoneal lavage. Rae1ε expression was assessed by labeling with anti-Rae1ε antibody and examined by flow cytometry. The RMAs/RMAs-Rae1ε ratio was obtained by comparing high and low CFSE-labeled cells. (G) Summary of RMAs/RMAs-Rae1ε ratios in six WT and six *KSR1*$^{-/-}$ mice. Horizontal bars indicate the mean ratios.

cific, as the polarized movement of lytic granules was inhibited by using inhibitors of phosphatidylinositol 3 kinase (data not shown). While the ability of KSR1-deficient NK cells to form conjugates with YAC-1 cells was not affected, lytic granule polarization was significantly reduced (Fig. 3B and C). These results suggest that KSR1 is also important in lytic granule polarization and that defects in granule polarization may be responsible for defects in cytolytic killing.

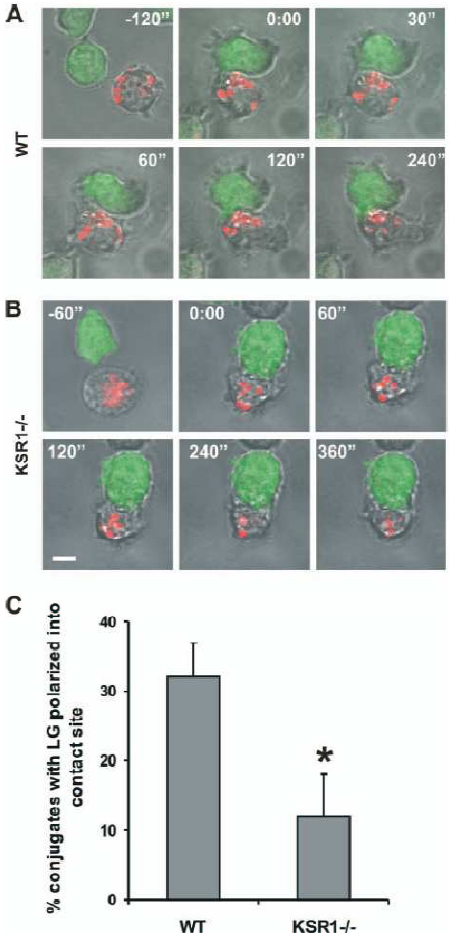


FIG. 3. Lytic granule polarization is impaired in NK cells from *KSR1*$^{-/-}$ mice. (A and B) Time-lapse experiment, extracted from a movie, showing representative images of the localization of lytic granules (red) in primary NK cells purified from spleens of WT (A) and *KSR1*$^{-/-}$ (B) mice and conjugated with CFSE-loaded YAC-1 target cells (green; time in seconds). Bar, 5 μm. (C) Lytic granule (LG) polarization at the contact site with the indicated target cells was quantitated after 5 min of incubation. Data represent the mean (±standard error of the mean) percentage of conjugates from three independent experiments with at least 40 conjugates. *, $P < 0.05$.

pERK recruitment into the NK IS is impaired in *KSR1*$^{-/-}$ mice. Recently it has been reported that pERK is recruited to the immunological synapse of CD8$^+$ T cells (58). Since it is postulated that the subcellular localization of signaling molecules is mediated by scaffold molecules (18, 39, 45), we wondered whether KSR1 might be involved in ERK localization to the IS.

We tested whether KSR1 plays a role in pERK localization by first imaging pERK localization using NK cells from KSR1-deficient mice. Purified NK cells from wild-type and *KSR1*$^{-/-}$ mice were conjugated with YAC-1 cells. Cells were stained with antibodies to pERK and analyzed by confocal microscopy. While pERK was detectable in both wild-type and KSR1-deficient NK cells, pERK localization at the synapses was infrequent in KSR1-deficient NK cells compared to wild-type cells (Fig. 4A and B).

So that we could dissect the mechanism of KSR1 function, we attempted to replicate these findings using Jurkat cells where KSR1 expression was suppressed using lentiviruses expressing two different KSR1-specific shRNAs. Bulk sorting of green fluorescent protein-positive cells showed that both shRNAs resulted in over 70% inhibition of expression (Fig. 4C). pERK recruitment to the synapse was then analyzed by forming conjugates between Jurkat cells and superantigen-coated APCs (Fig. 4D and E). While pERK was easily detected at the IS of conjugates formed using wild-type Jurkat cells (Fig. 4D, upper panels, and E), suppression of KSR1 expression significantly impaired the recruitment of pERK to the IS (Fig. 4D, lower panels, and E). Similar results were obtained when the same shRNAs were used in the human NK cell line NK92 (data not shown).

Consistent with results from KSR-deficient T cells (30), suppression of KSR1 expression in the Jurkat cells attenuated ERK activation (Fig. 5A). This suggested that our inability to detect pERK at the synapse could be due to a generalized defect in ERK activation. This seemed unlikely, as strong staining with the pERK antibody was easily detected in some of the KSR1-deficient cells (Fig. 4D). Germain and coworkers have demonstrated that ERK activation by the T-cell receptor is all or none in individual CD8$^+$ T cells (1). What they found was as the strength of T-cell receptor (TCR) signaling increases, there is not a graded increase in ERK activation. Rather, at the individual cell level, ERK is either fully activated in cells or not activated at all. We, therefore, hypothesized that in the absence of KSR1, cells could still be activated but that the total number of activated cells was lower. To confirm this, we used flow cytometry to compare ERK activation in control versus KSR1 shRNA-treated cells. As we expected, KSR1 shRNA cells were able to activate ERK to levels similar to wild-type cells but the number of cells that were activated was much lower (Fig. 5B). This suggests that KSR1 functions to increase the sensitivity of TCR-mediated activation of ERK. In addition, it suggests that the lack of pERK at the synapse is not due to a generalized defect in ERK activation and supports the hypothesis that KSR1 is required for the synapse localization of pERK.

KSR1 recruitment is required for pERK accumulation into the immunological synapse. To determine whether KSR1 itself is recruited to the IS, Jurkat T cells were transduced with a construct encoding KSR1 fused to YFP (KSR1-YFP). After conjugation with superantigen-coated APCs, KSR1-YFP was easily detected at the IS (Fig. 6A, lower panel, and B). As a control, YFP by itself was distributed homogenously throughout Jurkat cells with or without stimulation by SEE (Fig. 6C). The KSR1-YFP recruitment was specific to T-cell activation, as conjugation with APCs in the absence of superantigen did

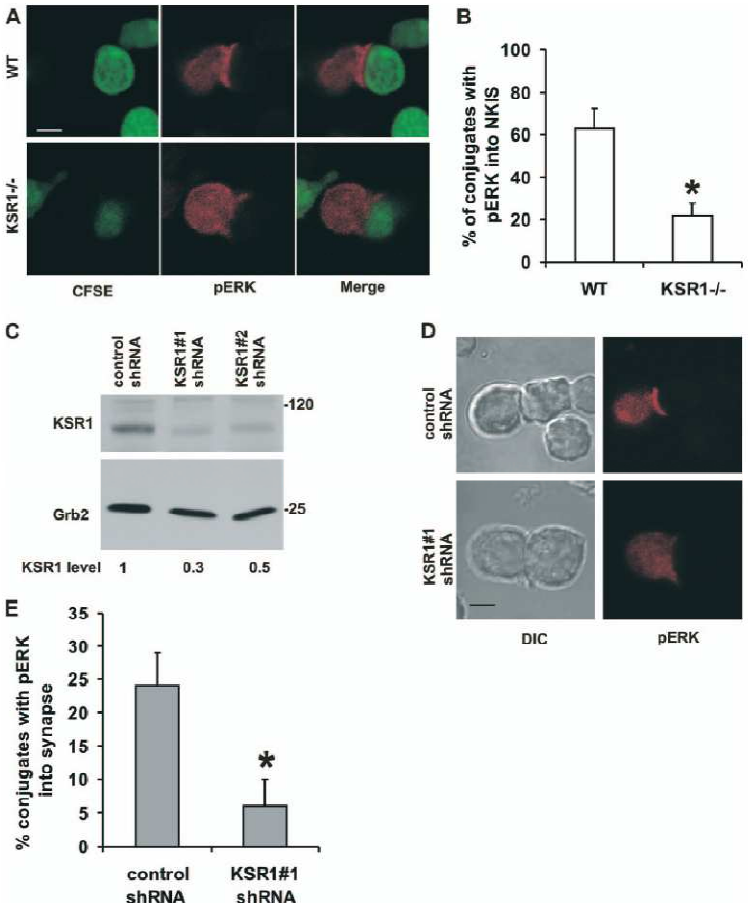


FIG. 4. Recruitment of pERK into the NK IS is impaired in KSR1-deficient mice. (A) Representative images showing the localization of pERK (red) in primary NK cells purified from spleens of WT and *KSR1*$^{-/-}$ mice that were conjugated with CFSE-loaded YAC-1 target cells (green). Bar, 5 μm. (B) pERK accumulation at the NK IS was quantitated by dividing the percentage of cells with pERK at the NK IS by the total number of pERK-positive cells imaged. Data represent the mean (±standard error of the mean) percentage of conjugates with an RRI (see Materials and Methods) of >1.1, from three independent experiments with at least 30 conjugates. *, $P < 0.05$. (C) KSR1 knockdown in Jurkat T cells. Immunoblotting results are for KSR1 and Grb2 expression in Jurkat T cells (3.5×10^6 cells/lane) transduced with the indicated shRNA. KSR1 expression levels were compared to control shRNA Jurkat T cells. (D) pERK recruitment into the contact site is impaired in KSR1 knockdown T cells. Representative differential interference contrast and Cy3 fluorescence images are shown for shRNA-expressing Jurkat T cells conjugated with Daudi B cells preloaded with 100 ng/ml of superantigen. Bar, 5 μm. (E) Percentage of conjugates with pERK recruited into the synapse, as described for panel D. Quantitative analysis was done for pERK accumulation at the contact site from three independent experiments with at least 40 conjugates. Data represent the mean (±standard error of the mean) percentage of conjugates with an RRI of >1.1. *, $P < 0.05$.

not result in any detectable KSR1 recruitment to the synapse (Fig. 6A, upper panel, and B).

After Ras activation, KSR1 is recruited to the plasma membrane via its CA3 domain (24). To verify that synapse recruitment of KSR1 is responsible for pERK localization in the synapse, we rescued KSR1-deficient Jurkat cells with either a CA3-mutated KSR1-YFP construct or a wild-type KSR1-YFP fusion. The CA3 domain has two conserved cysteine residues that can be mutated to disrupt the structure of the domain and its ability to bind to membranes (60). Cell sorting was used to isolate a population of cells with similar expression levels (Fig. 7A). Since high-level expression of KSR1 is known to inhibit ERK activation (20, 21), we first verified that the level of KSR1 expression in the cells that we isolated was able to restore ERK activation. Flow cytometric analysis showed that the level of wild-type KSR1 was sufficient to reconstitute ERK activation.

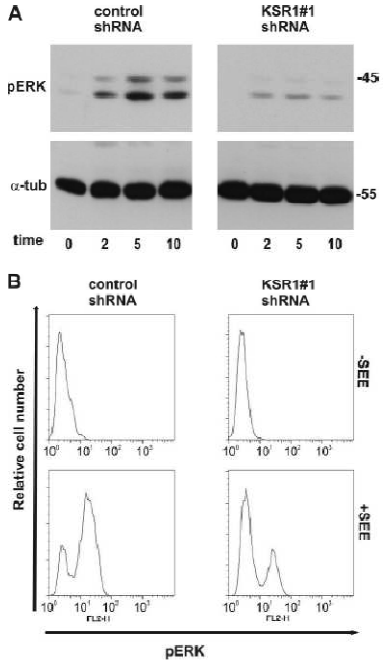


FIG. 5. Antigen-induced T-cell activation is regulated by KSR1. (A) ERK activation is impaired in KSR1 knockdown T cells. shRNA-transduced Jurkat T cells were stimulated with superantigen-coated Daudi cells (100 ng/ml) for the indicated times (in minutes) and analyzed for pERK1/2 by Western blotting. Blotting for α-tubulin (α-tub) was used to demonstrate equal loading of each sample. (B) Suppression of KSR1 expression inhibits the number of activated Jurkat cells. Representative histograms show the distribution of pERK as measured by flow cytometry of Jurkat T cells after activation by Daudi preloaded with or without superantigen (10 ng/ml SEE). In the absence of stimulation, no shift in the *x* axis of histograms was observed in either control or KSR1 shRNA-expressing Jurkat cells. Notably, after stimulation the change in area under the peak indicates that ERK activation was observed in a higher number of control shRNA cells.

Interestingly, the CA3 mutant was able to partially rescue ERK activation, suggesting that KSR1 can facilitate ERK activation in the absence of membrane recruitment (Fig. 7B).

We next tested whether cells expressing the CA3-mutated KSR1-YFP could rescue pERK localization at the IS. Imaging experiments showed that the wild-type KSR1-YFP was recruited to the synapse and was able to rescue pERK localization at the IS. In contrast, the CA3 mutant was not recruited to the IS, nor was pERK detectable at the IS (Fig. 7C and D). Together, these results demonstrate that KSR1 recruitment to the IS is mediated by its CA3 domain and that KSR1 recruitment to the IS is required for pERK localization at the immunological synapse.

Phosphorylation of the Lck PXSP motif is regulated by KSR1-mediated ERK activation. The requirement for active ERK recruitment to the IS suggests that it is required for the phosphorylation of proteins that are present in the synapse. Since Lck is a known substrate for ERK during T-cell activation (47, 55, 57), we tested whether KSR1 was required for Lck phosphorylation. A KSR1-specific shRNA-expressing lentivirus was used to inhibit endogenous KSR1 expression in a human NK cell line (Fig. 8A). We confirmed that suppression of KSR1 reduced ERK activation in the human NK cell line after stimulation with target cells (K562 cells) (Fig. 8B). Lck immunoprecipitates were prepared from both wild-type and KSR1 shRNA-expressing cells and blotted with an antibody that recognizes ERK phosphorylation sites (PXSP). The induction of Lck phosphorylation after target cell incubation was reduced in KSR1 shRNA-treated NK cells compared to wild-type cells (Fig. 8C). This supports the hypothesis that KSR1 recruitment of ERK facilitates the phosphorylation of ERK substrates at the synapse.

DISCUSSION

Here we examined the role of KSR1 on the cytolytic function of NK cells and found that KSR1-deficient NK cells exhibit a defect in killing. The defect appeared to be related to an inability to polarize cytolytic granules. Since pERK recruitment to the immunological synapse was recently reported during the activation of $CD8^+$ T cells (58), and because ERK activation is required for killing (6, 56), we explored the hypothesis that pERK recruitment to the synapse might be facilitated by KSR1. Indeed, we found that in KSR1-deficient T cells, pERK recruitment to the immunological synapse was defective.

KSR1 is thought to function as a scaffold for the Ras/MAP kinase pathway (18, 27, 28, 44). This scaffold molecule regulates the intensity and duration of growth factor-induced ERK activation to modulate a cell's proliferative, oncogenic, and adipogenic potential (20, 21, 40). It binds to all three kinases of the ERK MAP kinase cascade, Raf-1, MEK, and ERK (19, 51, 53), and is recruited to the plasma membrane during Ras activation, where it presumably facilitates the interaction between active Ras and Raf-1 (18, 28, 35). More recent data suggest that KSR may have additional roles facilitating phosphorylation of the activation loop of Raf (9). Previously, we showed that in KSR1-deficient T cells the activation of ERK was still detectable but highly attenuated (30). We interpreted this to mean that KSR1 is required for the efficient activation of ERK.

In this study, we analyzed the ERK activation defect in more detail. We previously used immunoblotting to measure ERK activation (10, 30). This method, because it relies on the lysis of millions of cells, averages the biochemical changes that occur at a specific moment in time. Using such a method, an attenuation of ERK activation could be due to attenuation of ERK activation in all cells or reflect a defect in ERK activation in some but not others. Using flow cytometry to analyze ERK activation at a single-cell level, we were surprised to find that in KSR1-deficient cells, the defect of ERK activation only affected some but not all cells. A small fraction of KSR1-deficient cells showed levels of ERK activation that are similar to wild-type cells.

Previous work had suggested that ERK activation in $CD8^+$ T cells is stochastic, that it is an all-or-none process (1). A weak stimulus results in only a few cells that are fully activated and

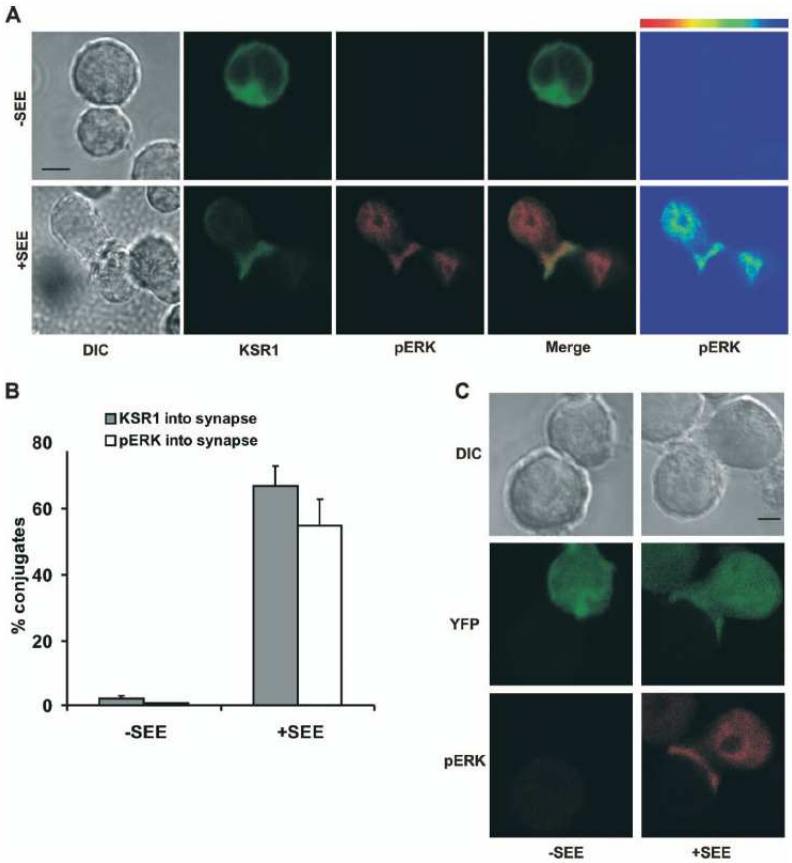


FIG. 6. KSR1 is recruited into the immunological synapse. (A) Representative differential interference contrast, YFP, and Cy3 fluorescence images of Jurkat T cells expressing KSR1-YFP after conjugation with Daudi B cells loaded with or without SEE (100 ng/ml). In the absence of SEE (-SEE), ERK (red) is not phosphorylated and KSR1 (green) is not recruited into the contact site. In the far right panel, the location of pERK is shown in false color. Bar, 5 μm. (B) KSR1 and pERK accumulation at the contact site was quantitated from three independent experiments with at least 50 conjugates. Data are represented as the average (±standard error of the mean) of conjugates with an RRI of >1.1 (see Materials and Methods). (C) Representative differential interference contrast, YFP, and Cy3 fluorescence images of Jurkat T cells expressing YFP conjugated with Daudi B cells and stimulated as for panel A. Images are representative of two independent experiments with at least 30 conjugates.

as the strength of a stimulus is increased, more and more cells show full ERK activation. In support of this idea, we found that the attenuation of ERK activation seen in KSR1-deficient cells is due to decreased numbers of activated cells, suggesting that KSR1 functions by lowering the threshold stimulus required for the stochastic activation of ERK. We speculate that by helping to recruit the Raf/MEK/ERK module to active Ras, KSR1 may function to enhance the activation of the pathway (22).

It is intriguing to speculate that recruitment of KSR1 and the ERK MAP kinase cascade to the immunological synapse may have functions in addition to simply facilitating ERK activation. By holding active ERK at the immunological synapse, KSR1 may function to allow ERK to phosphorylate specific substrates at the plasma membrane essential for T-cell function. For example, it has been proposed that ERK phosphorylation of Lck may play an important role in facilitating a positive feedback loop that is important for enhancing T-cell activation (47). Our immunoprecipitation data indicated that ERK-dependent phosphorylation of the PXSP motif in Lck was diminished after KSR1 suppression, supporting the role of KSR1 on ERK substrate phosphorylation into the synapse. Other important substrates at the immunological synapse include stathmin, a molecule that plays a key role in helping to regulate microtubule polymerization (4). It seems possible that the granule polarization defect seen in the KSR1-deficient cells

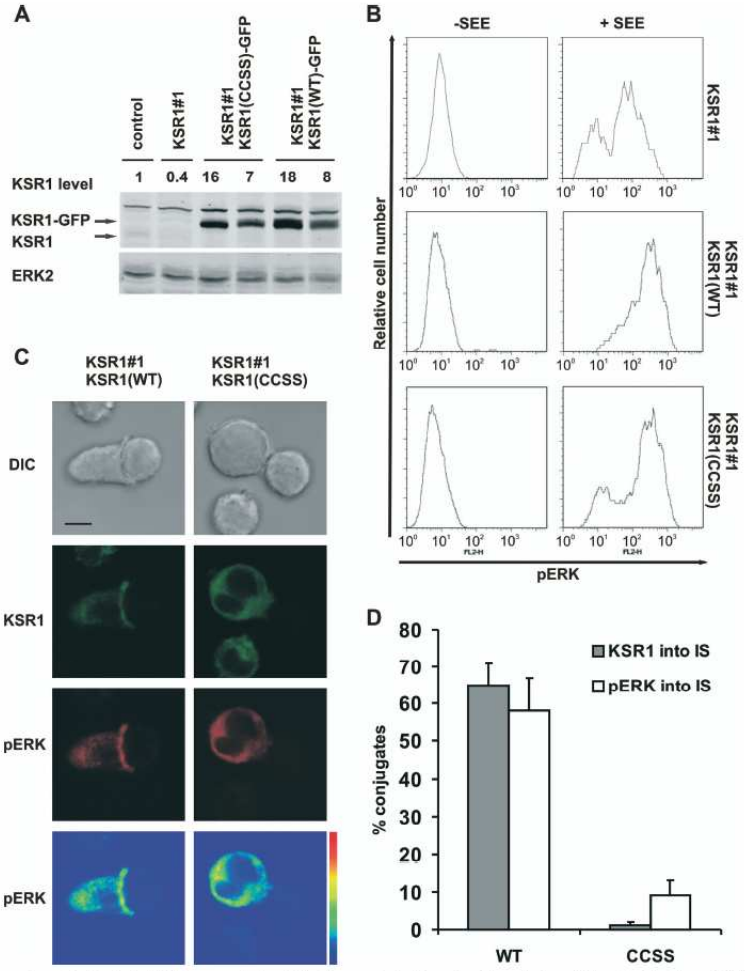


FIG. 7. KSR1 recruitment affected pERK accumulation into the contact site and antigen-induced T-cell activation. (A) Immunoblotting for KSR1 expression in Jurkat T cells (600×10^3 cells/lane) transduced with control shRNA, KSR1#1 shRNA, or rescue KSR1#1 shRNA lentivector containing KSR1(CCSS)-YFP or KSR1(WT)-YFP. Transduced Jurkat T cells with rescued CCSS mutant and WT KSR1 were sorted to achieve a pool of cells with similar expression levels (low and high) of KSR1-YFP. The KSR1 expression level compared to control shRNA transduced Jurkat T cells is indicated. (B) Representative histograms of the distribution of pERK as measured by flow cytometry of the indicated transduced Jurkat T cells after activation by Daudi cells preloaded without (-SEE) or with 1,000 ng/ml of superantigen (+SEE). (C) KSR1 is required for pERK recruitment into the IS. Images shown are representative of differential interference contrast (DIC), YFP (KSR1), and Cy3 (pERK) fluorescence images of KSR1#1 shRNA-KSR1(WT)-YFP or KSR1#1 shRNA-KSR1(CCSS)-YFP Jurkat T cells conjugated with Daudi B cells preloaded with SEE (100 ng/ml). In the far right panel, the location of pERK is shown in false color. Bar, 5 μm. (D) Quantitative analysis of KSR1 and pERK accumulation levels at the contact site from three independent experiments with at least 40 conjugates. Data are presented as the average (±standard error of the mean) of conjugates with an RRI (see Materials and Methods) of >1.1.

is due to defects in ERK phosphorylation of critical substrates at the immunological synapse.

The localization of the MAP kinase cascade at different sites in the cell has been suggested to play an important role in T-cell biology (26). While it was originally thought that Ras activation of the MAP kinase cascade could only be initiated at the plasma membrane, it has now become clear that different Ras isoforms are localized and activated at distinct intracellular membranes (2, 8, 37). At steady state, while K-Ras is mainly localized to the plasma membrane and N-Ras and H-Ras are

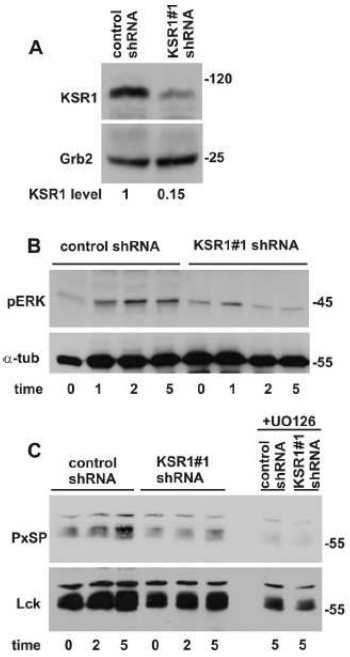


FIG. 8. ERK phosphorylation of Lck is facilitated by KSR1 in NK cells. (A) Inhibition of KSR1 expression after KSR1-specific shRNA transduction in the human NK92 cell line (3×10^6 cells/lane). Immunoblotting was performed with antibodies to KSR1 and Grb2. (B) Defective ERK activation in KSR1 knockdown NK92 cells. NK92 cells were stimulated with target cells (K562) for the indicated times (in minutes) and analyzed for pERK1/2 by Western blotting. Blotting with α-tubulin (α-tub) was used to demonstrate equal loading. (C) Serine phosphorylation of the Lck PXSP motif is facilitated by KSR1. Control and KSR1 shRNA-expressing NK92 cells were incubated with K562 cells as described for panel B. Lck immunoprecipitates were prepared at the indicated times (in minutes) and were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with a phospho-specific antibody to the sequence PXpSP. The membrane was then stripped and reprobed with monoclonal anti-Lck to confirm equal loading of Lck.

mainly localized to the Golgi apparatus (36). Philips and coworkers showed that TCR stimulation alone resulted mainly in Golgi complex activation of Ras, while costimulation with anti-LFA-1 allowed for plasma membrane and Golgi complex activation (25). In other systems, Ras signaling has been shown to occur on endosomes as well as in endoplasmic reticulum membranes (14, 38). Inherent in these studies is the idea that localized Ras signaling is important in the activation of location-specific effectors, but it is also possible that this plays a role in localizing active ERK close to the location-specific substrates. Unfortunately, in these studies, the localization of active ERK was not determined.

Whether KSR1 localizes to sites other than the plasma membrane is not yet known. It would be interesting if KSR1 and KSR2 were recruited to distinct membranes. Unfortunately, little is known about the expression pattern of KSR2. Probe sets for KSR2 are available on commercial microarrays for both human and mouse, but there are no data documenting any level of expression in any tissue. In our own hands, we have been unable to detect KSR2 message using a variety of different methods in any lymphoid or myeloid compartment.

ACKNOWLEDGMENTS

We thank G. Longmore and Y. Feng for providing reagents. We are grateful to Angela Fusello and Erin Filbert for helping with KSR1-deficient mice. We thank Aleksey Karpitskiy for technical assistance.

REFERENCES

1. **Altan-Bonnet, G., and R. N. Germain.** 2005. Modeling T cell antigen discrimination based on feedback control of digital ERK responses. PLoS Biol. **3:**e356.
2. **Apolloni, A., I. A. Prior, M. Lindsay, R. G. Parton, and J. F. Hancock.** 2000. H-ras but not K-ras traffics to the plasma membrane through the exocytic pathway. Mol. Cell. Biol. **20:**2475–2487.
3. **Boles, K. S., W. Barchet, T. Diacovo, M. Cella, and M. Colonna.** 2005. The tumor suppressor TSLC1/NECL-2 triggers NK-cell and $CD8^+$ T-cell responses through the cell-surface receptor CRTAM. Blood **106:**779–786.
4. **Budhachandra, K., R. K. Brojen Singh, and G. I. Menon.** 2008. Microtubule dynamics regulated by stathmin. Comput. Biol. Chem. **32:**141–144.
5. **Cella, M., K. Fujikawa, I. Tassi, S. Kim, K. Latinis, S. Nishi, W. Yokoyama, M. Colonna, and W. Swat.** 2004. Differential requirements for Vav proteins in DAP10- and ITAM-mediated NK cell cytotoxicity. J. Exp. Med. **200:**817–823.
6. **Chen, X., D. S. Allan, K. Krzewski, B. Ge, H. Kopcow, and J. L. Strominger.** 2006. CD28-stimulated ERK2 phosphorylation is required for polarization of the microtubule organizing center and granules in YTS NK cells. Proc. Natl. Acad. Sci. USA **103:**10346–10351.
7. **Chen, X., P. P. Trivedi, B. Ge, K. Krzewski, and J. L. Strominger.** 2007. Many NK cell receptors activate ERK2 and JNK1 to trigger microtubule organizing center and granule polarization and cytotoxicity. Proc. Natl. Acad. Sci. USA **104:**6329–6334.
8. **Choy, E., V. K. Chiu, J. Silletti, M. Feoktistov, T. Morimoto, D. Michaelson, I. E. Ivanov, and M. R. Philips.** 1999. Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. Cell **98:**69–80.
9. **Douziech, M., M. Sahmi, G. Laberge, and M. Therrien.** 2006. A KSR/CNK complex mediated by HYP, a novel SAM domain-containing protein, regulates RAS-dependent RAF activation in Drosophila. Genes Dev. **20:**807–819.
10. **Fusello, A. M., L. Mandik-Nayak, F. Shih, R. E. Lewis, P. M. Allen, and A. S. Shaw.** 2006. The MAPK scaffold kinase suppressor of Ras is involved in ERK activation by stress and proinflammatory cytokines and induction of arthritis. J. Immunol. **177:**6152–6158.
11. **Giblett, S. M., D. J. Lloyd, Y. Light, R. Marais, and C. A. Pritchard.** 2002. Expression of kinase suppressor of Ras in the normal adult and embryonic mouse. Cell Growth Differ. **13:**307–313.
12. **Gilfillan, S., E. L. Ho, M. Cella, W. M. Yokoyama, and M. Colonna.** 2002. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. Nat. Immunol. **3:**1150–1155.
13. **Giurisato, E., M. Cella, T. Takai, T. Kurosaki, Y. Feng, G. D. Longmore, M. Colonna, and A. S. Shaw.** 2007. Phosphatidylinositol 3-kinase activation is required to form the NKG2D immunological synapse. Mol. Cell. Biol. **27:**8583–8599.
14. **Gomez, G. A., and J. L. Daniotti.** 2005. H-Ras dynamically interacts with recycling endosomes in CHO-K1 cells: involvement of Rab5 and Rab11 in the trafficking of H-Ras to this pericentriolar endocytic compartment. J. Biol. Chem. **280:**34997–35010.
15. **Gong, J. H., G. Maki, and H. G. Klingemann.** 1994. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. Leukemia **8:**652–658.
16. **Harding, A., T. Tian, E. Westbury, E. Frische, and J. F. Hancock.** 2005. Subcellular localization determines MAP kinase signal output. Curr. Biol. **15:**869–873.
17. **Jiang, K., B. Zhong, D. L. Gilvary, B. C. Corliss, E. Hong-Geller, S. Wei, and J. Y. Djeu.** 2000. Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. Nat. Immunol. **1:**419–425.
18. **Kolch, W.** 2005. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat. Rev. Mol. Cell Biol. **6:**827–837.
19. **Kornfeld, K., D. B. Hom, and H. R. Horvitz.** 1995. The ksr-1 gene encodes a novel protein kinase involved in Ras-mediated signaling in C. elegans. Cell **83:**903–913.
20. **Kortum, R. L., D. L. Costanzo, J. Haferbier, S. J. Schreiner, G. L. Razidlo, M. H. Wu, D. J. Volle, T. Mori, H. Sakaue, N. V. Chaika, O. V. Chaika, and R. E. Lewis.** 2005. The molecular scaffold kinase suppressor of Ras 1 (KSR1) regulates adipogenesis. Mol. Cell. Biol. **25:**7592–7604.

21. **Kortum, R. L., and R. E. Lewis.** 2004. The molecular scaffold KSR1 regulates the proliferative and oncogenic potential of cells. Mol. Cell. Biol. **24:**4407–4416.
22. **Locasale, J. W., A. S. Shaw, and A. K. Chakraborty.** 2007. Scaffold proteins confer diverse regulatory properties to protein kinase cascades. Proc. Natl. Acad. Sci. USA **104:**13307–13312.
23. **Lyubchenko, T. A., G. A. Wurth, and A. Zweifach.** 2001. Role of calcium influx in cytotoxic T lymphocyte lytic granule exocytosis during target cell killing. Immunity **15:**847–859.
24. **Michaud, N. R., M. Therrien, A. Cacace, L. C. Edsall, S. Spiegel, G. M. Rubin, and D. K. Morrison.** 1997. KSR stimulates Raf-1 activity in a kinase-independent manner. Proc. Natl. Acad. Sci. USA **94:**12792–12796.
25. **Mor, A., G. Campi, G. Du, Y. Zheng, D. A. Foster, M. L. Dustin, and M. R. Philips.** 2007. The lymphocyte function-associated antigen-1 receptor co-stimulates plasma membrane Ras via phospholipase D2. Nat. Cell Biol. **9:**713–719.
26. **Mor, A., and M. R. Philips.** 2006. Compartmentalized Ras/MAPK signaling. Annu. Rev. Immunol. **24:**771–800.
27. **Morrison, D. K.** 2001. KSR: a MAPK scaffold of the Ras pathway? J. Cell Sci. **114:**1609–1612.
28. **Morrison, D. K., and R. J. Davis.** 2003. Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annu. Rev. Cell Dev. Biol. **19:**91–118.
29. **Muller, J., A. M. Cacace, W. E. Lyons, C. B. McGill, and D. K. Morrison.** 2000. Identification of B-KSR1, a novel brain-specific isoform of KSR1 that functions in neuronal signaling. Mol. Cell. Biol. **20:**5529–5539.
30. **Nguyen, A., W. R. Burack, J. L. Stock, R. Kortum, O. V. Chaika, M. Afkarian, W. J. Muller, K. M. Murphy, D. K. Morrison, R. E. Lewis, J. McNeish, and A. S. Shaw.** 2002. Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. Mol. Cell. Biol. **22:**3035–3045.
31. **Nosaka, T., T. Kawashima, K. Misawa, K. Ikuta, A. L. Mui, and T. Kitamura.** 1999. STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. EMBO J. **18:**4754–4765.
32. **Ohmachi, M., C. E. Rocheleau, D. Church, E. Lambie, T. Schedl, and M. V. Sundaram.** 2002. C. elegans ksr-1 and ksr-2 have both unique and redundant functions and are required for MPK-1 ERK phosphorylation. Curr. Biol. **12:**427–433.
33. **Orange, J. S.** 18 August 2008, posting date. Formation and function of the lytic NK-cell immunological synapse. Nat. Rev. Immunol. [Epub ahead of print.] doi:10.1038/nri2381.
34. **Orange, J. S., K. E. Harris, M. M. Andzelm, M. M. Valter, R. S. Geha, and J. L. Strominger.** 2003. The mature activating natural killer cell immunologic synapse is formed in distinct stages. Proc. Natl. Acad. Sci. USA **100:**14151–14156.
35. **Ory, S., M. Zhou, T. P. Conrads, T. D. Veenstra, and D. K. Morrison.** 2003. Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. Curr. Biol. **13:**1356–1364.
36. **Perez de Castro, I., T. G. Bivona, M. R. Philips, and A. Pellicer.** 2004. Ras activation in Jurkat T cells following low-grade stimulation of the T-cell receptor is specific to N-Ras and occurs only on the Golgi apparatus. Mol. Cell. Biol. **24:**3485–3496.
37. **Plowman, S. J., and J. F. Hancock.** 2005. Ras signaling from plasma membrane and endomembrane microdomains. Biochim. Biophys. Acta **1746:**274–283.
38. **Prior, I. A., and J. F. Hancock.** 2001. Compartmentalization of Ras proteins. J. Cell Sci. **114:**1603–1608.
39. **Raabe, T., and U. R. Rapp.** 2002. KSR—a regulator and scaffold protein of the MAPK pathway. Sci. STKE **2002:**PE28.
40. **Razidlo, G. L., R. L. Kortum, J. L. Haferbier, and R. E. Lewis.** 2004. Phosphorylation regulates KSR1 stability, ERK activation, and cell proliferation. J. Biol. Chem. **279:**47808–47814.
41. **Rechavi, O., I. Goldstein, H. Vernitsky, B. Rotblat, and Y. Kloog.** 2007. Intercellular transfer of oncogenic H-Ras at the immunological synapse. PLoS ONE **2:**e1204.
42. **Robertson, L. K., L. R. Mireau, and H. L. Ostergaard.** 2005. A role for phosphatidylinositol 3-kinase in TCR-stimulated ERK activation leading to paxillin phosphorylation and CTL degranulation. J. Immunol. **175:**8138–8145.
43. **Rocheleau, C. E., K. Cullison, K. Huang, Y. Bernstein, A. C. Spilker, and M. V. Sundaram.** 2008. The Caenorhabditis elegans ekl (enhancer of ksr-1 lethality) genes include putative components of a germline small RNA pathway. Genetics **178:**1431–1443.
44. **Roy, F., G. Laberge, M. Douziech, D. Ferland-McCollough, and M. Therrien.** 2002. KSR is a scaffold required for activation of the ERK/MAPK module. Genes Dev. **16:**427–438.
45. **Sacks, D. B.** 2006. The role of scaffold proteins in MEK/ERK signalling. Biochem. Soc. Trans. **34:**833–836.
46. **Shaul, Y. D., and R. Seger.** 2007. The MEK/ERK cascade: from signaling specificity to diverse functions. Biochim. Biophys. Acta **1773:**1213–1226.
47. **Stefanova, I., B. Hemmer, M. Vergelli, R. Martin, W. E. Biddison, and R. N. Germain.** 2003. TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. Nat. Immunol. **4:**248–254.
48. **Stinchcombe, J. C., G. Bossi, S. Booth, and G. M. Griffiths.** 2001. The immunological synapse of CTL contains a secretory domain and membrane bridges. Immunity **15:**751–761.
49. **Stinchcombe, J. C., and G. M. Griffiths.** 2003. The role of the secretory immunological synapse in killing by $CD8^+$ CTL. Semin. Immunol. **15:**301–305.
50. **Stinchcombe, J. C., E. Majorovits, G. Bossi, S. Fuller, and G. M. Griffiths.** 2006. Centrosome polarization delivers secretory granules to the immunological synapse. Nature **443:**462–465.
51. **Sundaram, M., and M. Han.** 1995. The C. elegans ksr-1 gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. Cell **83:**889–901.
52. **Sundaram, M., J. Yochem, and M. Han.** 1996. A Ras-mediated signal transduction pathway is involved in the control of sex myoblast migration in Caenorhabditis elegans. Development **122:**2823–2833.
53. **Therrien, M., H. C. Chang, N. M. Solomon, F. D. Karim, D. A. Wassarman, and G. M. Rubin.** 1995. KSR, a novel protein kinase required for RAS signal transduction. Cell **83:**879–888.
54. **Trambas, C. M., and G. M. Griffiths.** 2003. Delivering the kiss of death. Nat. Immunol. **4:**399–403.
55. **Watts, J. D., J. S. Sanghera, S. L. Pelech, and R. Aebersold.** 1993. Phosphorylation of serine 59 of p56lck in activated T cells. J. Biol. Chem. **268:**23275–23282.
56. **Wei, S., A. M. Gamero, J. H. Liu, A. A. Daulton, N. I. Valkov, J. A. Trapani, A. C. Larner, M. J. Weber, and J. Y. Djeu.** 1998. Control of lytic function by mitogen-activated protein kinase/extracellular regulatory kinase 2 (ERK2) in a human natural killer cell line: identification of perforin and granzyme B mobilization by functional ERK2. J. Exp. Med. **187:**1753–1765.
57. **Winkler, D. G., I. Park, T. Kim, N. S. Payne, C. T. Walsh, J. L. Strominger, and J. Shin.** 1993. Phosphorylation of Ser-42 and Ser-59 in the N-terminal region of the tyrosine kinase $p56^{lck}$. Proc. Natl. Acad. Sci. USA **90:**5176–5180.
58. **Yachi, P. P., J. Ampudia, T. Zal, and N. R. Gascoigne.** 2006. Altered peptide ligands induce delayed CD8-T cell receptor interaction—a role for CD8 in distinguishing antigen quality. Immunity **25:**203–211.
59. **Yu, W., W. J. Fantl, G. Harrowe, and L. T. Williams.** 1998. Regulation of the MAP kinase pathway by mammalian Ksr through direct interaction with MEK and ERK. Curr. Biol. **8:**56–64.
60. **Zhou, M., D. A. Horita, D. S. Waugh, R. A. Byrd, and D. K. Morrison.** 2002. Solution structure and functional analysis of the cysteine-rich C1 domain of kinase suppressor of Ras (KSR). J. Mol. Biol. **315:**435–446.

3.3 Conclusions

This work shows that KSR1-deficient mice display reduction of NK cell cytotoxicity. The defect was not mediated by defective cell-cell adhesion, since the absence of KSR1 did not affect the ability of NK cells to form conjugates with target cells. Instead, it might be mediated by defective polarization of lytic granules, which required KSR1 function. Search for the mechanism causing this defect showed that KSR1 recruitment to the membrane is required for recruitment of active ERK to the immunological synapse, which may be crucial to allow ERK to phosphorylate specific substrates at the plasma membrane.

It is intriguing to speculate that recruitment of KSR1 and the ERK MAP kinase cascade to the immunological synapse may have functions in addition to simply facilitating ERK activation. By holding active ERK at the immunological synapse, KSR1 may function to allow ERK to phosphorylate specific substrates at the plasma membrane essential for T-cell function. For example, it has been proposed that ERK phosphorylation of Lck may play a role in facilitating a positive feedback loop that is important for enhancing T-cell activation (Stefanova et al, 2003). Our immunoprecipitation data indicated that ERK-dependent phosphorylation of the PXSP motif in Lck was diminished after KSR1 suppression, supporting the role of KSR1 on ERK substrate phosphorylation into the synapse.

It seems possible that the granule polarization defect seen in the KSR1-deficient cells is due to defects in ERK phosphorylation of critical substrates at the immunological synapse. The localization of the MAP kinase cascade at different sites in the cell has been suggested to play an important role in T-cell biology (Mor et al, 2006). While it was originally thought that Ras activation of the MAP kinase cascade could only be initiated at the plasma membrane, it has now become clear that different Ras isoforms are localized and activated at distinct intracellular membranes (Apolloni et al, 2000; Choy et al, 1999; Plowman et al, 2005). These studies suggest that localized Ras signaling is important in the activation of location-specific effectors, but it is also possible that this plays a role in localizing active ERK close to the location-specific substrates. Unfortunately, in these studies, the localization of active ERK was not determined.

Whether KSR1 localizes to sites other than the plasma membrane is not yet known. It would be interesting if KSR1 and KSR2 were recruited to distinct membranes. Unfortunately, little is known about the expression pattern of KSR2.

In conclusion these data suggest that KRS1 may be crucial for cell-mediated cytotoxicity and may be a promising candidate molecule involved in diseases caused by defective cell-mediated cytotoxicity.

CONCLUSIONS

Autoimmune lymphoproliferative diseases are disorders of lymphocyte homeostasis caused primarily by defects in the Fas apoptotic pathway. These disorders include autoimmune lymphoproliferative syndrome (ALPS) and Dianzani autoimmune lymphoproliferative disease (DALD). In most cases, ALPS is due to deleterious mutations of the Fas gene (TNFRSF6) and is classified as ALPS type-Ia . Fas mutations are usually heterozygous, their penetrance depends on their effect on Fas function. Mutations hitting the intracellular death domain of Fas, involved in recruitment of FADD and caspase-8/10 and initiating the death signal, are often the most severe. They exert a dominant-negative effect and display high penetrance. By contrast, mutations hitting the extracellular portion or causing haploinsufficiency have weaker penetrance. Most ALPS type-Ia patients are heterozygous for the Fas mutation, but the parent carrying the mutation is generally healthy. Other complementary factors may thus be required to the development of the disease. One possibility is that mild Fas mutations only induces ALPS when cooperate with mutations of other genes impairing function of the Fas system itself. In line with this hypotesis we identified two unrelated ALPS patients carrying heterozygous mutations both in TNFR6 and CASP10 gene. The TNFR6 mutations were the weakly penetrant mutations Q47X and IVS3-2a>g and were not sufficient for development of ALPS. In these two patients a concurrent factor may be the co-present mutations of CAP10 (Y466C and P501L) which decreased the activity of caspase-10 and were per se insufficient to induce ALPS development.

Another mechanism involved in downmodulation of the immune response is the cell-mediated cytotoxicity. Cytotoxic T lymphocytes and natural killer cells are able to kill the target cell through both the Fas/FasL or perforin/granzymes systems. This cytotoxicity is crucial to kill virus-infected cells and clear viral infections but may also be involved in downmodulation of the immune response by fratricide of effector lymphocytes and antigen-presenting cells. The possibility of the involvement of perforin in the pathogenesis of autoimmune disease was first suggested by the observation of an ALPS patient with a Fas gene mutation inherited from the father and with a PRF1 mutation inherited from the mother. Since both parents and the patient’s brother, who carried the Fas mutation only, were healthy, it appeared that both mutations contributed to the development of ALPS (Clementi et al, 2005). We confirmed this result in a larger

group of ALPS/DALD patients, where we detected two HLH-associated amino acid substitutions of PRF1 N252S and A91V. We found that the frequency of N252S was increased in typical forms of ALPS and increased the risk of its development by about 62-fold, whereas that of A91V was increased in an incomplete variant of ALPS and increased this risk by about three fold. This risk was significantly increased by copresence of the OPN^{high} gene variants, but even their combination with A91V and the Fas defect was not sufficient to induce DALD in 4 of 8 DALD family members. These results underlined the complexity of the pathogenesis of ALPS, in which different mechanism may be involved affecting downmodulation of the immune response and /or viral clearance (Su et al, 2004; Badovinac et al, 2003; Zhou et al 2002).

Involvement of cell-mediated cytotoxicity defects in autoimmunity and defective viral clearance has been shown in other several diseases. Insight into how the lytic synapse is formed in cells from patients with these diseases has been gained mostly from T cell studies, but functional defects have been established in NK cells too. Protein involved on release of lytic granules by cytotoxic cell might be candidate molecules involved in diseases caused by defective cell-mediated cytotoxicity. The diseases that provide specific insight into the NK-cell lytic synapse are normally divided into two groups. Diseases in the first group affect steps that are involved in the initiation stage or the activation steps of the effector stage of synapse formation. Diseases in the second group affect steps in lytic-granule trafficking to the synapse in the effector stage. We can speculate that defect in KSR1 function may be involved in the pathogenesis of the second group of disease. In fact, the defect in NK cell cytotoxicity displayed by KSR1-deficient mice was not due to defective cell-cell adhesion, since the absence of KSR1 did not affect the ability of NK cells to form conjugates with target cells. Instead, it is mediated by defective polarization of lytic granules to the immunological synapse. Searching for the mechanism causing this defect, we demonstrated that KSR1 recruitment to the membrane is required for recruitment of active ERK to the immunological synapse, which may be crucial to allow ERK to phosphorylate specific substrates at the plasma membrane essential for NK-cell function.
It might be interestingly take in consideration a systematic evaluation of the role of KSR1 in the pathogenesis of autoimmune and immunodeficiency disease, due to cell-mediated cytotoxicity defects.

REFERENCES

Abo, T., Roder, J. C., Abo, W., Cooper, M. D. & Balch, C. M. 1982. Natural killer (HNK1+) cells in Chediak–Higashi patients are present in normal numbers but are abnormal in function and morphology. *J. Clin. Invest.* 70, 193–197

Adrain C, Murphy BM, Martin SJ. 2005. Molecular ordering of the caspase activation cascade initiated by the cytotoxic T lymphocyte/natural killer (CTL/NK) protease granzyme B. *J Biol Chem* 280: 4663–4673.

Alferink J, Tafuri A, Vestweber D, Hallmann R, Hammerling GJ, Arnold B. 1998. Control of neonatal tolerance to tissue antigens by peripheral T cell trafficking. *Science* 282(5392):1338–41

Algeciras-Schimnich A, Shen L, Barnhart BC, Murmann AE, Burkhardt JK, et al. 2002. Molecular ordering of the initial signaling events of CD95. *Mol. Cell. Biol.* 22:207–20

Alimonti JB, Shi L, Baijal PK, Greenberg AH. 2001. Granzyme B induces Bid-mediated cytochrome c release and mitochondrial permeability transition. *J. Biol. Chem.* 276: 6974–6982.

Almeida, C. R. & Davis, D. M. 2006. Segregation of HLA‐C from ICAM‐1 at NK cell immune synapses is controlled by its cell surface density. *J. Immunol.* 177, 6904–6910

Apolloni, A., I. A. Prior, M. Lindsay, R. G. Parton, and J. F. Hancock. 2000. H-ras but not K-ras traffics to the plasma membrane through the exocytic pathway. *Mol. Cell. Biol.* 20:2475–2487.

Arneson, L. N. et al, 2007. Cutting Edge: syntaxin 11 regulates lymphocyte‐mediated secretion and cytotoxicity. *J. Immunol.* 179, 3397–3401

Asano M, Toda M, Sakaguchi N, Sakaguchi S. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med.* 184(2):387–96

Awata T, Kuzuya T, Matsuda A, Iwamoto Y, Kanazawa Y. 1992. Genetic analysis of HLA class II alleles and susceptibility to type 1 (insulin-dependent) diabetes mellitus in Japanese subjects. *Diabetologia* 35(5):419–24

Badenhoop K, Walfish PG, Rau H, et al. 1995. Susceptibility and resistance alleles of human leukocyte antigen (HLA) DQA1 and HLA DQB1 are shared in endocrine autoimmune disease. *J. Clin. Endocrinol. Metab.* 80(7):2112–7

Badovinac VP, Hamilton SE, Harty JT. 2003. Viral infection results in massive CD8_ T-cell expansion and mortality in vaccinated perforin-deficient mice. *Immunity* 18:463–474

Bankovich AJ, Girvin AT, Moesta AK, Garcia KC. 2004. Peptide register shifting within the MHC groove: theory becomes reality. *Mol. Immunol.* 40(14–15):1033–9

Barry M, Heibein JA, Pinkoski MJ, Lee SF, Moyer RW, Green D et al. 2000. Granzyme B short circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol. Cell. Biol.* 20: 3781–3794.

Bi LL, Pan G, Atkinson TP, Zheng L, Dale JK, Makris C, Reddy V, McDonald JM, Siegel RM, Puck JM, Lenardo MJ, Straus SE. 2007. Dominant Inhibition of Fas Ligand-Mediated Apoptosis due to a Heterozygous Mutation Associated with Autoimmune Lymphoproliferative Syndrome (ALPS) Type Ib. *BMC Med. Genet.*8:41.

Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P. & Salazar‐Mather, T. P. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol.* 17, 189–220

Bleesing JJ, Brown MR, Dale JK, Straus SE, Lenardo MJ, et al. 2001. TcR-α/β+ CD4−CD8− T cells in humans with the autoimmune lymphoproliferative syndrome express a novel CD45 isoform that is analogous to murine B220 and represents a marker of altered O-glycan biosynthesis. *Clin. Immunol.* 100:314–24

Bleesing JJ, Brown MR, Novicio C, Guarraia D, Dale JK, et al. 2002. A composite picture of TcR α/β+ CD4−CD8− T cells (α/β-DNTCs) in humans with autoimmune lymphoproliferative syndrome. *Clin. Immunol.* 104:21–30

Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, Ricci JE, Edris WA, Sutherlin DP, Green DR, Salvesen GS. 2003. A unified model for apical caspase activation. *Mol Cell* 2:529-41.

Boatright KM, Salvesen GS. 2003. Mechanisms of caspase activation. *Curr. Opin. Cell Biol.* 15:725–31

Brocke S, Gaur A, Piercy C, et al. 1993. Induction of relapsing paralysis in experimental autoimmune encephalomyelitis by bacterial superantigen. *Nature* 365(6447):642–4

Bryceson, Y. T. et al. 2007. Defective cytotoxic lymphocyte degranulation in syntaxin‐11 deficient familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients. *Blood* 110, 1906–1915

Bryceson, Y. T., March, M. E., Ljunggren, H. G. & Long, E. O. 2006. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol. Rev.* 214, 73–91

Campagnoli MF, Garbarini L, Quarello P, Garelli E, Carando A, Baravalle V, Doria A, Biava A, Chiocchetti A, Rosolen A, Dufour C, Dian- zani U, Ramenghi U. 2006. The broad spectrum of autoimmune lymphoproliferative disease: molecular bases, clinical features and long-term follow-up in 31 patients. *Haematologica* 91:538-41.

Cappellano G, Orilieri E, Comi C, Chiocchetti A, Bocca S, Boggio E, Bernardone IS, Cometa A, Clementi R, Barizzone N, D'Alfonso S, Corrado L, Galimberti D, Scarpini E, Guerini FR, Caputo D, Paolicelli D, Trojano M, Figà-Talamanca L, Salvetti M, Perla

F, Leone M, Monaco F, Dianzani U. 2008. Variations of the perforin gene in patients with multiple sclerosis. *Genes Immun.* 9(5):438-44

Cerutti E, Campagnoli MF, Ferretti M, Garelli E, Crescenzio N, Rosolen A, Chiocchetti A, Lenardo MJ, Ramenghi U, Dianzani U. 2007. Co-inherited mutations of Fas and caspase-10 in development of the autoimmune lymphoproliferative syndrome. *BMC Immunol.* 13;8:28

Chen X, Allan DS, Krzewshi K, Ge B, Hopcow H and Strominger JL. 2006. CD28-stimulated ERK2 phosphorylation is required for polarization of the microtubule organizing center and granules in YTS NK cells. *Proc Natl Acad Sci USA* 103:10346-10351

Chiocchetti A, Indelicato M, Bensi T, Mesturini R, Giordano M, et al. 2004. High levels of osteopontin associated with polymorphisms in its gene are a risk factor for development of autoimmunity/lymphoproliferation. *Blood* 103:1376–82

Chowdhury D, Beresford PJ, Zhu P, Zhang D, Sung JS, Demple B, Perrino FW, Lieberman J. 2006. The exonuclease TREX1 is in the SET complex and acts in concert with NM23-H1 to degrade DNA during granzyme A-mediated cell death. *Mol. Cell.* 23:133-142.

Choy, E., V. K. Chiu, J. Silletti, M. Feoktistov, T. Morimoto, D. Michaelson, I. E. Ivanov, and M. R. Philips. 1999. Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. *Cell* 98:69–80.

Clark, R. H. et al. 2003. Adaptor protein 3-dependent microtubule-mediated movement of lytic granules to the immunological synapse. *Nature Immunol.* 4, 1111–1120

Clementi R, Chiocchetti A, Cappellano G, Cerutti E, Ferretti M, Orilieri E, Dianzani I, Ferrarini M, Bregni M, Danesino C, Bozzi V, Putti MC, Cerutti F, Cometa A, Locatelli F, Maccario R, Ramenghi U, Dianzani U. 2006. Variations of the perforin gene in patients with autoimmunity/lymphoproliferation and defective Fas function. *Blood.* 108(9):3079-84

Clementi R, Dagna L, Dianzani U, et al. 2004. Inherited perforin and Fas mutations in a patient with autoimmune lymphoproliferative syndrome and lymphoma. *New Eng. J. Med.* 351:1419-1424.

Clementi R, Locatelli F, Dupre L, Garaventa A, Emmi L, Bregni M, Cefalo G, Moretta A, Danesino C, Comis M et al. 2005. A proportion of patients with lymphoma may harbor mutations of the perforin gene. *Blood* 105:4424-4428.

Cohen PL, Eisenberg RA. 1991. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol.* 9:243–69

Criswell LA, Pfeiffer KA, Lum RF, et al. 2005. Analysis of families in the multiple autoimmune disease genetics consortium (MADGC) collection: the *PTPN22* 620W allele associates with multiple autoimmune phenotypes. *Am. J. Hum. Genet.* 76(4):561–71

Darmon AJ, Nicholson DW, Bleackley RC. 1995. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature* 377: 446–448.

Davidson WF, Giese T, Fredrickson TN. 1998. Spontaneous development of plasmacytoid tumors in mice with defective Fas-Fas ligand interactions. *J. Exp. Med.* 187:1825–38

Davis JE, Sutton VR, Smyth MJ, Trapani JA. 2000. Dependence of granzyme B-mediated cell death on a pathway regulated by Bcl-2 or its viral homolog, BHRF1. *Cell. Death. Differ.* 7: 973–983.

Del-Rey M, Ruiz-Contreras J, Bosque A, Calleja S, Gomez-Rial J, Roldan E, Morales P, Serrano A, Anel A, Paz-Artal E, Allende LM. 2006. A homozygous Fas ligand gene mutation in a patient causes a new type of autoimmune lymphoproliferative syndrome. *Blood* 108:1306-12.

Dianzani U, Bragardo M, DiFranco D, Alliaudi C, Scagni P, Buonfiglio D, Redoglia V, Bonissoni S, Correra A, Dianzani I, Ramenghi U. 1997. Deficiency of the Fas apoptosis pathway without Fas gene mutations in pediatric patients with autoimmunity/ lymphoproliferation. *Blood* 89:2871-2879.

Dianzani U, Chiocchetti A, Ramenghi U. 2003. Role of inherited defects decreasing Fas function in autoimmunity. *Life Sciences* 72:2803-2824.

Djeu, J. Y., Jiang, K. & Wei, S. 2002. A view to a kill: signals triggering cytotoxicity *Clin. Cancer Res.* 8, 636–640

Dong C, Juedes AE, Temann UA, et al. 2001. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409(6816):97–101

Duan H, Orth K, Chinnaiyan AM, Poirier GG, Froelich CJ, HeWWet al. 1996. ICE-LAP6, a novel member of the ICE/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B. *J. Biol. Chem.* 271: 16720–16724.

Evavold BD, Sloan-Lancaster J, Wilson KJ, Rothbard JB, Allen PM. 1995. Specific T cell recognition of minimally homologous peptides: evidence for multiple endogenous ligands. *Immunity* ;2(6):655–63

Enders, A. et al. 2006. Lethal hemophagocytic lymphohistiocytosis in Hermansky–Pudlak syndrome type II. *Blood* 108, 81–87

Feldmann, J. et al. 2003. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell* 115,461–473

Fontana, S. et al. 2006. Innate immunity defects in Hermansky–Pudlak type 2 syndrome. *Blood* 107, 4857–4864

Fernandes-Alnemri T, Armstrong RC, Krebs J, Srinivasula SM, Wang L, et al. 1996. In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc. Natl. Acad. Sci. USA* 93:7464–69

Fesik SW. 2000. Insights into programmed cell death through structural biology. *Cell* 103:273–82

Fischer, A. M., Katayama, C. D., Pages, G., Pouyssegur, J. & Hedrick, S. M. 2005. The role of Erk1 and Erk2 in multiple stages of T cell development. *Immunity* 23, 431–443

Fisher GH, Rosenberg FJ, Straus SE, Dale JK, Middleton LA, Lin AY, Strober W, Lenardo MJ, Puck JM. 1995. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell* 81:935–46

Ford ML, Evavold BD. 2004 Degenerate recognition of T cell epitopes: impact of T cell receptor reserve and stability of peptide:MHC complexes. *Mol. Immunol.* 40(14–15):1019–25

Freeman GJ, Long AJ, Iwai Y, et al. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp. Med.* 192(7):1027–34

Fusello, A. M. et al. 2006. The MAPK scaffold kinase suppressor of Ras is involved in ERK activation by stress and proinflammatory cytokines and induction of arthritis. *J. Immunol.* 177, 6152–6158

Fuss IJ, Strober W, Dale JK, Fritz S, Pearlstein GR, et al. 1997. Characteristic T helper 2 T cell cytokine abnormalities in autoimmune lymphoproliferative syndrome, a syndrome marked by defective apoptosis and humoral autoimmunity. *J. Immunol.* 158:1912–18

Gismondi, A. et al. 2004. Impaired natural and CD16‐mediated NK cell cytotoxicity in patients with WAS and XLT: ability of IL‐2 to correct NK cell functional defect. *Blood* 104, 436–443

Giurisato E, Cella M, Takai T, Kurosaki T, Feng Y, Longmore GD, Colonna M, Shaw AS. 2007. Phosphatidylinositol 3-kinase activation is required to form the NKG2D immunological synapse. *Mol Cell Biol.* 27:8583-8599

Giurisato E, Lin J, Harding A, Cerutti E, Cella M, Lewis RE, Colonna M, Shaw AS. 2009. The mitogen-activated protein kinase scaffold KSR1 is required for recruitment of extracellular signal-regulated kinase to the immunological synapse. *Mol. Cell Biol.* 29(6):1554-64.

Grakoui, A. Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML. 1999 The immunological synapse: a molecular machine controlling T cell activation. *Science* 285, 221–227

Gronbaek K, Dalby T, Zeuthen J, Ralfkiaer E, Guldberg P. 2000. The V410I (G1228A) variant of the caspase-10 gene is a common polymorphism of the Danish population. *Blood*, 95:2184-2185.

Grossman, Z. & Paul, W. E. 2000. Self-tolerance: context-dependent tuning of T cell antigen recognition. *Semin. Immunol*. 12, 197–203

Grossman WJ, Revell PA, Lu ZH, Johnson H, Bredemeyer AJ, Ley TJ. 2003. The orphan granzymes of humans and mice. *Curr. Opin. Immunol.* 2003; 5: 544–552.

Groux H, O'Garra A, Bigler M, et al. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389(6652):737–42

Gu Y, Sarnecki C, Fleming MA, Lippke JA, Bleackley RC, Su MS. 1996. Processing and activation of CMH-1 by granzyme B. *J. Biol. Chem.* 271: 10816–10820.

Haliotis, T. *et al.* Chediak–Higashi gene in humans I. 1980. Impairment of natural‐killer function. *J. Exp. Med.* 151, 1039–1048

Hang L, Theofilopoulos AN, Balderas RS, Francis SJ, Dixon FJ. 1984. The effect of thymectomy on lupus-prone mice. *J. Immunol.* 132:1809–13

Healy, J. I. & Goodnow, C. C. 1998. Positive versus negative signaling by lymphocyte antigen receptors. *Annu. Rev. Immunol*. 16, 645–670

Heibein JA, Goping IS, Barry M, Pinkoski MJ, Shore GC, Green DR et al. 2000. Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. *J. Exp. Med.* 192: 1391–1402.

Henter, J. I.et al. 2007.. HLH‐2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr. Blood* Cancer 48, 124–131

Hill MM, Adrain C, Duriez PJ, Creagh EM, Martin SJ. 2004. Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. *EMBO J*. 23:2134–2145.

Holzelova E, Vonarbourg C, Stolzenberg MC, Arkwright PD, Selz F, et al. 2004. Autoimmune lymphoproliferative syndrome with somatic Fas mutations. *N. Engl. J. Med.* 351:1409–18

Hull KM, Drewe E, Aksentijevich I, Singh HK, Wong K, McDermott EM, Dean J, Powell RJ, Kastner DL. 2002. The TNF receptor-associated periodic syndrome (TRAPS): emerging concepts of an autoinflammatory disorder. *Medicine (Baltimore)* 81**:**349-68.

Hundt M, Posovszky C, Schmidt RE. 2002. A new disorder of lymphocyte apoptosis: combination of autoimmunity, infectious lymphadenopathy, double negative T cells, and impaired activation-induced cell death. *Immunobiology* 206:514–18

Huizing, M., Anikster, Y. & Gahl, W. A. 2001. Hermansky–Pudlak syndrome and Chediak–Higashi syndrome: disorders of vesicle formation and trafficking. *Thromb. Haemost.* 86, 233–245

Ignatowicz, L., Kappler, J. & Marrack, P. 1996. The repertoire of T cells shaped by a single MHC/peptide ligand. *Cell* 84, 521–529

Introne, W., Boissy, R. E. & Gahl, W. A. 1999. Clinical, molecular, and cell biological aspects of Chediak–Higashi syndrome. *Mol. Genet. Metab.* 68, 283–303

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, et al. 1997. Inhibition of death receptor signals by cellular FLIP. *Nature* 388:190–95

Jacobson, D. L., Gange, S. J., Rose, N. R. & Graham, N. M. 1997. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin. Immunol. Immunopathol*. 84, 223–243

Janeway CA, Jr. 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol. Today* 13(1):11–6

Jiang K, Zhong B, Gilvary DL, Corliss BC, Hong-Geller E, Wei S, and Djeu JY. 2000. Pivotal role of phosphatidylinositol 3-kinase in regulation of cytotoxicity in Natural Killer cells. *Nat Immunol*. 1:419-425

Jiang X, Wang X. 2004. Cytochrome C-mediated apoptosis. *Annu. Rev. Biochem.* 73:87–106

Jiang X, Wang X. 2000. Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *J. Biol. Chem.* 275: 31199–31203.

Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM, Hengartner H. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* 369:31-37.

Kagi D, Ledermann B, Burki K, Zinkernagel RM, Hengartner H. 1996. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. *Annu. Rev. Immunol.* 14:207-232.

Kasahara Y, Wada T, Niida Y, Yachie A, Seki H, Ishida Y, Sakai T, Koizumi F, Koizumi S, Miyawaki T, Taniguchi N. 1998. Novel Fas (CD95/ APO-1) mutations in infants with a lymphoproliferative disorder. *Int Immunol* **10:**195-202.

Katano H, Cohen JI 2005. Perforin and lymphohistiocytic proliferative disorders. *Br. J. Haematol.* 128:739-750.

Katz, P., Zaytoun, A. M. & Fauci, A. S. 1982. Deficiency of active natural killer cells in the Chediak–Higashi syndrome. Localization of the defect using a single cell cytotoxicity assay. *J. Clin. Invest.* 69, 1231–1238

Keir ME, Sharpe AH. 2005. The B7/CD28 costimulatory family in autoimmunity. *Immunol Rev.* 204:128–43

Kelly JM, Waterhouse NJ, Cretney E, BrowneKA, Ellis S, Trapani JA, Smyth MJ: 2004. Granzyme M mediates a novel form of perforindependent cell death. *J. Biol. Chem.* 279:22236-22242.

Kimura M, Matsuzawa A. 1994. Autoimmunity in mice bearing lprcg: a novel mutant gene. *Int. Rev. Immunol.* 11:193–210

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, et al. 1995. Cytotoxicitydependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J.* 14:5579–88

Kishimoto, T. K., Hollander, N., Roberts, T. M., Anderson, D. C. & Springer, T. A. 1987. Heterogeneous mutations in the β subunit common to the LFA‐1, Mac‐1, and p150, 95 glycoproteins cause leukocyte adhesion deficiency. *Cell* 50, 193–202

Klein, C. et al. 1994. Partial albinism with immunodeficiency (Griscelli syndrome). *J. Pediatr.* 125, 886–895

Klein, M. et al. 1980. Chediak–Higashi gene in humans. II. The selectivity of the defect in natural killer and antibody dependent cell mediated cytotoxicity function. *J. Exp. Med.* 151, 1049–1058

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275:1132–1136.

Kohl, S., Springer, T. A., Schmalstieg, F. C., Loo, L. S. & Anderson, D. C. 1984. Defective natural killer cytotoxicity and polymorphonuclear leukocyte antibody dependent cellular cytotoxicity in patients with LFA‐1/ OKM‐1 deficiency. *J. Immunol.* 133, 2972–2978

Kopcow, H. D., Allan, D. S., Chen, X., Rybalov, B., Andzelm, M. M., Ge, B. & Strominger, J. L. 2005. Human decidual NK cells form immature activating synapses and are not cytotoxic. *Proc. Natl. Acad. Sci. USA* 102, 15563–15568

Kornfeld, K., Hom, D. B. & Horvitz, H. R. 1995. The *ksr-1* gene encodes a novel protein kinase involved in Ras mediated signaling in *C. elegans*. *Cell* 83, 903–913

Krensky, A. M.et al. 1985. Heritable lymphocyte function associated antigen‐1 deficiency: abnormalities of cytotoxicity and proliferation associated with abnormal expression of LFA‐1. *J. Immunol.* 135, 3102–3108

Krueger A, Fas SC, Baumann S, Krammer PH. 2003. The role of CD95 in the regulation of peripheral T-cell apoptosis. *Immunol. Rev.* 193:58–69

Krueger A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S. 2001. Cellular FLICE inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *J. Biol. Chem.* 276:20633–40

Kuchroo VK, Das MP, Brown JA, et al. 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* 80(5):707–18

Kuniyasu Y, Takahashi T, Itoh M, Shimizu J, Toda G, Sakaguchi S. 2000. Naturally anergic and suppressive CD25(+)CD4(+) T cells as a functionally and phenotypically distinct immunoregulatory T cell subpopulation. *Int. Immunol* 12(8):1145–55

Kurts C, Miller JF, Subramaniam RM, Carbone FR, Heath WR. 1998. Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. *J. Exp. Med.*188(2):409–14

Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R et al. 2002. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* 111: 331–342.

Kyewski B, Derbinski J. 2004. Self-representation in the thymus: an extended view. *Nat Rev Immunol.* 4(9):688–98

Lang HL, Jacobsen H, Ikemizu S, et al. 2002. A functional and structural basis for TCR cross-reactivity in multiple sclerosis. *Nat. Immunol.* 3(10):940–3

Lanzavecchia A. 1995. How can cryptic epitopes trigger autoimmunity? *J. Exp. Med.* 181(6):1945–8

Latchman Y, Wood CR, Chernova T, et al. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat. Immunol.* 2(3):261–8

Laufer, T. M., DeKoning, J., Markowitz, J. S., Lo, D. & Glimcher, L. H. 1996. Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex. *Nature* 383, 81–85

Legembre P, Barnhart BC, Zheng LX,Vijayan S, Straus SE, et al. 2004. Induction of apoptosis and activation of NF-κB by CD95 require different signalling thresholds. *EMBO Rep.* 5:1084–89

Li H, Zhu H, Xu C, Yuan J. 1998. Cleavage of BID by caspase-8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94: 491–501.

Locksley RM, Killeen N, Lenardo MJ. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 104:487–501

Lopatin U, Yao X,Williams RK, Bleesing JJ, Dale JK, et al. 2001. Increases in circulating and lymphoid tissue interleukin-10 in autoimmune lymphoproliferative syndrome are associated with disease expression. *Blood* 97:3161–70

Lowin, B., Peitsch, M.C. & Tschopp, J. 1995. Perforin and granzymes: crucial effector molecules in cytolytic T lymphocyte and natural killer cell-mediated cytotoxicity. *Curr.Top. Microbiol. Immunol*. 198, 1–24

Lu H, Hou Q, Zhao T, Zhang H, Zhang Q, Wu L, Fan Z: 2006. Granzyme M directly cleaves inhibitor of caspase-activated DNase (CAD) to unleash CAD leading to DNA fragmentation. *J. Immunol.* 177:1171-1178.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. 1998. Bid, a Bcl-2 protein, mediates the release of cytochrome c from mitochondria in response to cell surface death receptors. *Cell* 94: 481–490.

Lyubchenko TA, Wurth GA and Zweifac A. 2001. Role of calcium influx in cytotoxic T lymphocyte lytic granule exocytosis during target cell killing. *Immunity* 15:847-859

Marcenaro, S. et al. 2006. Analysis of natural killer cell function in familial hemophagocytic lymphohistiocytosis (FHL). Defective CD107a surface expression heralds Munc13‑4 defect and discriminates between genetic subtypes of the disease. *Blood* 108, 2316–2323

Martin SJ, Aramante-Mendes GP, Shi L, Chuang TH, Casiano CA, O'Brien GA et al. 1996. The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism. *EMBO J* 15: 2407–2416.

Masson D, Tschopp J. 1987. A family of serine esterases in lytic granules of cytolytic T lymphocytes. *Cell* 49: 679–685.

Matzinger P. 1994. Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* 12:991–1045

McHugh RS, Shevach EM. 2002. Cutting edge: depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. *J. Immunol.* 168(12):5979–83

Medema JP, Toes RE, Scaffidi C, Zheng TS, Flavell RA, Melief CJ et al. 1997. Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T lymphocyte-induced apoptosis. *Eur. J. Immunol.* 27: 3492–3498.

Mehta PA, Davies SM, Kumar A, Devidas M, Lee S, Zamzow T, Elliott J, Villanueva J, Pullen J, Zewge Y et al. 2006. Perforin polymorphism A91V and susceptibility to B-precursor childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group. *Leukemia* 20:1539-1541.

Menasche, G. et al. 2000. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. *Nature Genet.* 25, 173–176

Michaud, N. R., M. Therrien, A. Cacace, L. C. Edsall, S. Spiegel, G. M. Rubin, and D. K. Morrison. 1997. KSR stimulates Raf-1 activity in a kinase-independent manner. *Proc. Natl. Acad. Sci. USA* 94:12792-6

Millard, P.J., Henkart, M.P., Reynolds,C.W. & Henkart, P.A. 1984 Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors. *J. Immunol*. 132, 3197–3204

Miyazaki T, Ono M, Qu WM, Zhang MC, Mori S, et al. 2005. Implication of allelic polymorphism of osteopontin in the development of lupus nephritis in MRL/lpr mice. *Eur. J. Immunol.* 35:1510–20

Molleran Lee S, Villanueva J, Sumegi J, Zhang K, Kogawa K, Davis J, Filipovich AH. 2004. Characterisation of diverse PRF1 mutations leading to decreased natural killer cell activity in North American families with haemophagocytic lymphohistiocytosis. *J. Med. Genet.* 41:137-144.

Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N. & Kupfer, A. 1998 Three‐dimensional segregation of supramolecular activation clusters in T cells. *Nature* 395, 82–86

Mor, A., and M. R. Philips. 2006. Compartmentalized Ras/MAPK signaling. *Annu. Rev. Immunol.* 24:771-800

Muschen M, Rajewsky K, Kronke M, Kuppers R. 2002 The origin of CD95-gene mutations in B-cell lymphoma. *Trends Immunol.* 23:75-80.

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J. 1996. FLICE, a novel FADD-homologous ICE/CED3-like protease, is recruited to the CD95 (Fas/Apo-1) deathinducing signalling complex (DISC). *Cell* 85: 817–827.

Nagata S. 1998. Human autoimmune lymphoproliferative syndrome, a defect in the apoptosis-inducing Fas receptor: a lesson from the mouse model. *J. Hum. Genet.* 43:2–8

Nakajima H, Park HL, Henkart PA. 1995. Synergistic roles of granzymes A and B in mediating target cell death by rat basophilic leukemia mast cell tumors also expressing cytolysin/perforin. *J. Exp. Med.* 181: 1037–1046.

Nedvetzki, S. et al. 2007. Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. *Blood* 109, 3776–3785

Nemazee, D. & Hogquist, K. A.2003. Antigen receptor selection by editing or downregulation of V(D)J recombination. *Curr. Opin. Immunol*. 15, 182–189

Nguyen, A. et al. 2002 Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation *in vivo*. *Mol. Cell. Biol.* 22, 3035–3045

Nicholson DW, Thornberry NA. 1997. Caspases: killer proteases. *Trends Biochem. Sci.* 22:299–306

Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Li P et al. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91: 479–489.

Nose M, Nishihara M, Kamogawa J, Terada M, Nakatsuru S. 2000. Genetic basis of autoimmune disease in MRL/lpr mice: dissection of the complex pathological manifestations and their susceptibility loci. *Rev. Immunogenet.* 2:154–64

O'Garra A, Vieira P. 2004. Regulatory T cells and mechanisms of immune system control. *Nat. Med.* 10(8):801–5

Oida T, Zhang X, Goto M, et al. 2003. CD4+CD25− T cells that express latency-associated peptide on the surface suppress CD4+CD45RB high-induced colitis by a TGF-beta-dependent mechanism. *J. Immunol.* 170(5):2516–22

Oldstone MB, Nerenberg M, Southern P, Price J, Lewicki H. 1991. Virus infection triggers insulindependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell* 65(2):319–31

Oliveira JB, Bidere N, Niemela JE, Zheng L, Sakai K, Nix CP, Danner RL, Barb J, Munson PJ, Puck JM, Dale J, Straus SE, Fleisher TA, Lenardo MJ. 2007. NRAS mutation causes a human autoimmune lymphoproliferative syndrome. *Proc. Natl. Acad. Sci. USA*104:8953–8

Orange, J. S. 2006. Human natural killer cell deficiencies. *Curr. Opin. Allergy Clin. Immunol.* 6, 399–409

Orange, J. S. 2002. Human natural killer cell deficiencies and susceptibility to infection. *Microbes Infect.* 4, 1545–1558

Orange, J. S. et al. 2002. Wiskott–Aldrich syndrome protein is required for NK cell cytotoxicity and colocalizes with actin to NK cell‑activating immunologic synapses. *Proc. Natl Acad. Sci. USA* 99, 11351–11356

Orange, J. S., Harris, K. E., Andzelm, M. M., Valter, M. M., Geha, R. S. & Strominger, J. L. 2003. The mature activating natural killer cell immunologic synapse is formed in distinct stages. *Proc. Natl. Acad. Sci. USA* 100, 14151–14156
Orange, J. S., Stone, K. D., Turvey, S. E. & Krzewski, K. 2004. Biomedicine and disease: the Wiskott–Aldrich syndrome. *Cell. Mol. Life Sci.* 61, 2361–2385

Orilieri E, Cappellano G, Clementi R, Cometa A, Ferretti M, Cerutti E, Cadario F, Martinetti M, Larizza D, Calcaterra V, D'Annunzio G, Lorini R, Cerutti F, Bruno G, Chiocchetti A, Dianzani U. 2008. Variations of the perforin gene in patients with type 1 diabetes. *Diabetes.* 57(4):1078-83.

Orth K, Chinnaiyan AM, Garg M, Froelich CJ, Dixit VM. 1996. The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *J. Biol. Chem.* 271: 16443–16446.

Pasic, S., Micic, D. & Kuzmanovic, M. 2003. Epstein–Barr virus‐associated haemophagocytic lymphohistiocytosis in Wiskott–Aldrich syndrome. *Acta Paediatr.* 92, 859–861

Pinkoski MJ, Waterhouse NJ, Heibein JA, Wolf BB, Kuwana T, Goldstein JC et al. 2001. Granzyme B-mediated apoptosis precedes predominantly through a Bcl-2-inhibitable mitochondrial pathway. *J. Biol. Chem.* 276: 12060–12067.

Plebani, A., Ciravegna, B., Ponte, M., Mingari, M. C. & Moretta, L. 2000. Interleukin 2 mediated restoration of natural killer cell function in a patient with Griscelli syndrome. *Eur. J. Pediatr.* 159, 713–714

Plowman, S. J., and J. F. Hancock. 2005. Ras signaling from plasma membrane and endomembrane microdomains. Biochim. *Biophys. Acta* 1746:274–283.

Podack, E.R.,Young, J.D. & Cohn, Z.A. 1985. Isolation and biochemical and functional characterization of perforin from cytolytic T cell granules. *Proc. Natl.Acad. Sci. USA* 82, 8629–8633

Porter, J. C., Bracke, M., Smith, A., Davies, D. & Hogg, N. 2002. Signaling through integrin LFA-1 leads to filamentous actin polymerization and remodeling, resulting in enhanced T cell adhesion. *J. Immunol.* 168, 6330–6335

Quan LT, Tewari M, O'Rourke K, Dixit V, Snipas SJ, Poirier GG et al. 1996. Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B. *Proc. Natl. Acad. Sci. USA* 93: 1972–1976.

Quaratino S, Thorpe CJ, Travers PJ, Londei M. 1995. Similar antigenic surfaces, rather than sequence homology, dictate T-cell epitope molecular mimicry. *Proc. Natl. Acad. Sci. USA* 92(22):10398–402

Ramenghi U, Bonissoni S, Migliaretti G, DeFranco S, Bottarel F, et al. 2000. Deficiency of the Fas apoptosis pathway without Fas gene mutations is a familial trait predisposing to development of autoimmune diseases and cancer. *Blood* 95:3176–82

Ramsdell F. 2003. Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity* 19(2):165–8

Rasper DM, Vaillancourt JP, Hadano S, Houtzager VM, Seiden I, et al. 1998. Cell death attenuation by 'Usurpin,' a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD-95 (Fas, APO-1) receptor complex. *Cell Death Differ.* 5:271–88

Rechavi, O., I. Goldstein, H. Vernitsky, B. Rotblat, and Y. Kloog. 2007. Intercellular transfer of oncogenic H-Ras at the immunological synapse. *PLoS ONE* 2:e1204

Rieux-Laucat F, Fischer A, Deist FL. 2003. Cell-death signaling and human disease. *Curr. Opin. Immunol.* 15:325–31

Rieux-Laucat F, Le Deist F, Hivroz C, Roberts IA, Debatin KM, Fischer A, de Villartay JP. 1995. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* 268:1347–9

Rieux-Laucat F, Le Deist F, De Saint Basile G. 2005. Autoimmune lymphoproliferative syndrome and perforin. *N. Engl. J. Med.*352:306-307.

Risma KA, Frayer RW, Filipovich AH, Sumegi J. 2006. Aberrant maturation of mutant perforin underlies the clinical diversity of hemophagocytic lymphohistiocytosis. *J Clin Invest* 116:182–192

Riteau, B., Barber, D. F. & Long, E. O. 2003. Vav1 phosphorylation is induced by β2 integrin engagement on natural killer cells upstream of actin cytoskeleton and lipid raft reorganization. *J. Exp. Med.* 198,469–474

Robertson LK, Mireau LR, Ostergaard HL. A role for Phosphatidylinositol 3-kinase in TCR-stimulated ERK activation leading to paxillin phosphorylation and CTL degranulation. *J Immunol.* 175: 8138-8145

Roder, J. C. et al. 1982. Further studies of natural killer cell function in Chediak–Higashi patients. *Immunology* 46, 555–560

Salemi S, Caporossi AP, Boffa L, Longobardi MG, Barnaba V. 1995. HIVgp120 activates autoreactive CD4-specific T cell responses by unveiling of hidden CD4 peptides during processing. *J. Exp. Med.*181(6):2253–7

Sarin A, Williams MS, Alexander-Miller MA, Berzofsky JA, Zacharchuk CM, Henkart PA. 1997. Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases. *Immunity* 6: 209–215.

Sauer, H., Pratsch, L.,Tschopp, J., Bhakdi, S. & Peters, R. 1991. Functional size of complement and perforin pores compared by confocal laser scanning microscopy and fluorescent microphotolysis. *Biochim. Biophys.Acta* 1063, 137–146

Schneider-BrachertW,Tchikov V, Neumeyer J, JakobM,Winoto-Morbach S, et al. 2004. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. *Immunity* 21:415–28

Schwartz, R. H. 2003. T cell anergy. *Annu. Rev. Immunol*. 21, 305–334

Shiver JW, Henkart PA. 1991. A non cytotoxic mast cell tumor line exhibits potent IgE-dependent cytotoxicity after transfection with the cytolysin/perforin gene. *Cell* 64: 1175–1181.

Shiver JW, Su L, Henkart PA. 1992. Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolysin and granzyme A. *Cell* 71: 315–322.

Siegel RM, Muppidi JR, Sarker M, Lobito A, Jen M, et al. 2004. SPOTS: signaling protein oligomeric transduction structures are early mediators of death receptor-induced apoptosis at the plasma membrane. *J. Cell Biol.* 167:735–44

Simitsek PD, Campbell DG, Lanzavecchia A, Fairweather N, Watts C. 1995. Modulation of antigen processing by bound antibodies can boost or suppress class II major histocompatibility complex presentation of different T cell determinants. *J. Exp. Med.* 181(6):1957–63

Simmonds MJ, Howson JM, Heward JM, et al. 2005. Regression mapping of association between the human leukocyte antigen region and Graves disease. *Am. J. Hum. Genet.* 76(1):157–63

Slee EA, Adrain C, Martin SJ. 1999. Serial killers: ordering caspase activation events in apoptosis. *Cell. Death. Differ.* 6: 1067–1074.

Smyth D, Cooper JD, Collins JE, et al. 2004. Replication of an association between the lymphoid tyrosine phosphatase locus (LYP/*PTPN22*) with type 1 diabetes, and evidence for its role as a general autoimmunity locus. *Diabetes* 53(11):3020–3

Sneller MC, Dale JK, Straus SE. 2003. Autoimmune lymphoproliferative syndrome. *Curr. Opin. Immunol.* 15:417–21

Sneller MC, Straus SE, Jaffe ES, Jaffe JS, Fleisher TA, et al. 1992. A novel lymphoproliferative/ autoimmune syndrome resembling murine lpr/gld disease. *J. Clin. Invest.* 90:334–41

Snover, D. C., Frizzera, G., Spector, B. D., Perry, G. S. 3rd & Kersey, J. H. 1981. Wiskott–Aldrich syndrome: histopathologic findings in the lymph nodes and spleens of 15 patients. *Hum. Pathol.* 12, 821–831

Stefanova, I., B. Hemmer, M. Vergelli, R. Martin, W. E. Biddison, and R. N. Germain. 2003. TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nat. Immunol.* 4:248–254.

Stepp, S. E. Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, Henter JI, Bennett M, Fischer A, de Saint Basile G, Jumar Vet al. 1999. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* 286, 1957–1959

Stinchcombe, J. C. et al. 2001. Rab27a is required for regulated secretion in cytotoxic T lymphocytes. *J. Cell Biol.* 152, 825–834

Stinchcombe, J. C., Bossi, G., Booth, S. & Griffiths, G. M. 2001 The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* 15, 751–761

Stinchcombe, J. C., Page, L. J. & Griffiths, G. M. 2000. Secretory lysosome biogenesis in cytotoxic T lymphocytes from normal and Chediak Higashi syndrome patients. *Traffic* 1, 435–444

Straus SE, Jaffe ES, Puck JM, Dale JK, Elkon KB, et al. 2001. The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis. *Blood* 98:194–200

Straus SE, Sneller M, Lenardo MJ, Puck JM, Strober W. 1999. Inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. *Ann. Int. Med.* 130:591-601.

Su MW, Pyarajan S, Chang JH, Yu CL, Jin YJ, Stierhof YD, Walden P, Burakoff SJ. 2004. Fratricide of CD8_ cytotoxic T lymphocytes is dependent on cellular activation and perforin-mediated killing. *Eur J Immunol* 34:2459–2470

Suda T, Takahashi T, Golstein P, Nagata S. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 75:1169–78

Sullivan, K. E., Mullen, C. A., Blaese, R. M. & Winkelstein, J. A. 1994. A multi institutional survey of the Wiskott–Aldrich syndrome. *J. Pediatr.* 125, 876–885

Sundaram, M. & Han, M. 1995. The *C. elegans ksr-1* gene encodes a novel Raf-related kinase involved in Ras mediated signal transduction. *Cell* 83, 889–901

Sundstedt A, O'Neill EJ, Nicolson KS, Wraith DC. 2003. Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo. *J. Immunol.* 170(3):1240–8

Sutton VR, Davis JE, Cancilla M, Johnstone RW, Ruefli AA, Sedelies K et al. 2000. Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B mediated caspase activation. *J. Exp. Med.* 192: 1403–1414.

Targan, S. R. & Oseas, R. 1983. The "lazy" NK cells of Chediak–Higashi syndrome. *J. Immunol.* 130, 2671–2674

Therrien, M. et al. 1995. KSR, a novel protein kinase required for RAS signal transduction. *Cell* 83, 879–888

Thomas DA, Scorrano L, Putcha GV, Korsmeyer SJ, Ley TJ. 2001. Granzyme B can cause mitochondrial depolarization and cell death in the absence of BID, BAX, and BAK. *Proc. Natl. Acad. Sci. USA* 98: 14985–14990

Thome M, Tschopp J. 2001. Regulation of lymphocyte proliferation and death by FLIP. *Nat. Rev. Immunol.* 1:50–58

Thornton AM, Shevach EM. 2000. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J. Immunol.* 164(1):183–90

Tibbetts MD, Zheng LX, Lenardo MJ. 2003. The death effector domain protein family: regulators of cellular homeostasis. *Nat. Immunol.* 4:404–9
Trambas, C. M. & Griffiths, G. M. 2003. Delivering the kiss of death. *Nat. Immunol.* 4, 399–403

Trambas C, Gallo F, Pende D, Marcenaro S, Moretta L, De Fusco C, Santoro A, Notarangelo L, Arico M, Griffiths GM. 2005. A single amino acid change, A91V, leads to conformational changes that can impair processing to the active form of perforin. *Blood* 106:932–937

Trapani JA, Jans DA, Jans PJ, Smyth MJ, Browne KA, Sutton VR. 1998. Efficient nuclear targeting of granzyme B and the nuclear consequences of apoptosis induced by granzyme B and perforin are caspase-dependent, but cell death is caspase-independent. *J. Biol. Chem.* 273: 27934–27938.

Tschopp, J., Massom,D. & Stanley, K.K. 1986. Structural-functional similarity between proteins involved in complement and cytotoxic T-lymphocyte-mediated cytolysis. *Nature* 322, 831–834

Tsuneyama K, Nose M, Nisihara M, Katayanagi K, Harada K, et al. 2001. Spontaneous occurrence of chronic non-suppurative destructive cholangitis and antimitochondrial autoantibodies in MRL/lpr mice: possible animal model for primary biliary cirrhosis. *Pathol. Int.* 51:418–24

Ueda H, Howson JM, Esposito L, et al. 2003. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 423(6939):506–11

Vaidya B, Imrie H, Perros P, et al. 1999. The cytotoxic T lymphocyte antigen-4 is a major Graves' disease locus. *Hum. Mol. Genet.* 8(7):1195–9.
Vaux DL, Strasser A. The molecular biology of apoptosis. 1996. *Proc. Natl. Acad. USA* 93: 2239-44

Voskoboinik I, Thia MC, Trapani JA. 2005. A functional analysis of the putative polymorphisms A91V and N252S and 22 missense perforin mutations associated with familial hemophagocytic lymphohistiocytosis. *Blood* 105: 4700–4706

Voskoboinik I, Smyth MJ, Trapani JA. 2006. Perforin-mediated target cell death and immune homeostasis. *Nat Rev Immunol* 6:940-952.

Vyas, Y. M., Maniar, H. & Dupont, B. 2002. Cutting Edge: differential segregation of the SRC homology 2‐containing protein tyrosine phosphatase‐1 within the early NK cell immune synapse distinguishes noncytolytic from cytolytic interactions. *J. Immunol.* 168, 3150–3154

Walker LS, Abbas AK. 2002. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat. Rev. Immunol.* 2(1):11–9

van den Broek ME, Kagi D, Ossendorp F, Toes R, Vamvakas S, Lutz WK, Melief CJ, Zinkernagel RM, Hengartner H. 1996. Decreased tumor surveillance in perforin-deficient mice. *J. Exp. Med.* 184:1781-1790.

Wang, I. J. et al. 2005. Epstein–Barr virus associated post transplantation lymphoproliferative disorder with hemophagocytosis in a child with Wiskott–Aldrich syndrome. *Pediatr. Blood Cancer* 45, 340–343

Wang J, Lenardo MJ. 1997. Essential lymphocyte function associated 1 (LFA-1): intercellular adhesion molecule interactions for T cell-mediated B cell apoptosis by Fas/APO-1/CD95. *J. Exp. Med.* 186:1171–76

Wang J, Lobito AA, Shen F, Hornung F, Winoto A, Lenardo MJ. 2000. Inhibition of Fas-mediated apoptosis by the B cell antigen receptor through c-FLIP. *Eur. J. Immunol.* 30:155–63

Wang J, Zheng L, Lobito A, Chan FK, Dale J, Sneller M, Yao X, Puck JM, Straus SE, Lenardo MJ. 1999. Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. *Cell* 98:47–58

Wardemann, H. 2003. Predominant autoantibody production by early human B cell precursors. *Science* 301, 1374–1377

Waterhouse P, Penninger JM, Timms E, et al. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 1995;270(5238):985–8

Wei S, Gamero AM, Liu JH, Daulton AA, Valkov NI, Trapani JA, Larner AC, Weber MJ, and Djeu JY. 1998. Control of lytic function by mitogen-activated protein kinase/extracellular regulatory kinase 2 (ERK2) in a human natural killer cell line: identification of perforin and granzyme B mobilization by functional ERK2. *J. Exp. Med.* 187:1753-1765

Wofsy D, Ledbetter JA, Roubinian JR, Seaman WE, Talal N. 1982. Thymic influences on autoimmunity in MRL-lpr mice. *Scand. J. Immunol.* 16:51–58

Wong CS, Arkwright PD, Rieux-Laucat F, Cant AJ, Stevens RF, et al. 2004. Childhood linear IgA disease in association with autoimmune lymphoproliferative syndrome. *Br. J. Dermatol.* 150:578–80

Wu J, Wilson J, He J, Xiang L, Schur PH, Mountz JD. 1996. Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. *J. Clin. Invest.* 98:1107–13

Wucherpfennig KW. 2001. Mechanisms for the induction of autoimmunity by infectious agents. *J. Clin. Invest.* 108(8):1097–104

Wucherpfennig KW, Strominger JL. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 80(5):695–705

Wulfing, C., Purtic, B., Klem, J. & Schatzle, J. D. 2003. Stepwise cytoskeletal polarization as a series of checkpoints in innate but not adaptive cytolytic killing. *Proc. Natl. Acad. Sci. USA* 100, 7767–7772

Yachi, P. P., J. Ampudia, T. Zal, and N. R. Gascoigne. 2006. Altered peptide ligands induce delayed CD8-T cell receptor interaction—a role for CD8 in distinguishing antigen quality. *Immunity* 25:203–211.

Zerrahn, J., Held, W. & Raulet, D. H. 1997. The MHC reactivity of the T cell repertoire prior to positive and negative selection. *Cell* 88, 627–636

Zhang J, Cado D, Chen A, Kabra NH, Winoto A. 1998. Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. *Nature* 392:296–300

Zheng L, Trageser CL, Willerford DM, Lenardo MJ. 1998. T cell growth cytokines cause the super induction of molecules mediating antigen-induced T lymphocyte death. *J. Immunol.* 160:763–69

Zhou S, Ou R, Huang L, Moskophidis D. 2002. Critical role for perforin-, Fas/FasL-, and TNFR1-mediated cytotoxic pathways in down-regulation of antigen-specific T-cells during persistent viral infection. *J Virol* 76:829–840

Zhu S, Hsu AP, Vacek MM, Zheng L, Schaffer AA, Dale JK, Davis J, Fischer RE, Straus SE, Boruchov D, Saulsbury FT, Lenardo MJ, Puck JM. 2006. Genetic alterations in caspase-10 may be causative or protective in autoimmune lymphoproliferative syndrome. *Hum. Genet.* 119:284–94

Zuniga-Pflucker JC, Lenardo MJ. 1996. Regulation of thymocyte development from immature progenitors. *Curr. Opin. Immunol.* 8:215–24

zur Stadt, U. et al. 2005. Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type 4 to chromosome 6q24 and identification of mutations in syntaxin 11. *Hum. Mol. Genet.* 14, 827–834

zur Stadt U, Beutel K, Kolberg S, Schneppenheim R, Kabisch H, Janka G, Hennies HC. 2006. Mutation spectrum in children with primary hemophagocytic lymphohistiocytosis: molecular and functional analyses of PRF1, UNC13D, STX11, and RAB27A. *Hum. Mutat.* 27:62-68.

ACKNOWLEDGMENTS

I am grateful to many people at the laboratory of Immunology, Department of Medical Science, Novara for their guidance and support during this PhD program. First and foremost, I would like to thank my advisor Umberto Dianzani, for providing me an opportunity to pursue research in an interesting area of Immunology and his counsel and tremendous support through out. I would also like to thank my "mentor" Annalisa Chiocchetti for her continuous support during these years of work, for her enthusiasm and for answering definitely to "all my question"!

Special thanks go to all my co-workers (not only) in the lab: Beppe, Betty, Thea, Max, Teo, Ilaria, Ric, Stefania and all the others for their strong encouragement and for their friendship during these years.

I am indebted to Andrey Shaw, Professor of Immunology at Washington University in St. Louis to give me the possibility to live this incredible experience working and learning in his lab. Thanks to everybody from the Shaw lab too.

Thanks to my best "old and new" friends for their emotional support: Francy, Chicca, Manu, Karel, Giulietta, Annalisa, Carlo, Barbara, Francesca, Domy and to everybody that I met during this period....all of you mean a lot to me!!!

Finally, I have been fortunate to have a wonderful family. Thanks to their incredible love, their confidence and encouragement I have been able to carry out my work! Thanks to mom and dad, to Lalla, Paolo, Laura, Dany...you have been always understanding....even of the distance from home!!! And, at the end, thanks to my little Richy because, even if he is not understanding right now, he has been very important to me....I'm so proud of him!!